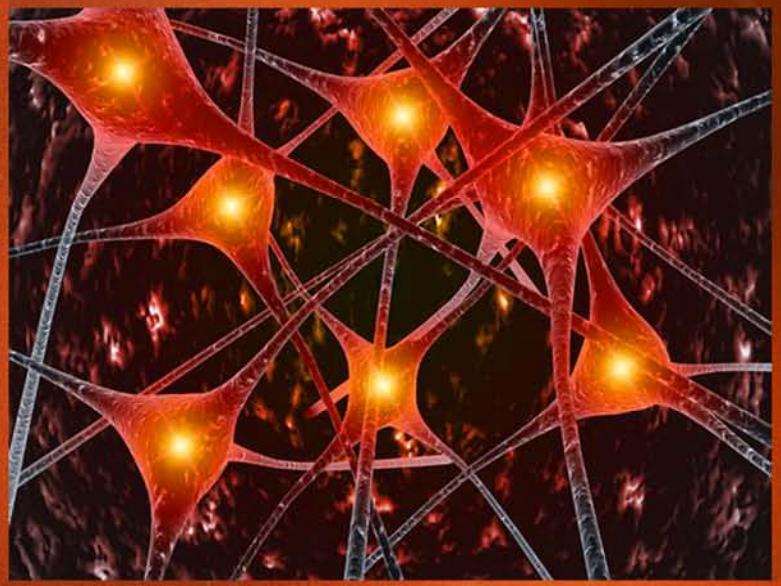
CHINTHU ROMEO

Ph.D. Thesis

AUGUST 2012

Cholinergic receptor subtypes functional regulation in spinal cord injured monoplegic rats: Effect of 5-HT, GABA and Bone Marrow Cells



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CENTRE FOR NEUROSCIENCE
DEPARTMENT OF BIOTECHNOLOGY
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

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CHOLINERGIC RECEPTOR SUBTYPES FUNCTIONAL REGULATION IN SPINAL CORD INJURED MONOPLEGIC RATS: EFFECT OF 5-HT, GABA AND BONE MARROW CELLS

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BY

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CERTIFICATE

This is to certify that the thesis entitled **"CHOLINERGIC RECEPTOR SUBTYPES FUNCTIONAL REGULATION IN SPINAL CORD INJURED MONOPLEGIC RATS: EFFECT OF 5-HT, GABA AND BONE MARROW CELLS"** is a bonafide record of the research work carried out by **Mrs. Chinthu Romeo,** under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has been presented for the award of any other degree.

Cochin – 682 022

(C. S. Paulose)

August 17, 2012

DECLARATION

I hereby declare that the thesis entitled "**Cholinergic receptor subtypes functional regulation in spinal cord injured monoplegic rats: Effect of 5-HT, GABA and Bone Marrow Cells"** is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, under the guidance of Prof. C. S. Paulose, Director, Centre for Neuroscience, Department of Biotechnology and no part thereof has been presented for the award of any other degree, diploma, associateship or other similar titles or recognition.

Cochin - 682 022
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Dedicated to Lord Almighty

The ultimate source of all knowledge and power. . .

ABBREVIATIONS

5-HT	Serotonin
ACh	Acetylcholine
AChE	Acetylcholine esterase
BDNF	Brain-Derived Neurotrophic Factor
B_{max}	Maximal binding
BMC	Bone marrow cells
BrdU	Bromodeoxyuridine
cAMP	3'-5'-cyclic adenosine monophosphate
cGMP	3'-5'-cyclic guanosine monophosphate
ChAT	Choline acetyl transferase
CNS	Central Nervous System
Cox	Cyclo oxygenase
CREB	cAMP regulatory element binding protein
CSF	Cerebrospinal fluid
DAG	Diacylglycerol
DNA	Deoxy ribonucleic acid
DRG	Dorsal Root Ganglion
EDTA	Ethylene diamine tetra acetic acid

GABA	Gamma amino butyric acid
GDNF	Glial Derived Neurotrophic Factor
GFR	Glial cell line-derived neurotrophic factor receptor
GPx	Glutathione peroxidase
GSH	Glutathione
IGF	Insulin like growth factor
IL	Inter leukine
IP_3	Inositol trisphosphate
iNOS	inducible Nitric Oxide Synthase
K_d	Dissociation constant
Mn SOD	Manganese Superoxide Dismutase
nAChRs	Nicotinic acetylcholine receptors
NeuN	Neuronal-specific nuclear protein
NF-κB	Nuclear factor-kappa B
NGF	Nerve growth factor
NTF	Neurotrophic factors
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Triton X- 100
PCR	Polymerase Chain Reaction

PFA	Paraformaldehyde
PI3	Phosphatidyl inositol-3
PI3-K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 3,4-Bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-Trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
QNB	Quinuclidinylbenzilate
ROS	Reactive oxygen species
SCI	Spinal cord injury
SEM	Standard error of mean
SOD	Superoxide dismutase
TNF	Tumour Necrosis factor
TNFR	Tumour Necrosis factor receptor

CONTENTS

Introduction

Spinal cord injury (SCI) is a major public health issue worldwide. It causes changes in all physical systems and functional abilities (Krause & Crewe, 1991). It is a devastating neurological injury, resulting in varying degrees of paralysis and sensory loss which are permanent and irreversible. In India, approximately fifteen lakh people are affected with SCI. Majority of them (82%) are males in the age group of 16-30 years (Gupta *et al.,* 2008). A recent survey reported that the prevalence of SCI ranges from 236 per million in India to 1800 per million in the USA (Hagen *et al.,* 2012). SCI is caused mainly from motor vehicle accidents, fall from heights, sports injuries (Dunn *et al.,* 2000). It also results from a gunshot or knife wound that penetrates and damages spinal cord. When the spinal cord is injured, the nerves above the level of the injury continue to work. However, below the level of the injury communication is disrupted which can result in loss of movement, sensation, bowel and bladder control. The spinal cord relay messages between the brain and various parts of the body. Disruption of the spinal cord leads to diminished transmission of descending control from the brain to motor neurons and ascending sensory information.

In medical terms, SCI is defined as “the occurrence of an acute, traumatic lesion of neural elements in the spinal canal resulting in temporary or permanent sensory deficit" (Thurman *et al.,* 1995). An injury to the spinal cord occurs when pressure is applied to the spinal cord or the blood supply, which carries oxygen to the spinal cord is disrupted. The consequence of injury depends on the site of injury and completeness of injury. The higher the level of lesion, the greater is the injury. The major conditions that result from injury to the spinal cord are quadriplegia, paraplegia and monoplegia. Quadriplegia is the paralysis of all four limbs, hands and the trunk. Paraplegia involves paralysis from the chest or waist downwards. Monoplegia is the paralysis of one limb or hand. A complete injury

results in no function below the level of injury, no sensation and no movement. An incomplete injury results in some functional disability below the level of injury. The spinal cord nerve tissue is like brain tissue in that it usually does not fully recover when damaged.

The pathophysiology of SCI is characterized by an initial primary injury followed by secondary deterioration. SCI causes destruction of sensory nerve fibres and also lead to loss of sensation such as touch, pressure and temperature. SCI often leads to a mutilation of the respiratory system (Beth *et al.,* 2007), secondary musculoskeletal deterioration (Shields & Dudley-Javoroski, 2003) and sexual function. The neurologic symptoms include pain, numbness, paresthesias, muscle spasm, weakness and bowel/bladder changes. Other effects of SCI also includes low postural blood pressure (postural hypotension), inability to regulate blood pressure effectively, reduced control of body temperature (poikilothermic) and inability to sweat below the level of injury.

Since SCI affects CNS (central nervous system), its understanding lead to new strategies to reverse the damage caused by SCI. Gamma Amino Butyric Acid (GABA) (Todd *et al.,* 1992), glycine (Todd & Sullivan, 1990), serotonin (5-HT) (Basbaum *et al.,* 1978), norepinephrine (Dahlström & Fuxe, 1965), dopamine (Fleetwood-Walker & Coote, 1981), choline acetyl transferase (ChAT) (Todd, 1991), acetyl choline esterase (AChE) (Kása, 1986) are distributed throughout the spinal cord. There are reports suggesting that neurotransmitter release from intra spinal grafts is a highly relevant parameter to evaluate the functional ability of transplanted cells (Leanza *et al.,* 1993b; Cenci *et al.,* 1994; Leanza *et al.,* 1999; Cenci & Kalén, 2000).

Acetylcholine (ACh) is the key neurotransmitter for para sympathetic nervous system. It modulates spinal sensory processing in the dorsal horn (Myslinski & Randic, 1977; Urban *et al.,* 1989) through the intrinsic cholinergic inter neurons found in the dorsal horn (Barber *et al.,* 1984; Todd, 1991). ACh is also found in the motor neurons. (Villégier *et al.,* 2010). Depletion in the motor

neurons causes a decrease in ACh concentration (Rosario *et al.,* 2007). In mammals rhythmic limb movement, such as walking is controlled by pattern-generating neurons within the spinal cord. During early development, motor neurons seem to become spontaneously active and they release ACh, which excites neighbouring cells as a form of cell-cell communication. Motor neurons thus mediate locomotion *via* ACh. Knock off model of mice that lacked the enzyme necessary for synthesising ACh resulted in development of defective spinal circuit that lacked the control of leg movements. This demonstrates the relevance of ACh in control of leg movements. Thus ACh is necessary for a proper neural circuit.

Necrosis or cell death is a pathophysiological process that occurs as a result of secondary damage after SCI. Cell death continues to occur over several days and weeks following SCI. In the secondary phase, lipid peroxidation and free-radical production also occurs. The invading inflammatory cells increase the local concentrations of cytokines and chemokines. SCI triggers apoptosis, which kills oligodendrocytes in injured areas of the spinal cord days to weeks after the injury. Oligodendrocytes are the cells that form the myelin sheath around axons and speeds the conduction of nerve impulses. Apoptosis strips myelin from intact axons in adjacent ascending and descending pathways, which further impairs the spinal cords ability to communicate with the brain. Thus free radicals and apoptosis increase the damage in SCI. Both neurons and glia die by apoptosis; the response of oligodendrocytes in long tracts undergoing Wallerian degeneration is particularly long lived and is responsible for chronic demyelination and some of the dysfunction in chronic SCI. After SCI in the rat, posttraumatic necrosis occurred and apoptotic cells were found from 6 hours to 3 weeks after injury (Maria *et al.,* 1997).

In the present scenario, basic research on SCI focuses on several areas that target functional restitution and regeneration of the injured neurons within the spinal cord. Stimulating the rejuvenation of axons is a key factor of spinal cord

repair because every axon in the injured spinal cord that can be reconnected and increases the chances for improvement of function. Previously, CNS neurons were thought to be incapable of regeneration. But, Liu and Chambers (1958) indicated that central projections of primary afferent fibres can develop in the spinal cord after injury. Subsequent work by Richardson *et al.,* (1980) demonstrated axonal elongation. Axonal growth alone is not sufficient for functional recovery. Axons have to make the proper connections and re-establish functioning synapses. Therefore, SCI research should focus on preventing the loss of function and on restoring lost functions, including sensory and motor functions with the ultimate goal of fully restoring to the individual levels of activity and function that a person had before injury. Targets for intervention for improving functional outcome in SCI include free radical reduction, prevention of neuronal populations from apoptosis and promotion of neurite outgrowth.

With recent molecular strategies and techniques, research in the understanding of neuronal injury and neural regeneration provide new promises for reversal of SCI that was thought to be permanent and irrevocable (Carlson & Gorden, 2002). A variety of tissues and cells have been implanted in the damaged spinal cord to restore function. These include bone marrow cells (BMCs), olfactory ensheathing cells, dorsal root ganglia, adrenal tissue, hybridomas, peripheral nerves or transplanted conduits of schwann cells. It is hypothesised that these cells would rescue, replace or provide a regenerative pathway for injured neurons, which would then integrate or promote the regeneration of the spinal cord and restore function after injury (Zompa *et al.,* 1997). Thus the promising treatment of SCI is cell-based therapy (Stanworth & Newland, 2001; Hipp & Atala, 2004) due to the limited success of pharmacological treatment. Cellular transplantation strategies have been used in various models of SCI (Eftekharpour *et al.,* 2008). The cell replacement approach has the advantage that it promotes regeneration and repair. Regeneration involves replacement of lost or damaged neurons and induces axonal regeneration. Repair involves replacement of

supportive cells such as oligodendrocytes in order to prevent progressive demyelination and induce remyelination (Totoiu & Keirstead, 2005). In addition, BMC transplantation promotes protection of endogenous cells from further cell damage by attenuation of secondary injury process. BMC can also generate endoderm and ectoderm derivates including neural cells (Jiang *et al.,* 2002; Kim *et al.,* 2002). Non-embryonic sources of adult stem cells, which are not of ethical and legal concerns usually associated with embryonic stem cell research, offer great promise for the advancement of spinal cord treatment (Moore *et al.,* 2006). BMCs support the repair of damaged tissues. Under specific experimental condition, BMCs differentiate into mature neurons or glial cells (Munoz *et al.,* 2003). Transplanted BMCs improvises neurological deficits in the CNS injury models by producing neural cells or myelin producing cells (Chopp *et al.,* 2000; Akiyama *et al.,* 2002). BMCs actively remyelinate spinal cord once administered directly or intravenously (Dezawa *et al.,* 2005).

Consequences of SCI are devastating and any strategy to alleviate neurological loss is attractive (Treherne *et al.,* 1992). Neurotransmitters relay, amplify and modulate signals between the neurons. 5-HT is known to play a facilitory role in locomotor circuit by increasing motoneuron excitability, modulating spinal central pattern generators (CPG) (Rossignol *et al.,* 1988) and improving locomotor behaviour following SCI (Kim *et al.,* 1999; Ribotta *et al.,* 2000). A small amount of 5-HT can activate super-sensitive motor neurons (Li *et al.,* 2007). Reports suggests that the cell-specific effect of 5-HT on regenerating neurons within the adult CNS by increasing the calcium concentration of the cells (Murrain *et al.,* 1990). GABA receptors are known to be involved in during neuronal development. The presence of GABA receptors in developing oligodendrocytes provides a new mechanism for neuronal–glial interactions during development and offers a novel target for promoting remyelination following white matter injury (Luyt *et al.,* 2007). GABA increases synaptic plasticity. Soltani *et al.,* (2011) reported that GABA promotes proliferation of β-

cells in pancreas. GABAergic inputs to hippocampal progenitor cells promote neuronal differentiation (Tozuka *et al.,* 2005). Continuous application of GABA could promote dendritic growth *in vivo*, influence ganglion sensitivity to ACh and alter development of pre synaptic specialisation (Wolf *et al.,* 1987). 5-HT and GABA can be also used as agents for cell proliferation and differentiation. Earlier reports from our lab showed that 5HT acting through specific receptor subtypes $5HT_2$ (Sudha & Paulose, 1998) and GABA acting through specific receptor subtypes $GABA_B$ (Biju *et al.,* 2002) control cell proliferation and act as co-mitogens. These reports have paved way to study of the effect of 5-HT and GABA in after SCI.

SCI is a major cause of concern and the role of cholinergic neurotransmitter system in SCI has not been widely studied. In the present study, we have chosen Wistar rats as our model for SCI. Rats have been chosen to study not only because they are readily available but also because the morphological, biochemical and functional changes that occur are similar to those seen in humans (McTigue *et al.,* 2000; Metz *et al.,* 2000; Norenberg *et al.,* 2004; Fleming *et al.,* 2006). Since ACh is the major neurotransmitter in the motor neurons, the study of cholinergic alteration during SCI will enlighten the signalling pathways that is involved in the SCI mediated motor deficits. This study further investigated the effect of regenerative cell proliferation and differentiation in SCI, when BMCs, 5-HT and GABA are supplemented individually and in combination. The present study also investigated the second messenger alterations by studying inositol triphosphate (IP3), 3'-5'-cyclic adenosine monophosphate (cAMP) and 3'-5'-cyclic guanosine monophosphate (cGMP) functional regulation and gene expression of Phospholipase C (PLC) and cAMP regulatory element binding protein (CREB). The changes in gene expression of anti oxidant enzymes like Superoxide dismutase (SOD) and Glutathione peroxidase (GPx) were investigated. Gene expression studies of apoptotic factors like Bax, Caspase-8, Tumour Necrosis factor α (TNFα) and Nuclear factor kappa-light-chain-enhancer of activated B

cells (NF-κB) were studied. The gene expression of neuronal survival factors Brain Derived Neurotrophic Factor (BDNF), Glial Derived Neurotrophic Factor (GDNF), insulin like growth factors (IGF-1), Akt-1 and Cyclin D2 were also studied. We also demonstrated the autologous differentiation of BMC to neurons using comitogenic 5-HT and GABA by confocal studies with Bromodeoxyuridine (BrdU) labelling and Neuronal-specific nuclear protein (NeuN) expression. Behavioural studies were planned to evaluate the locomotor function in control and experimental rats. Our present study on 5-HT, GABA and BMC dependent regulation of muscarinic receptors in the spinal cord and brain will certainly enlighten novel therapeutic possibilities for the treatment of SCI.

OBJECTIVES OF THE PRESENT STUDY

1. To induce spinal cord injury in rats and to study the effect of 5-HT, GABA and BMC treatment individually and in combination.

2. To investigate the behavioural changes in control and experimental rats using rotarod test, grid walk test and narrow beam test.

3. To study cholinergic receptors alterations and in the spinal cord and brain regions of control and experimental rats.

4. To analyse the muscarinic receptors - muscarinic M1, M2, M3, nicotinic receptor - α7 nAChR, cholinergic enzymes - AChE and ChAT gene expression in the spinal cord and brain regions of control and experimental rats using real time PCR.

5. To study gene expression studies of second messenger enzyme - PLC; transcription factor - CREB; apoptotic factors - Bax, Caspase-8, TNFα and NF-κB; anti-oxidant enzymes SOD and GPX; cell survival factors - BDNF, GDNF, IGF-1, Akt-1 and Cyclin D2 in the spinal cord and brain regions of control and experimental rats using real time PCR.

6. To study the second messenger - IP3, cGMP and cAMP content in the spinal cord and brain regions of control and experimental rats.

7. To study the localization and expression status of muscarinic M1, M3 and α7 nAChR using confocal microscope by immunofluorescent specific

antibodies in the spinal cord and brain sections of control and experimental rats using Confocal microscope.

8. To study neuronal regeneration using Brdu and NeuN in spinal cord using confocal microscope.

Literature Review

Spinal cord injury is a devastating clinical problem that has permanent consequences. It has many medical, emotional and social consequences. It results in irreversible functional loss and life time disability (Sekhon & Fehlings, 2001). SCI is seen mostly in younger age group people (O'Connor & Murray, 2005). In the western countries, it is estimated that about 5 per 100,000 people suffer from the disabilities caused by SCI. The reasons of SCI are vehicular accidents (44.8%), fall from heights (21.7%), acts of violence like gun shots (16%) and sports injuries (13%). Since 80% of cases occur in younger age group between the ages of 16 to 30 years, SCI causes a significant cost in terms of lifetime care and loss of productivity.

Damage to motor nerves results in paralysis or loss of control of movement. Damage to somatosensory nerves results in loss of sensation and perception; one can no longer feel touch, pain, temperature or be able to tell without looking where in space the nerve damaged body part is positioned. After injury, the spinal cord undergoes a series of pathologic changes, including micro haemorrhage, cytotoxic edema, neuronal necrosis, axonal fragmentation, demyelination, secondary cellular destruction and eventually cyst formation (Balentine, 1978; Balentine & Greene, 1984; Coutts & Keirstead, 2008). The most frequent neurological deficit associated with SCI is incomplete tetraplegia (30.6%), followed by complete paraplegia (25.8%), complete tetraplegia (22.1%) and incomplete paraplegia (19.3%).

An injury to the spinal cord affects the brain regions (Gomez *et al.*, 2012). When spinal cord is injured, there occurs a primary mechanical injury followed by a secondary injury. Primary injury is the initial mechanical damage, whereas secondary injury is progressive cell injury that begins in the gray matter and progresses into the white matter (Ballentine, 1978). Primary mechanical injury is

caused by the direct compression of spinal cord. Primary injury mechanisms include acute compression, impact, missile and distraction forces. The severity and the site of injury determine the effect of primary injury. The primary mechanical injury disrupts axons, blood vessels and cell membranes. Damage to blood vessels can be toxic to the CNS (Asano *et al.,* 1980). It results in pathogenesis.

The concept of secondary injury was first put forward by Allen (1911). However, our knowledge of the exact mechanism through which primary injury triggers secondary injury is not very specific (Simon *et al.,* 2009). The secondary injury phase involves vascular dysfunction, oedema, ischemia, excitotoxicity, electrolyte shifts, free radical production, inflammation and delayed apoptotic cell death. After SCI, the mammalian CNS fails to adequately regenerate due to intrinsic inhibitory factors expressed on central myelin and the extracellular matrix of the posttraumatic gliotic scar. Secondary injury disrupts the blood-spinal cord barrier and generates inflammatory response. Both barrier disruption and inflammation perturb the microenvironment and expose neurons to plasma-derived cells and molecules that can be injurious to intact, neighbouring tissue (Schlosshauer, 1993). Inflammation is considered to be an important element in secondary damage after SCI. This secondary damage leads to tissue loss and functional impairments. The immune responses are triggered by SCI and are mediated by a variety of factors that have both detrimental and beneficial effects. Inflammation that results from secondary injury is characterized by the accumulation of activated microglia, macrophages and contributes to secondary pathogenesis (Blight, 1992; Hirschberg *et al.,* 1994; Popovich *et al.,* 1994; Blight *et al.,* 1995; Bethea *et al.,* 1998). Inflammatory cells are coupled with delayed neuronal death and demyelination (Blight, 1985; Davis *et al.,* 1990; Dijkstra *et al.,* 1994; Hirschberg *et al.,* 1994). Therefore, strategies have focused on diminishing the secondary effects of SCI (Dumont *et al.,* 2001; Hall & Traystman, 2009;

Fehlings & Nguyen, 2010). SCI also leads to a range of alterations that are cytotoxic to both nerve cells and glial cells. Reports suggest that neurons and glial cells are changed permanently after SCI. Changes occur to segments above and below the SCI and these persistent changes lead to dysfunction. Hence, comprehension of secondary injury mechanisms and their complexities in SCI are invaluable requisite for planned therapeutic strategies: to stimulate axonal regrowth (regeneration), to arrest the self-perpetuating degeneration (neuroprotection), and the generation of new neurons and glia that will repopulate the site of injury and functionally integrate into the surviving neural tissue.

The most important physical consequences of a SCI are motor and sensory loss and impairments of bladder, bowel and sexual function leading to widespread disabilities in activities of daily life. There are certain health problems that arise secondary to the SCI. They are pain, spasms, pressure sores, urinary problems, bowel problems, respiratory failure, oedema and excessive sweating (Post *et al.,* 1998). SCI is associated with respiratory complications. In case of acute SCI, 80% of cases are associated with respiratory complications (Tollefson & Fondenes, 2012). The common respiratory complications are atelectasis, pneumonia and respiratory failure (Jackson & Groomes, 1994). Pulmonary dysfunction is the cause for the largest portion of morbidity after SCI (Fishburn *et al.,* 1990; Linn *et al.,* 2000). SCI causes instant damage of nervous tissue followed by the loss of motor and sensory function. Due to the restricted self-repair ability of damaged nervous tissue, there underlies the need for reparative interventions to restore function after SCI. Without control from the brain, movements produced by a spinal CPG were not likely to be useful in restoring successful walking without regulation from the brain.

Magnetic Resonance Imaging (MRI) is the method to evaluate patients who have a persistent neurological deficit following SCI as it allows direct visualization of the injured cord, bony intervertebral and ligamentous structures,

and paraspinal soft tissues. MRI has replaced myelography and Computer Tomography myelography as the primary imaging preference available to assess compression of the spinal cord and is also a vital diagnostic modality in cases of SCI without radiographic abnormality. MRI also provides information regarding prognosis and neurological recovery.

CURRENT TREATMENTS AND ITS SIDE EFFECTS IN SPINAL CORD INJURY

There are various treatments available for SCI. Corticosteroids are used for the pharmacological treatment of SCI. They act by improving the blood flow in the spinal cord, restore impulse transmission, regulates calcium metabolism and enhance functional neurological recovery (Anderson *et al.*, 1982; Bracken, 1992; Hall, 1993; Constantini, 1994). Methyl prednisolone is a cortico steroid that has anti-oxidant activity and is used in SCI treatment (Bracken, 1990). A study by Yu *et al.,* (2004) reported that early repeated methyl prednisolone sodium succinate treatment allows greater recovery from SCI. Sharma *et al.*, (2004) suggested that methyl prednisolone sodium succinate was of use in promoting post traumatic clinical recovery when given 1h after trauma. Methyl prednisolone sodium succinate proved to be more effective than dexamethasone in reducing edema when both are given after an interval of 1h (Sharma *et al.*, 2004). Methylprednisolone also has a neuro protective effect (Amar & Levy, 1999). It improves neurologic function when given within 8 hours after injury (Bracken *et al.*, 1990). It also has various side effects. It causes severe allergic reactions (rash, hives, itching, difficulty breathing, tightness in the chest, swelling of the mouth, face, lips, or tongue, unusual hoarseness) bloody black or tarry stools, changes in body fat, chest pain, fainting, fever, chills or sore throat, increased hunger, thirst or urination, mental or mood changes (eg, depression, personality or behavioural changes), muscle pain, weakness or wasting, seizures, severe nausea or vomiting, shortness of breath, slow fast or irregular heartbeat, slow wound healing, stomach

pain, sudden severe dizziness or headache, swelling of the feet or legs, tendon bone or joint pain, thinning or discoloration of the skin, unusual bruising or bleeding, unusual skin sensation, unusual weight gain, vision changes or other eye problems and vomit that looks like coffee grounds.

Lazeroids lack gluco corticoid activity and inhibits free radical formation. They also inhibit lipid peroxidation and arachidonic acid formation (Quarles *et al.*, 1990). Endogenous opioids, acting through opiate receptors within the spinal cord, mediate certain secondary pathophysiological changes that contribute to irreversible tissue injury. Opiate receptor antagonists also reduces the secondary damage that occurs after SCI (Faden & Salzman, 1992). Hyperglycaemia increases reactive acidosis and triggers biochemical events such as increase in calcium levels and break down of cell membrane that lead to neuronal death (Sala *et al.*, 1999). Calcium plays a key role in neuronal injury. Therefore calcium channel blockers are used to treat SCI. Nimodipine is one such calcium channel blocker that increases the rat spinal cord blood flow. Free radical scavengers and anti-oxidants are also used to treat SCI (Hall, 1992). GM1 Ganglioside, a complex acidic glycolipid compound present in the neuronal membrane is also used in the treatment of SCI (Geisler *et al.*, 1991). Adenosine also has a neuro protective effect on SCI (Sulfianova *et al.*, 2002). Levetiracetam prolonged the survival and the function of spinal motor neurons and have a therapeutic potential for several diseases that kill or degenerate the spinal motor neurons, including SCI (Yasuhiro *et al.*, 2012).

Clinically existing treatments provide modest benefit; therefore present research is aimed at developing more effective therapies for spinal cord repair and regeneration (Kwon *et al.*, 2004; Baptiste & Fehlings, 2007; Ali & Bahbahani, 2010; Fehlings & Nguyen, 2010). None of the human trials has produced a major progress in neurological recovery or a meaningful increase in function (Tator, 2006; Simon *et al.*, 2009; Wang *et al.*, 2009; Jablonska *et al.*, 2010). Cell-based

strategies to remyelinate spared axons is an attractive emerging approach in the treatment of SCI.

NEUROTRANSMITTERS AND ITS RECEPTORS IN SPINAL CORD INJURY

In the adult nervous system, neurotransmitters mediate cellular communication within neuronal circuits. Neurons within the spinal cord represent a primary site for the integration of somatosensory input. Spinal sensory integration is a dynamic process regulated by factors that include multisensory convergence and pathway selection (Lundberg, 1979; Baldissera *et al.,* 1981; Jankowska, 1992). The transmission of the sensory information begins with activation of the peripheral receptors of primary afferent neurons whose cell bodies lie within the dorsal root ganglia (DRG) and whose central terminals project to secondary neurons in the dorsal horn of the spinal cord. Several neurotransmitters and a large variety of receptors have been found in the superficial laminae of the dorsal horn. Transmission of the somatosensory information from the primary afferent fibers to the secondary dorsal horn neurons depends on the balance between the excitatory effects of excitatory amino acids and the inhibitory actions of several other transmitter systems. Neurotransmitter signalling has profound influence on the normal sequence of events involved in development of the spinal cord and hence locomotion. Neurotransmitters that promote cell proliferation include ACh, 5-HT, GABA.

5-HT

5-HT is present in the axons and terminals of raphe-spinal neurons in the dorsal horn, especially in the superficial laminae, laminae I-III. The origin of serotonergic projection to the dorsal horn is mainly the nucleus raphe magnus (Dahlström & Fuxe, 1965; Fuxe, 1965; Basbaum *et al.,* 1978; Miletic *et al.,* 1984). 5-HT and several peptides may be co-localized in the same raphe neurons and in

their terminals. 5-HT may also be co-localized with GABA (Millhorn *et al.*, 1987a,b). Molecular cloning has identified seven distinct families of 5-HT receptors (5-HT1-7). The 5-HT3 family consists of ligand gated ion channel receptors. The other 6 families interact with G-proteins and are coupled to second messengers. Three 5-HT receptor subtypes influence the dorsal horn somatosensory processing: 5-HT_1, 5-HT_2 and 5-HT_3. There are three major sources of 5-HT receptors to the spinal cord dorsal horn: the DRG cells, the intrinsic spinal neurons and the descending systems. Neonatal capsaicin treatment or dorsal rhizotomy decrease 5-HT_{1A} and 5-HT_3 receptor binding in laminae I and II, but some still remains, indicating both pre and post synaptic localizations. A large majority of the 5-HT receptors in the dorsal horn do not participate in classic synapses, but are found in extra synaptic sites along the dendrites and somas.

The 5-HT systems are widespread throughout the brain, with most of the cell bodies of serotonergic neurons located in the raphe nuclei of the midline brain stem (Palacios *et al.*, 1990). The largest collections of 5-HT neurons are in the dorsal and median raphe nuclei of the caudal midbrain (Jacobs & Azmitia, 1992). The neurons of these nuclei project widely over the thalamus, hypothalamus, basal ganglia, basal forebrain and the entire neocortex. Interestingly, these 5-HT neurons also provide a dense subependymal plexus throughout the lateral and third ventricles. Activation of this innervations result in 5-HT release into the cerebrospinal fluid (CSF) and measurement of 5-HT content in CSF in disease states will largely reflect this pool (Chan-Palay, 1976).

The activation of 5-HT receptors can produce multiple physiological events, as 5-HT receptor families can either promote or inhibit different second messenger systems. Intrathecally administered 5-HT can either inhibit or stimulate (Hylden & Wilcox, 1983; Clatworthy *et al.*, 1988) nociceptive reflexes. Iontophoretic application in the vicinity of dorsal horn neurons generally causes inhibition (Griersmith & Duggan, 1980), although excitatory effects have also

been reported (Todd & Millar, 1983). It has been suggested that the 5-HT_{1B} and 5-HT_{1D} receptor subtypes mediate selective inhibition of nociceptive neurons, whereas 5-HT_{1A} agonists facilitate nociceptive responses (El-Yassir *et al.,* 1988; Alhaider & Wilcox, 1993). Spinal 5-HT_3 mediated analgesia involves GABA receptors, probably through the excitation of GABAergic interneurons (Alhaider & Wilcox, 1991).

A variety of neuro modulators play various roles in the activation of different neuron types, aiding in locomotion. 5-HT is known to play a facilitory role in locomotor circuitry by increasing motoneuron excitability, modulating spinal CPG (Salles *et al.,* 1979; Sillar *et al.,* 1997; Rossignol *et al.,* 1998) and improving locomotor behaviour following spinal injury (Kim *et al.,* 1999; Ribotta *et al.,* 2000). A small amount of 5-HT can activate super-sensitive motor neurons (Li *et al.,* 2007). The 5-HT system surrounds the corticospinal tract in the lateral funiculus, which explains the correlation between losses of 5-HT and motor deficit after SCI (Shapiro, 1997). Muscle paralysis after SCI is partly caused by a loss of brain stem derived 5-HT, which normally maintains motoneuron excitability by regulating crucial persistent calcium currents (Murray *et al.,* 2010).

5-HT AS CO-MITOGEN

In rats, 5-HT neurons in the brain stem raphe are among the first neurons to differentiate in the brain and play a key role in regulating neurogenesis (Kligman & Marshak, 1985). Lauder and Krebs (1978) reported that para chloro phenyl alanine, a 5-HT synthesis inhibitor, retarded neuronal maturation, while mild stress, a releaser of hormones, accelerated neuronal differentiation. These workers defined differentiation as the cessation of cell division measured by incorporation of [^{3}H] thymidine. Since then, many other workers have shown a role for 5-HT in neuronal differentiation (Marois & Croll, 1992; Hernandez, 1994). The effects of 5-HT on morphology have long been known. For more than

50 years, 5-HT has been known to constrict blood vessels (Page, 1968) and induce shape changes in skeletal muscle (at both the light and electron microscope level) (O'Steen, 1967), platelets (Leven *et al.,* 1983), endothelial cells (Welles *et al.,* 1985), and fibroblast (Boswell *et al.,* 1992). In the periphery, 5-HT originates largely from mast cells, which can produce, release and reuptake 5-HT. The released 5-HT, then act as a chemotactic, increase vascular permeability, vasodilatation and smooth muscle spasm (Metcalfe *et al.,* 1981). In addition to its role in morphological changes, 5-HT also has been shown to play a role in cell proliferation. In cultured rat pulmonary artery smooth muscle cells, 5-HT induces DNA synthesis and potentiates the mitogenic effect of platelet-derived growth factor-BB (Eddahibi *et al.,* 1999). 5-HT effects on cell proliferation are involved with phosphorylation of GTPase activating protein an intermediate signal in 5-HT induced mitogenesis of smooth muscle cells (Lee *et al.,* 1997). Earlier studies from our laboratory showed that 5HT acting through specific receptor subtypes $5HT_2$ (Sudha & Paulose, 1998) control cell proliferation and act as co-mitogens. Previous work also suggests that 5-HT can also trigger the cell division in the dopaminergic neurons in substantia nigra (Nandhu, 2011). Thus, there is evidence that 5-HT is involved in a variety of cellular processes involved in regulating metabolism, proliferation and morphology.

GABA

GABA have been localized in the spinal cord dorsal horn (Price *et al.,* 1984; Towers *et al.,* 2000). GABAergic neurons are found throughout the gray matter of the spinal cord, with a higher frequency in the superficial laminae (laminae I-III) (Magoul *et al.,* 1987; Todd & Sullivan, 1990; Powell & Todd, 1992; Spike & Todd, 1992; Todd *et al.,* 1992). It is an inhibitory neurotransmitter in the dorsal horn as they increase Cl^- conductance through neuronal cell membranes and hence produce inhibitory postsynaptic potentials. It functions both pre and post synaptically. GABAergic neurons play an important role in spinal

cord function and dysfunction (Coull *et al.,* 2003) and SCI (Craig, 2002; Finnerup & Jensen, 2004). After SCI, a reduction occurs in the number of inhibitory synapses related to GABA.

Three GABA receptor subtypes have been identified: the $GABA_A$ receptor subtype, which mediates rapid ionotropic transmission; the $GABA_B$ receptor subtype, which mediates a variety of metabotropic responses; and the $GABA_C$ subtype, which has not yet been found in the dorsal horn. There is significantly more $GABA_B$ than $GABA_A$ receptor ligand binding in the dorsal horn. Both receptor subtypes can be found in great numbers on the primary afferent terminals. There is a heavy concentration of GABA receptors exists in lamina II and much thoughts has therefore been given to the presynaptic modulation of the fine caliber, presumably nociceptive, primary afferent input. $GABA_B$ receptors are found in abundance in laminae I, III and IV and accordingly are thought to be involved in the presynaptic control of Aδ and Aβ primary afferent fibers. Activation of presynaptic $GABA_B$ receptors decreases glutamate release from primary afferent terminals in the spinal cord (Iyadomi *et al.,* 2000). Loss of GABA leads to neuropathic pain after SCI (Meisner *et al.,* 2010; Young & Claire 2011; Lee *et al.,* 2012)

Almost all cell bodies in the DRG are positively stained with an antibody to the subunits of the $GABA_A$ receptors. Moreover, intrinsic dorsal horn neurons contribute significantly to the $GABA_A$ receptor population in the dorsal horn. Baclofen (a GABA agonist), a chlorophenyl derivative of GABA and selective ligand for $GABA_B$ receptors, depresses both monosynaptic and polysynaptic transmission in the dorsal horn possibly through a decrease in transmitter release rather than by any antagonism at postsynaptic receptors. It has been reported that $GABA_B$ sites, unlike $GABA_A$ sites, are present in high concentrations in laminae I, II, III and IV of the dorsal horn and that after the neonatal administration of capsaicin this binding is reduced by 40-50% (Price *et al.,* 1984). It has been

hypothesized that $GABA_A$ sites regulate presynaptic glutamate release (Ishikawa *et al.,* 2000). $GABA_A$ receptors induce neuronal changes from hyperpolarization to depolarization and further mediate transmembrane calcium influx (Shulga *et al.,* 2008).

The inputs to the basal ganglia portion of the motor circuit are focused principally on the putamen, whereas the caudate nucleus and the nucleus accumbens are the principal input sites of the limbic circuit depicts a simplified scheme of the 'motor circuit' (Albin *et al.,* 1989). This postulates that in the normal brain there exists a balance between direct inhibitory input (GABA, co-localised with substance P) and indirect excitatory input (aspartate/glutamate) to the medial globus pallidus, which in turn controls thalamocortical activation.

In the GABAergic neurons in the lumbar spinal cord, GABA coexists with glycine or ACh (Rosemary *et al.,* 1993). It is assumed that chronic SCI increases GABA receptor sensitivity in the spinal cord, possibly due to down regulation of the inhibitory GABAergic mechanism (Minoru *et al.,* 2008). GABA administration has been found to cause locomotor hyper activity. GABA within the CNS is involved in the control of locomotor activity. This makes GABA supplementation more important for the development of therapeutics for SCI.

GABA AS CO-MITOGEN

GABA, the most abundant and important inhibitive neurotransmitter in the CNS, plays a regulatory role in regeneration of various nerve cells (Ben-Ari *et al.,* 1989; Baher *et al.,* 1996; Chiba *et al.,* 1997; Behar *et al.,* 2000; Haydar *et al.,* 2000; Luyt *et al.,* 2007). Through embryonic development, GABA was demonstrated as acting as a chemo-attractant and being involved in the regulation of progenitor cell proliferation. For example, GABA induces migration and motility of acutely dissociated embryonic cortical neurons (Baher *et al.,* 1996; Behar *et al.,* 2000). In addition, the neurotransmitters GABA and glutamate

reportedly reduce the number of proliferating cells in dissociated or organotypic cultures of neocortex (LoTurco *et al.,* 1995). In contrast, GABA was shown to promote cell proliferation in cultures of cerebellar progenitors (Fiszman *et al.,* 1999). GABA also dramatically increases proliferation in the ventricular zone of the embryonic cerebrum in organotypic cultures by shortening the cell cycle. However, a reverse effect was observed in the subventricular zone (Haydar *et al.,* 2000). Thus, during embryonic neurogenesis, GABA emerges as an important signal for cell proliferation and migration, but its precise regulation is depend on the region and cell type affected. GABA affects the proliferation of embryonic stem cells (Michael *et al.,* 2008).

Cellular response to GABA is mediated through its known receptors and the intracellular signals associated with them. The contribution of $GABA_A$ to both chemo-attraction (Behar *et al.,* 2000) and cell proliferation (Haydar *et al.,* 2000) was indicated. However, in some aspects of cell motility there is an apparent involvement of GABA dependent G protein indicating a role of $GABA_B$ (Behar *et al.,* 2000). GABA acts as a trophic factor not solely during prenatal neurogenesis but also in the postnatal period in injured tissue. The effect of GABA involves stimulation of cell proliferation and Nerve growth factor (NGF) secretion (Ben-Yaakov & Golan, 2003).

GABA increases synaptic plasticity. Soltani *et al.,* (2011) reported that GABA promotes proliferation of β-cells in pancreas. GABAergic inputs to hippocampal progenitor cells promote neuronal differentiation (Tozuka *et al.,* 2005). There has been evidence that baclofen, GABA agonist increase 5-HT secretion in lumbar spinal tract and rat striatum (Waldmeier & Fehr, 1978). Also, baclofen is seen to act as a potent co-mitogen, triggering DNA synthesis in primary cultures of rat hepatocytes, mediated through the G(i) protein-coupled $GABA_B$ receptors (Biju *et al.,* 2002).

ACETYLCHOLINE

ACh, one of the monoamines, is found in the motor nuclei of the cranial nerves and in the motor neurons of the spinal cord. In these areas it serves as the chemical messenger for neuromuscular transmission. ACh is also present in certain pathways within the CNS. Cholinergic neurons project in a widespread ascending system from the medial septal nuclei to the hippocampus and from the nucleus basalis of Meynert to the cerebral cortex. The basal ganglia are rich in this monoamine and enzymes responsible for its synthesis and break down (ChAT and AChE, respectively). Large cholinergic neurons have been found in the human striatum. ACh mediates its facilitatory actions predominantly post synaptically. The presumed postsynaptic action of ACh is consistent with the dominant postsynaptic localization of cholinergic receptors in the spinal cord dorsal horn (Gillberg & Askmark, 1991). Intrinsic cholinergic innervation has been demonstrated in the spinal cord. The most prominent cholinergic system consists of cholinergic cells associated with cranial nerve nuclei and motoneurons of the spinal cord (Ribeiro-da-Silva & Cuello, 1990; Wetts & Vaughn, 1994). ACh exerts its actions through mainly two kinds of receptors: muscarinic and nicotinic receptors.

Preganglionic neurons that originate in the brain stem and sacral spinal cord communicate with postganglionic neurons by extending very long axons that release the neurotransmitter, ACh. The postganglionic neurons have very short axons that release ACh onto the targeted organ to modulate the intrinsic activity of the eye, lacrimal gland, salivary gland, heart, bronchi and lungs, small intestine, stomach, gallbladder, liver, pancreas, large intestine, rectum, genitalia, blood vessels, bladder, legs and hands. Each of these targeted organs expresses ACh receptors to respond to the parasympathetic nervous system.

At the cellular level, cholinergic neurotransmission from motoneurons and interneurons is involved in modulating neuronal excitability in the spinal cord of many vertebrates. Other cholinergic neurons in the mammalian spinal cord include central canal cells (lamina X) and the partition cells, the latter of which extend from the central canal to the lateral edge of the gray matter and have been proposed to participate in locomotor activity on the basis of c-fos staining and electrophysiological recordings (Barber *et al.,* 1984; Borges & Iversen, 1986; Sherriff & Henderson, 1994; Carr *et al.,* 1995; Huang *et al.,* 2000). Recently, evidence has come forth that medial partition cells give rise to the large cholinergic C terminals on motoneurons (Miles *et al.,* 2007). Activation of muscarinic cholinergic receptors on motoneurons increases excitability through reduction of the after-spike hyperpolarization (Chevallier *et al.,* 2006; Miles *et al.,* 2007). In addition to motoneurons, many other spinal neurons of the dorsal and ventral horns and lamina X are responsive to ACh (Zieglga¨nsberger & Reiter, 1974; Jiang & Dun, 1986; Urban *et al.,* 1989; Bordey *et al.,* 1996a,b) indicating that both motoneuron and interneuron excitability are modulated by ACh.

ACh was found to exert powerful modulation of locomotor network activity, cellular properties and synaptic strength in the spinal cord of lamprey (Katharina *et al.,* 2008). It has been reported that cholinergic modulation of the lamprey spinal locomotor network is likely produced by both motoneurons and cholinergic interneurons acting *via* combined postsynaptic and presynaptic actions (Katharina *et al.,* 2008, Quinlan & Buchanan, 2008). The CPG for swimming in the lamprey spinal cord has provided a model system to study the vertebrate locomotor network. During fictive swimming, sufficient ACh is present in the isolated spinal cord to provide ongoing modulation of the locomotor network through both nicotinic and muscarinic receptors (Quinlan *et al.,* 2004).

In hatchling Xenopus, cholinergic feedback from spinal motoneurons provides excitation to both motoneurons and interneurons of the locomotor

network and cholinergic excitation during locomotion in Xenopus embryos constitutes a significant portion of the depolarizing drive to these neurons (Perrins & Roberts, 1995a,b,c). In other organisms, such as turtles, salamander and neonatal rats and mice, cholinergic input contributes to excitatory drive during locomotion, inducing or promoting the induction of rhythmic activity (Cowley & Schmitt, 1994; Perrins & Roberts, 1995 a,b,c; Kiehn *et al.,* 1996; Zhao & Roberts, 1998; Guertin & Hounsgaard, 1999; Myers *et al.,* 2005; Carlin *et al.,* 2006; Chevallier *et al.,* 2006; Miles *et al.,* 2007), although cholinergic effects on network activity are diverse.

Evidence suggesting that ACh function as a synaptic transmitter in spinal cord has come from biochemical studies demonstrating its presence in the spinal cord (Barnes & Worrall, 1968) and from the evidence that stimulation of appropriate spinal structures causes the release of ACh (Kuno & Rudomin, 1966). ACh plays a role in morphogenic cell movments, cell proliferation, growth and differentiation. ACh released from growing axons regulates growth, differentiation and plasticity of developing CNS neurons. Growth promoting actions of endogenous ACh are evident from the severe growth defects observed in ChAT deficient nematode and drosophila mutant (Rand & Russel, 1984). ACh regulates neurite outgrowth (Lopton & Kater, 1989). Cholinergic neurons appear earliest in the rat spinal cord and brain at embryonic stage (Semba, 1992). ACh also modulates spinal sensory processing in the dorsal horn (Myslinski & Randic, 1977; Urban *et al.,* 1989). As it appears that there are no descending cholinergic systems in the rat (Bowker *et al.,* 1983; Willis & Coggeshall, 1991), these actions probably arise from a population of intrinsic cholinergic interneurons found in the dorsal horn (Barber *et al.,* 1984; Todd, 1991). Coronas, *et al.,* (2000) found that ACh neuritic outgrowth in the rat olfactory bulb.

Neuromodulation through cholinergic receptors is widespread in the CNS and the effects of ACh on the neural network that generates locomotor like

activity in the spinal cord are diverse. Starting with its proper development, the spinal locomotor network depends on cholinergic transmission (Hanson & Landmesser, 2003; Myers *et al.,* 2005). During drug-induced locomotor activity in neonatal rat and mouse, exposure of the isolated spinal cord to ACh or cholinergic agents alters the amplitude and frequency of locomotor activity (Myers *et al.,* 2005; Miles *et al.,* 2007) and exposure to ACh alone can induce rhythmic activity in the isolated cord (Cowley & Schmidt, 1994). ACh, carbachol (a mixed muscarinic/nicotinic agonist) and nornicotine (a nicotine metabolite with agonist properties) also had neuroprotective properties in striatal cultures (Marin *et al.,* 1994).

It is reported that functional activity of grafted spinal cord transforms into motor neurons (Demierre *et al.,* 1990; Sieradzan & Vrbová, 1991; Clowry & Vrbová, 1992) and it can be evaluated by ACh release (Rosario *et al.,* 2007). ACh levels in the ventral spinal cord are likely to reflect motoneuronal activity. Cholinergic interneurons in the lumbar spinal cord are involved in the production of fictive locomotion (Huang *et al.,* 2000). Cholinergic inputs from the mesencephalic locomotor region to reticulospinal cells play a substantial role in the initiation and the control of locomotion (Le ray *et al.,* 2003). Since ACh serve as excitatory neurotransmitter within the spinal cord, it could contribute to the functional neurologic impairment that follows injury (Faden *et al.,* 1986). Cholinergic receptor density is affected in SCI (Jay, 2002).

MUSCARINIC RECEPTORS

Dale (1914) is the first one to divide the actions of ACh into nicotinic and muscarinic. Muscarinic receptors have been demonstrated to be present in spinal cord of human, rat and cat using invitro autoradiography with a variety of tritiated ligands, including [3H]-QNB (Kayaalp & Neff, 1980; Yamamura *et al.,* 1983; Gillberg *et al.,* 1984; Scatton *et al.,* 1984; Seybold & Elde, 1984; Villiger & Faull,

1985), [3H] pirenzepine (Villigerand Faull, 1985), [3H]-ACh (Gillberg *et al.,* 1988) and [3H] N Methyl Scopalamine (Wamsley *et al.,* 1984). Muscarinic receptors have also been detected by radioligand autoradiography in the prenatal rat brain (Lichtensteiger, 1988). They are expressed two to three times higher than nicotinic acetyl choline receptor (nAChR) in the spinal cord (Gillberg *et al.,* 1988).

The muscarinic class of ACh receptors are widely distributed throughout the body and serve numerous vital functions in both the brain and autonomic nervous system (Lefkowitz *et al.,* 1990). Within the nervous system, muscarinic receptors are present on some axon endings (heteroreceptors and autoreceptors), regulating neurotransmitter release (Raiteri *et al.,* 1984; Akaike *et al.,* 1988; Vizi *et al.,* 1989; Raiteri *et al.,* 1990). These receptors are also on the soma and dendrites of many types of neurons, including cholinergic and noncholinergic neurons (Wamsley *et al.,* 1984; Raiteri *et al.,* 1990).

Muscarinic ACh receptors are known to regulate numerous fundamental physiological processes, including central sensory, vegetative and motor functions (Wess *et al.,* 1990; Levin *et al.,* 1995; Brown *et al.,* 1996; Levin *et al.,* 1997). It is reported that muscarinic cholinergic effects of ACh are important in the normal function of both the sensory and motor systems (James *et al.,* 1981). Muscarine stimulated neural activity lead to locomotion. Activation of spinal muscarinic receptors increases the intraspinal release of ACh and that inhibition of these receptors decreases ACh release (Höglund *et al.,* 2000). The stimulation of neuronal muscarinic receptors induce neurite outgrowth in chick dorsal root ganglia, neuroblastoma cells and in a rat pheochromocytoma neuronal cell line transfected with the muscarinic M1 receptor (Kathryn *et al.,* 2009).

Activation of muscarinic receptors in the periphery causes a decrease in heart rate, a relaxation of blood vessels, a constriction in the airways of the lung,

an increase in the secretions and motility of the various organs of the gastrointestinal tract, an increase in the secretions of the lacrimal and sweat glands, and a constriction in the iris sphincter and ciliary muscles of the eye. In the brain, muscarinic receptors participate in many important functions such as learning, memory and the control of posture. Muscarinic receptors indirectly stimulate GABA release. For instance, Urban *et al.,* (1989) reported an increase in excitability of spinal cord dorsal horn neurons by ACh, while Baba *et al.,* (1998) reported a muscarinic induced facilitation of GABA release in substantia gelatinosa neurons of the rat.

mRNAs encoding five genetically distinct muscarinic ACh receptors are present in the CNS (Kubo *et al.,* 1986; Bonner *et al.,* 1987). Because of their pharmacological similarities, it has not been possible to detect the individual encoded proteins; thus, their physiological functions are not well defined. These receptor subtypes had marked differences in regional and cellular localization as shown by immunocytochemistry. By convention, molecularly identified subtypes are referred to as m1 to m5, and pharmacologically identified subtypes are designated M1 to M4 (Birdsall *et al.,* 1989). The M1 to M4 subtypes generally correspond to the m1 to m4 subtypes (Waelbroeck *et al.,* 1990; Caulfield, 1993). Wamsley and colleagues (1984) showed that high and low affinity muscarinic binding sites are present in spinal cord. Villiger and Faull (1985) named these receptors M1 and M2, respectively.

Studies suggest that M2 binding sites were distributed throughout the dorsal and ventral horns, whereas M3 binding sites were localized to laminae I to III of the dorsal horn. Only background levels of M1 binding sites were detected. The finding that M2 and M3 binding sites were localized to the superficial laminae of the dorsal horn where nociceptive Ad and C fibers terminate which suggest the possibility that either or both of these muscarinic receptor subtypes modulate anti nociception. The present demonstration of M4 binding sites in

spinal cord is consistent with the possibility that M2 and/or M4 receptors are involved in the regulation of blood pressure at the spinal level (Hoglund & Baghdoyan, 1997).

In particular, muscarinic M1, M3 and M4 subtypes are abundantly and broadly detected in rat brain, including the cerebral cortex, striatum and hippocampus. Next in abundance is mRNA for the m2 receptor. The muscarinic M1 protein is present in cortex and striatum and was localized to cell bodies and neurites, consistent with its role as a major postsynaptic muscarinic receptor. The muscarinic M2 receptor protein is abundant in basal forebrain, scattered striatal neurons, mesopontine tegmentum and cranial motor nuclei; this distribution is similar to that of cholinergic neurons and suggests that muscarinic M2 is an autoreceptor. However, muscarinic M2 receptor was also present in noncholinergic cortical and subcortical structures, providing evidence that this subtype presynaptically modulate release of other neurotransmitters and/or function postsynaptically. Heart is a rich source for the muscarinic M2 receptor and exocrine glands. The abundance of muscarinic M3 mRNA was greatest in the cerebral cortex and hippocampus, but low in the caudate putamen and in caudal regions of the brain. Smooth muscles are rich in the muscarinic M3 subtype. The muscarinic M4 receptor was enriched in neostriatum, olfactory tubercle and islands of Calleja, indicating an important role in extrapyramidal function. The muscarinic M5 receptor message is least abundant in brain (Liao *et al.,* 1989; Vilaro *et al.*, 1990).

All five muscarinic receptors are homologous proteins consisting of between 460 and 590 amino acids for the human receptors (Peralta *et al.,* 1987; Bonner *et al.,* 1988). There is a very high degree of homology at the amino acid level for each receptor across species (for example, human m1 has 98.9% identity with porcine m1) (Peralta *et al.,* 1987). Analysis of the primary sequences of the muscarinic subtypes shows that these receptors are members of a superfamily of

genes including the opsins and numerous receptors which signal through G proteins (Kubo *et al.,* 1986; Bonner *et al.,* 1987). These receptors are typified by the presence of seven hydrophobic regions in their sequence which form alpha helixes that span the membrane. The transmembrane segments of the muscarinic receptor represent the regions of highest homology among the different subtypes and across other members of this large family of G-protein-linked receptors. If the sequences of the five subtypes of the muscarinic receptor are aligned to achieve maximum identity, it can be seen that differences in the lengths of the sequences arise from differences in the extracellular amino terminis, the cytoplasmic carboxy terminis and the third intracellular loop. The remaining portions of the protein—namely, the seven transmembrane segments, the three extracellular loops and the first two cytoplasmic loops are all the same length. There is 63% identity among the amino acids of seven transmembrane segments of the human M1–M5 subtypes and most of the remaining residues in these segments are conservative replacements. The greatest divergence arises from the third cytoplasmic loop. This loop varies in length from 156 (M1) to 239 (M3) residues in the five human sequences and it accounts for 34–45% of the total number of amino acids. A comparison of the sequences shows that the muscarinic M1, M3 and M5 subtypes show maximum homology with each other, whereas the muscarinic M2 and M4 subtypes constitute a separate homologous group.

MUSCARINIC RECEPTORS IN SPINAL CORD INJURY AND REGENERATION

The cholinergic system is important for regulation of neuronal activity and body movement (Franchi, 2000; Lloyd & Williams, 2000; Sun *et al.,* 2002). 80% or more of muscarinic binding sites in the spinal cord cell cultures are on neuron (Brookes & Burt 1990). The muscarinic receptors mediate increment of intracellular calcium concentrations following nerve injury which suggests that the cholinergic system is associated with nerve regeneration and repair following

injury (Dawei *et al.,* 2010). Intracellular free calcium levels are closely related to regeneration in the rat hippocampus and spinal cord cortical neurons (Sahly *et al.,* 2006; Shulga *et al.,* 2008; Kamber *et al.,* 2009). Neuronal damage can lead to a transient increase of free calcium ion levels at the injury site, thereby increasing neuronal regeneration and repair (Kamber *et al.,* 2009). Similarly, increased levels of calcium ions in hippocampal neurons are necessary for repair of injured central neurons (Shulga *et al.,* 2008). ACh induced increase of calcium ion levels has been shown following muscarinic M1 and/or M3 cholinergic receptor mediated release of intracellular calcium in a retinal regeneration study (Ohmasa & Saito, 2003). M2 and M4 receptors are expressed during retinal regeneration which suggests their role in neuronal regeneration (Cheon *et al.,* 2001).

Traumatic SCI in rats and ischemic SCI in rabbits are associated with localized decreases in muscarinic receptor binding (Faden *et al.,* 1986). The spinal cholinergic system and muscarinic receptors are important in nociception. Intrathecal administration of cholinergic muscarinic agonists or AChE inhibitors produces analgesia in both animals and humans (Iwamoto & Marion, 1993; Naguib & Yaksh, 1994; Hood *et al.,* 1997). Importantly, the muscarinic M2 subtype is the predominant muscarinic receptor in the spinal cord dorsal horn (Hoglund & Baghdoyan, 1997; Yung & Lo, 1997). Studies performed in muscarinic receptor knockout mice provide further evidence that the muscarinic M2 receptors play an essential role in cholinergic analgesia (Gomeza *et al.,* 1999).

NICOTINIC ACETYL CHOLINE RECEPTORS

nAChRs receptors are characterised through their interaction with nicotine in tobacco. The binding of nicotine can activate nAChRs, modifying the neurons in two ways: the depolarisation of the membrane through the movement of cations results in an excitation of the neuron, while the influx of calcium acts through intracellular cascades affect the regulation of certain genes and the release of neurotransmitters.

Radioactive nAChR ligands preferentially bind to the substantia gelatinosa, laminae III or IV, with the emphasis on laminae III and IV (Wamsley *et al.*, 1981a; Gillberg & Aquilonius, 1985; Gillberg & Wiksten, 1986). It is not clear whether nAChRs are restricted to any particular cell type or sensory modality. Both nicotinic and muscarinic ligands bind to DRG cells and dorsal rhizotomy significantly reduces their binding sites in the dorsal horn (Wamsley *et al.*, 1981a,b; Seybold & Elde, 1984; Seybold, 1985).

Signal transduction is relatively simple at nAChRs. The nAChRs consisting of five subunits surrounding an internal channel is its own ligand-gated ion channel (Popot *et al.*, 1976; Conti-Tronconi *et al.*, 1982). The binding of ACh to nAChRs brings about their activation. When two molecules of ACh bind a nAChR, a conformational change occurs in the receptor, resulting in the formation of an ion pore. At the neuromuscular junction, the opening of a pore produces a rapid increase in the cellular permeability of sodium and calcium ions, resulting in the depolarisation and excitation of the muscle cell, thereby producing a muscular contraction. The activation of neuronal nAChRs also causes the movement of cations through the opening of an ion channel, with the influx of calcium ions affecting the release of neurotransmitters. nAChRs on a postganglionic neuron are responsible for the initial fast depolarisation of that neuron.

nAChRs are composed of five types of subunits: alpha (α1-α10), beta (β2-β5), delta, epsilon and gamma. These receptors span the membrane, containing extracellular, transmembrane and cytoplasmic domains, the latter being the most variable. nAChRs are always pentamers, with the subunits arranged symmetrically around a central receptor channel. The receptors always contain two or more alpha subunits, which are critical in ACh binding. The ACh binding site is comprised of a dimer formed by the α subunits (principal component) plus an adjacent subunit (complementary component), where binding to both sites is required for the channel to open.

nAChRs are involved in a wide range of physiological processes and can be either neuronal or muscle type. Muscle type nAChRs are localised at neuromuscular junctions, where an electrical impulse from a neuron to a muscle cell signals contraction and is responsible for muscle tone; as such, these receptors are targets for muscle relaxants. The many types of neuronal nAChRs are located at synapses between neurons, such as in the CNS where they are involved in cognitive function, learning and memory, arousal, reward, motor control and analgesia.

nAChRs appear to have a predominant presynaptic location and facilitate the release of a number of different neurotransmitters and hormones including ACh, GABA, glutamate, dopamine, 5-HT and norepinephrine (O'neill *et al.*, 2002). Increased expression of calcium binding proteins by nicotine could protect the cells from a number of insults that cause cellular damage secondary to increases in cytoplasmic calcium overload (Prendergast *et al.*, 2001, Freir & Herron, 2003). nAChR mediate communication across synapses.

In mammals, nAChR play a crucial role in motor control (Marc *et al.*, 1991). Following peripheral nerve injury, the expression of numerous receptors involved in nociceptive processing is altered in the superficial dorsal horn of the spinalcord. Activation of nAChR promotes survival of chicken spinal motoneurons that would otherwise undergo apoptosis when deprived of trophic factors (Messi *et al.*, 1997). It is suggested that spinal ACh release is regulated by different nAChR ACh receptors. These receptors tonically regulate spinal ACh release either directly or indirectly *via* inhibitory interneurons. Peripheral nerve injury produces a variety of changes within the spinal cord both ipsilaterally and contralaterally, including changes in the expression of nAChRs (Yang *et al.*, 2004).

Abundant evidence for the presence of nAChRs in the spinal cord, particularly in the superficial laminae of the dorsal horn where nociceptive Ad and C fibres terminate (Gillberg *et al.,* 1988; Khan *et al.,* 1994b; Roberts *et al.,* 1995; ; Khan *et al.,* 1996; Khan *et al.,* 1997; Marubio *et al.,* 1999) and in other parts of the nociceptive system (Adem *et al.,* 1989; Iwamoto, 1991; Bitner *et al.,* 1998; Flores, 1998; Ryan & Loiacono, 2000). In the spinal cord, nAChRs are expressed on primary afferents (Roberts *et al.,*1995; Li *et al.,* 1998; Genzen & McGehee, 2003;Miao *et al.,*2004;; Khan *et al.,*2004), descending noradrenergic (Li *et al.,* 2000) and serotoninergic (Cordero-Erausquin & Changeux, 2001) fibers presynaptically, as well as postsynaptically on spinal inhibitory and excitatory neurons (Bradaia & Trouslard, 2002a,b; Cordero-Erausquin *et al.,* 2004; Genzen & McGehee, 2005). Previous studies suggest that the α4β2 and α7 nAChRs on primary afferent C-fibers are likely responsible for the nociceptive responses while an α3β4 or a previously undescribed nAChRs are responsible for the antinociceptive properties (Rueter *et al.,* 2000; Khan *et al.,* 2001).

nAChR have been detected by radioligand autoradiography in the prenatal rat brain (Lichtensteiger, 1988; Schlumpf, 1991). The two most prominent nAChRs expressed in the mammalian CNS are those consisting of either α4/ β2 subunits or homomeric α7 receptors (Marks *et al.,* 1986; Lukas *et al.,* 1999). The brain alpha-bungarotoxin receptor is a homomeric receptor consisting of only α7 nAChR subunits (Hogg *et al.,* 2003). The α7 receptor is unique because of its high permeability to calcium. Numerous studies have shown that α7 receptors are involved with cognition, synaptic plasticity and presynaptic neurotransmitter release and regulation of immune function (Kem, 2000).The cellular mechanisms involved with nicotine-induced neuroprotection against excitotoxins are not well established, but previous studies have pointed to up regulation of intracellular calcium binding proteins, (Prendergast *et al.,* 2001;Stevens *et al.,* 2003) changes in nitric oxide mediated signaling (Shimohama *et al.,* 1993) and other signaling

events downstream from nAChR activation (O'neill *et al.,* 2002; Dajas & Wonnacott, 2004).

Cholinergic stimulation of the α7 homo pentameric nAChR inhibits production of pro-inflammatory cytokines (Bernik *et al.,* 2002; Borovikova *et al.,* 2000; Wang *et al.,* 2003; Saeed *et al.,* 2005; Nizri *et al.,* 2006), expression of endothelial cell adhesion molecules and leukocyte recruitment during inflammation (Saeed *et al.,* 2005). The anti-inflammatory effect of α7 nAChR stimulation is partly related to the regulation of cytokine production by macrophages (Borovikova *et al.,* 2000; Bernik *et al.,* 2002; Wang *et al.,* 2003; Shytle *et al.,* 2004; Saeed *et al.,* 2005; Ulloa, 2005; RosasBallina *et al.,* 2009) and T lymphocytes (Nizri *et al.,* 2006). The α7 nAChR subunit is considered a regulator of macrophage/monocyte activation for the release of molecules (TNFα, Inter leukine (IL-1β) involved in inflammatory responses and apoptotic pathways (Wang *et al.,* 2003; Pavlov & Tracey, 2005; Ulloa, 2005; Yoshikawa *et al.,* 2006; Tracey, 2007).

NICOTINIC RECEPTORS IN SPINAL CORD INURY AND REGENERATION

nAChR agonists and antagonists have been shown to be neuroprotective in a variety of *in vivo* and *in vitro* experimental models (O'neill *et al.,* 2002; Dajas & Wonnacott, 2004). There is solid evidence for involvement of both α4/β 2 and α7 nAChRs in nicotine-mediated neuroprotection (Prendergast *et al.,* 2001; O'neill *et al.,* 2002; Dajas & Wonnacott, 2004). Nicotine has been shown to reduce apoptotic cell death in a wide variety of model systems including neurons, lung cancer cell lines and the cardiovascular system (Garrido *et al.,* 2001; Opanashuk *et al.,* 2001; Suzuki *et al.,* 2003; Weilgus *et al.,* 2004). Audesirk and Cabell (1999) reported that nicotine not only did not influence neuronal survival or neurite production but increases the branching of both axons and dendrites.

nAChR are up regulated during optic nerve regeneration (Hieber *et al.*, 1992). Spinal nerve ligation increases the numbers of cells expressing the α3 subunit and the number of fibers expressing the α5 subunit (Vincler & Eisenach, 2004). The survival of newborn neurons can be controlled by the activation of β2-containing nAChR (Naguib *et al.*, 2004). α7 nAChRs regulate proliferation of neurons in the hippocampus (Naguib *et al.*, 2004). α7 subunit is reported to have an important role in motor control (Villégie *et al.*, 2010).

CHOLINERGIC ENZYMES – ChAT & AChE

Preganglionic autonomic neuron and somatic motoneurons are cholinergic and make up a significant proportion of the total neuronal pool. ChAT, the marker enzyme for cholinergic neurons which synthesizes ACh, is abundant in the spinal dorsal horn, especially in the superficial laminae (Kása & Morris, 1972; Kimura *et al.*, 1981; Barber *et al.*, 1984; Kása, 1986; Phelps *et al.*, 1988; Houser, 1990; Ribeiro-da-Silva & Cuello, 1990; Todd, 1991). ChAT has not been found in DRG cells or in their axons (Barber *et al.*, 1984; Borges & Iversen, 1986). AChE, the enzyme which removes ACh by hydrolysis, has also been localized in the dorsal horn, with highest concentrations in laminae IIII (Kása, 1986). The anatomical organization of cholinergic systems has been extensively studied by mapping the distribution of AChE using AChE histochemistry and by immunohistochemical staining for the ChAT. ChAT and AChE are cholinergic markers.

The enhancement of spinal cord ChAT activity by K-252a and staurosporine defines a new neurotrophic activity for these small organic molecules and raises the possibility that they activate some regulatory elements in common with the ciliary neurotrophic factor and leukemia inhibitory factor family of neurotrophic proteins. It is known that neuronal depolarization can influence ChAT activity levels and therefore ACh production. Neurons treated with depolarizing agents or stimulated directly by electric current exhibit an increase in

ChAT enzyme activity (Ishida & Deguchi, 1983). Nishi and Berg (1981) reported that a high KCl concentration enhanced ChAT activity, protein synthesis, and other activities in neurons cultured from chick ciliary ganglion. These same phenomena were observed in mouse spinal cord (Ishida & Deguchi, 1983).

In addition to its role in cholinergic neurotransmission, AChE has been implicated in several non-cholinergic actions such as cell proliferation (Appleyard, 1994) neurite outgrowth (Chacón *et al.,* 2003) and haematopoiesis (Silman & Sussman, 2005). Interestingly, AChE responds to various insults including oxidative stress, an important event that has been related to the pathogenesis and progression of a variety of CNS disorders, such as stroke (Ozkul *et al.,* 2007), Alzheimer's diseases (Chauhan & Chauhan, 2006) and diabetes mellitus (Kuhad *et al.,* 2008). Acute stress is known to induce expression of the AChE gene and to increase brain AChE activity (Kaufer *et al.,* 1998). In transgenic mice, overexpression of human AChE is accompanied by progressive cognitive deterioration (Beeri *et al.,* 1995). Decreases in AChE and ChAT have been reported in axotomised cholinergic neurons (Liberman, 1971; Ducker, 1985). AChE activity in cerebral cortex is mainly due to expression of this enzyme in cholinergic neurons and their axons. Neurobehavioural deficit can be caused by increase in cerebellum and cortex AChE activity (Mohamed *et al.,* 2001). Takada (2003) reported that the AChE inhibitor, donepezil, was neuroprotective in cultured cortical neurons exposed to excitotoxins.

SIGNAL TRANSDUCTION THROUGH SECOND MESSENGERS

The term signal transduction refers to the mechanism used by the first messenger (the neurotransmitter, neuromodulator, or hormone) of the transmitting cell to convert its information into a second messenger within the receiving cell. Signal transduction will involve a receptor for the first messenger and involve both transducers and effectors. In the field of receptors, a transducer is defined as

a molecule that translates one form of "energy" (e.g., the neurotransmitter) into another form, the second messenger. Effector is a molecule that mediates a specific effect (e.g., an ion channel). Prominent second messengers in brain include cAMP, cGMP and IP_3. Altered levels of second messengers mediate the actions of neurotransmitter-receptor activation on some types of ion channels, as well as on numerous other physiological responses.

Inositol 1,4,5-trisphosphate

IP_3 is a molecule that functions to transfer a chemical signal received by the cell, such as from a hormone, neurotransmitters, growth factors and hypertrophic stimuli such as angiotensin-II, β adrenergic receptor agonists, and Endothelin-1 to various signaling networks within the cell. Two essential signaling pathways are involved in the intracellular generation of IP_3. The first signaling pathway is initiated by PLC. PLC are soluble proteins that are partly cytosolic and partly associated with membrane. When a ligand binds to a G protein-coupled receptor that is coupled to a Gq heterotrimeric G protein, the α-subunit of Gq can bind to and induce activity in the PLC isozyme PLC-β, which results in the cleavage of PIP_2 into IP_3 and Diacylglycerol (DAG) (Biaggioni *et al.*, 2011). IP_3 diffuses to the endoplasmic reticulum, where it triggers release of Ca^{2+} ions into the cytosol. Subsequently, the released Ca^{2+} and DAG activate protein kinase C. The second signaling pathway involving IP_3 generation is initiated by Phosphoinositide 3-Kinase, an enzyme that phosphorylates inositol lipids generating two signaling molecules, PIP_2 (Phosphatidylinositol 3,4-Bisphosphate) and PIP_3 (Phosphatidylinositol 3,4,5-Trisphosphate). PIP_2 and PIP_3 function as activators of protein kinases and may regulate G proteins.

There is a very close association between the activation of muscarinic receptors and the formation of IP_3 and cGMP. The two events are linked to one another and that one (cGMP response) could be dependent on the other (IP_3).

However, with rat brain tissue, there is evidence to suggest that different subtypes of the muscarinic receptor mediate these responses independently (Kendall, 1986; Tonnaer *et al.,* 1991). M1, M3, and M5 subtypes stimulate phosphoinositide hydrolysis. This results in the release of ca^{2+} ions (Power & Sah, 2000). The rise in calcium regulates a number of process including synaptic plasticity (Bardo *et al.,* 2006; Rose & Konnerth, 2000) that occur in dendritic spines.

In the nervous system, IP_3 serves as a second messenger, with the cerebellum containing the highest concentration of IP_3 receptors (Worley *et al.,* 1989). There is evidence that IP_3 receptors play an important role in the induction of plasticity in cerebellar Purkinje cells (Sarkisov & Wang, 2008). IP_3, generated from PIP_2 has a vital role in the control of cellular and physiological processes as diverse as cell division, cell proliferation, apoptosis, fertilization, development, behaviour, memory and learning.

3'-5'-cyclic guanosine monophosphate

cGMP formation mediated by ACh was first reported over two decades ago with rat heart (George *et al.,* 1970). Shortly thereafter, muscarinic responses in other tissues were reported. The dependence of the response on ca^{2+} was established early (Schultz *et al.,* 1973). All the major target organs of parasympathetic cholinergic fibers contain muscarinic receptors that mediate an increase in cGMP (Goldberg & Haddox, 1973). Muscarinic receptors in sympathetic ganglia and in brain also mediate cGMP synthesis. The role of G proteins in muscarinic receptor-mediated cGMP synthesis has not been defined. However, it is synthesis of Nitric oxide and subsequently, cGMP following receptor activation is secondary to the increase in intracellular ca^{2+}, resulting from the release of IP_3 from PIP_2 by the action of PLC.

3'-5'-cyclic adenosine monophosphate

Neurotransmitters stimulate or inhibit proliferation by activating receptors coupled to different G-proteins and second messenger pathways (Lauder, 1993). It has also been noted that muscarinic M1, M3 and M5 receptors stimulate cAMP accumulation in intact cells (Peralta *et al.,* 1988; Lai *et al.,* 1992). However, this response is downstream from the phosphoinositide response, resulting from calcium or protein kinase C activation of adenylate cyclase. The discovery that the β γ subunits of heterotrimeric G proteins activate the type II and IV adenylate cyclases (Gao *et al.,* 1991; Tang & Gilman, 1991) provides another mechanism for muscarinic enhancement of adenylate cyclase activity that could be demonstrable in a broken cell preparation. This mechanism is dependent upon simultaneous activation by the α subunit of G_S (stimulatory guanine-nucleotide-binding protein) and represent the mechanism by which muscarinic M4 receptors stimulate adenylate cyclase activity in homogenates of the olfactory tubercle (MC, Onali P, 1991).

Phospho Lipase C

Activation of PLC leads to a cascade of events. It results in the breakdown of PIP_2 into DAG and IP_3 (Fisher, 1987; Berridge, 1983). Stimulation of muscarinic receptors causes DNA synthesis. These mitogenic responses are correlated with increased activity of PLC (Ashkenazi *et al.,* 1989; Gutkind *et al.,* 1991; Mckenzie *et al.,* 1992). Activation of Gq coupled muscarinic M1, M3 and M5 mAChR receptor subtypes stimulates mitogen activated protein kinase C by PLC dependent and PLC independent mechanisms (Wotta *et al.,* 1998). Muscarinic receptor dependent activation of PLC has been reported (Larocca *et al.,* 1994). Receptor-mediated activation of PLC is by no means exclusive to muscarinic subtypes m1, m3 and m5 or to muscarinic receptors in general. The odd numbered muscarinic receptors are most efficiently coupled to this response

and give the most robust responses, compared to those of the even-numbered receptors (Peralta *et al.,* 1988; Ashkenazi *et al.,* 1989).

cAMP regulatory element binding protein

CREB is a plasticity-associated transcription factor, mediating responses to various neurotransmitters, mitogenic factors and differentiating factors (Harris, 2002). Calcium ions act as second messenger in CNS (Clapham, 1995). Extra cellular signals can increase intracellular calcium and activates signal transduction pathways. CREB is a critical mediator for calcium dependent gene expression (Sheng *et al.,* 1990). CREB becomes phosphorylated during some forms of synaptic activity (Deisseroth *et al.,* 1996) and is required for several learning processes and adaptive responses in the brain (Bourtchuladze *et al.,* 1994; Maldonado *et al.,* 1996). CREB is involved in glial cell fate determination (Bayatti & Engele., 2001; Harris, 2002). CREB promotes proliferation and survival of neurons and glia in the injured brain (Ong *et al.,* 2000) and mediates cell viability during early embryonic development (Bleckmann *et al.,* 2002). However, in smooth muscle cells, CREB activation (by Ser-133 phosphorylation) associates with suppressed expression of multiple cell cycle regulatory genes and reduced proliferation (Bleckmann *et al.,* 2002; Harris, 2002). Thus, CREB operate either as an inducer or as a suppressor of gene expression, depending on the signal pathway promoting its activation. CREB production up regulates muscarinic M1 receptor (Hu *et al.,* 2010)

CREB is involved in many functions in the nervous system, including neurogenesis and neuronal survival, development, differentiation, neuroprotection, axonal outgrowth and regeneration, synaptic plasticity (Mioduszewska *et al.*, 2003; Persengiev & Green, 2003; Dragunow, 2004; Barco & Kandel, 2006)**.** Genes whose transcription is regulated by CREB include: c-fos, BDNF, tyrosine

hydroxylase and neuropeptides such as somatostatin, enkephalin and corticotropin-releasing hormone (Lauren, 2005).

CREB is a downstream target of cAMP signaling. Multiple lines of evidence define a role for CREB in proliferation and differentiation of certain cells and tissues (Heasley *et al.,* 1991; Spaulding, 1993; Iyengar, 1996). Disruption of CREB activity, using expression of a dominant-negative CREB slows neurite outgrowth and blocks adipocyte differentiation (Engelman *et al.,* 1998; Shimomura *et al.,* 1998).

APOPTOSIS & SPINAL CORD INJURY

Apoptosis is a morphologically defined form of programmed cell death. It is a vital component of normal cellular differentiation, development and tissue homeostasis (Wyllie *et al.,* 1980; Ellis *et al.,* 1991; Raff, 1992). Apoptosis is also a key mechanism for removal of damaged, infected or mutated cells that would otherwise present a risk to the organism. Characteristic features of apoptosis include surface membrane blebbing, dilation of the endoplasmic reticulum, externalization of phosphatidylserine at the cell surface, nuclear and cytoplasmatic condensation and DNA fragmentation.

Apoptosis is seen after ischemic or traumatic injury to the CNS (Li *et al.,* 1996; Johnson *et al.,* 1997; Rink *et al.,* 1997), suggesting that active cell death as well as passive necrosis may mediate damage after CNS injury. Both secondary degeneration at the site of SCI and the chronic demyelination of tracts away from the injury appear to be due in part to apoptosis. At the original site of injury, apoptosis occurs about eight hours after the injury in glial cells. A second wave of apoptosis comes about seven days after the injury in the oligodendrocytes of the white matter and the effect is much broader, expanding far away from the original location of the injury.

SCI leads to multifaceted cellular and molecular interactions within the CNS in an effort to repair the initial tissue damage (Thuret *et al.,* 2006). The pathophysiology of SCI is marked by cell death, immune cell transmigration, shearing of cell membranes and axons, disruption of the blood-spinal cord barrier and myelin degradation (Dumont *et al.,* 2001). SCI involves an initial mechanical or primary injury which is then followed by a series of cellular and molecular secondary events resulting in secondary injury that augment the extent of the initial damage and results in the progressive devastation of spinal cord tissue. Secondary injury mediated by multiple injury processes including inflammation, free radical induced cell death and gliosis. Secondary insult immediately after injury is marked by destruction of neuronal and glial cells. It also results in permanent motor and sensory deficits (Taoka & Okajima, 1998; Hagg & Oudega, 2006). Inflammation as a result of secondary injury results in apoptosis of neurons and oligodendrocytes as well as in scar formation and finally in the reduction of neuronal function (Shen *et al.,* 2009). Damage to the spinal cord result in extensive proliferation of microglia and macrophages in and around the injury epicenter. After SCI, cells die by post-traumatic necrosis or by apoptosis (Byrnes *et al.,* 2007). Apoptosis, dependent on active protein synthesis contributes to the neuronal and glial cell death, as well as to the neurological dysfunction, induced by mild-to-moderate severity traumatic insults to the rat spinal cord (Liu *et al.,* 1997). Apoptosis of neurons and oligodendrocytes result in paralysis of patients with SCI (Mizuno, 1998; Mattson, 2000). There are several proteins involved in apoptosis.

CASPASES

Apoptosis begins with the activation of a family of proteins known as caspases. Caspases break down normal cellular substrates that are used for such functions as cytoskeleton formation and DNA repair. There are different types of caspases. The caspase-8 is a key enzyme at the top of the apoptotic cascade, both

involved in the extrinsic or death receptors pathway and in the intrinsic mitochondrial pathway. Caspase-8 also known as MACH, FLICE and Mch5 is synthesized as an inactive single polypeptide chain zymogen procaspase. At least three different mechanisms exist for caspase activation in mammalian cells: recruitment activation, in which a type 1 procaspase is sequestered into an oligomeric activating complex by way of interactions through its extensive prodomain; transactivation, in which the caspase is activated by another caspase and auto activation, in which a caspase initiates its own activating cleavage (Nicholson, 1991). Upon activation by proteolytic cleavage, effector caspases cleave their substrates and inactivate proteins essential for survival, leading to the disintegration of cells (Hengartner, 2000). Knockout data indicate that caspase-8 is required for killing induced by the death receptors Fas, TNF-R1 (TNF receptor 1) and death receptor 3 (Juo *et al.,* 1998; Varfolomeev *et al.,* 1998).

Caspase-8 can directly activate downstream effector caspases including procaspase 3, 6 and 7 (Cohen, 1997). Active caspase-8 initiates downstream cleavage of caspase-3 by direct or mitochondrial-dependent mechanisms leading to apoptosis (Kuwana *et al.,* 1998; Stennicke *et al.,* 1998). In addition, activated caspase-3 cleave procaspase-8 (Slee *et al.,* 1999; Woo *et al.,* 1999), thereby amplifying the death process. It has been reported that caspase-3, an effector caspase, was marked in a few fragmented cells at 24 h following injury. Activated caspase-8 is known to propagate the apoptotic signal either by directly cleaving and activating downstream caspases or by cleaving the BH3 Bcl2-interacting protein, which leads to the release of cytochrome C from mitochondria (Oh, 2005), triggering activation of caspase-9 in a complex with dATP and Apaf-1. Activated caspase-9 then activates further downstream caspases, including caspase-8.

In SCI, some cells die by a mechanism resembling apoptosis, as is evident by caspase activation (Hara *et al.,* 1997; Endres *et al.,* 1998; Namura *et al.,* 1998;

Springer *et al.,* 1999). Procaspase-8 cleavage was reported by Velier *et al.,* (1999) in mouse cortical gray matter neurons after permanent middle cerebral artery occlusion. Hence, ischemia triggers caspase 8 cleavage in spinal cord as well as within brain. As early as 1.5 hr after transient ischemia, activated caspase-8 mRNA appeared within neurons in intermediate gray matter and in medial ventral horn (Matsushita *et al.,* 2000). Oxidative stress can lead to the activation of caspase 8 (Baumgartner *et al.,* 2007).

Bax

Apoptosis can be controlled by the degradation rate of proapoptotic proteins such as Bax (Li and Dou, 2000). Bax is otherwise known as Bcl-2 associated protein X. Bax belongs to Bcl-2 family of proteins. It was the first identified pro-apoptotic member of the Bcl-2 protein family (Oltvai *et al.,* 1993). Bax is found in the cytosol, but upon initiation of apoptotic signaling, it undergoes a conformation shift and inserts into organelle membranes, primarily the outer mitochondrial membrane (Wolter *et al.,* 1997). Thus, it is a major player in the mitochondrion form of apoptosis (Kroemer and Reed, 2000). Mitochondria mediated cell death involve down-modulation of Bax antagonists such as Bcl-X_L or Bcl-2 (Vander & Thompson, 1999) or the translocation of Bax from the cytosol to mitochondria (Khaled *et al.,*1999). Mitochondria dysfunction promoted by Bax translation leads to the leakage of cytochrome C from mitochondria (Costantini *et al.,* 2000). Bax promote apoptosis by the release of downstream apoptogenic factors (Wei, 2001). Bax is activated by tumor suppressor protein p53.

Knockout studies in mice have shown that the presence of Bax is necessary for the execution of the apoptotic program (Cheng *et al.,* 2001; Wei *et al.,* 2001; Zong *et al.,* 2001). Bax has been reported to be up regulated following ischemia induced retinal injury in rat (Kaneda, 1999). It is also elevated in intraocular pressure in murine glaucoma model (Ji, 2005). An increase in Bax

oligomerization leads to mitochondria mediated caspase activation (D Lee *et al.*, 2008). Bax can be an important mediator of anticancer drug-induced cell death. Neurons that are lacking in Bax are protected against apoptosis (White *et al.*, 1998). The decrease in Bax protein can reduce apoptosis in hemisection induced SCI (Han *et al.*, 2012; Wang *et al.*, 2012).

Tumour Necrosis Factor α

TNFα, a proinflammatory cytokine which is best known for its role in immune and vascular responses, can induce apoptosis in nonimmune tissues *via* the death domain of its cell surface receptor, TNF-R1. TNFα is activated during extrinsic pathway of apoptosis. It is found either as a 27-kDa membrane-bound precursor or a 17-kDa mature soluble form produced by the protease action of TNFα converting enzyme and MMP-9 (Kherif *et al.*, 1999; Mullberg *et al.*, 2000). 17-kDa TNFα form, which correlates with higher proteolytic cleavage is increased during active myonecrosis (Leite *et al.*, 2010). One of the most important biological triggers of oligodendrocyte apoptosis in SCI is TNFα. In particular, it has been shown that TNFα induces apoptosis in oligodendrocytes both *in vitro* and *in vivo* (Muzio *et al.*, 1997) by the activation of caspase-3 and caspase-8 (Hisahara *et al.*, 1997).

There is a large amount of evidence that TNFα and IL-1β also play an important role in the induction of iNOS (inducible Nitric Oxide Synthase), which is known to play an important role in the development of SCI (Matsuyama *et al.*, 1998). The inflammatory cytokine mRNAs were shown to be induced as early as 15 min following contusion of rat spinal cord, with increased TNFα (Pan *et al.*, 2002; Lammertse *et al.*, 2004). Previous studies suggests that 30-45 min post SCI, TNFα positive cells are seen over the injured spinal cord segment and from 3 to 24 h, TNFα was strongly up regulated around the contused area (Habgood *et al.*, 2007). TNFα could potentiate glutamate mediated neuronal cell death in the rat

spinal cord (Bracken *et al.,* 1997; Hermann *et al.,* 2001), while TNFα antagonist reduced the development of inflammation and tissue injury events associated with SCI (Xu *et al.,* 1998; Genovese *et al.,* 2006). Etanercept (TNF α antagonist) reduces the associated tissue damage of SCI, improves hindlimb locomotor function, and facilitates myelin regeneration. This positive effect of etanercept on SCI is attributable to the suppression of TNFα and caspase-8, there by inhibiting neuronal and oligodendroglial apoptosis. (Chen *et al.,* 2007).However, there are conflicting reports as to the role of cell death in SCI that probably reflect the known capacity of TNF to be both pro and anti apoptotic (Inukai *et al.,* 2009; Cantarella *et al.,* 2010; Genovese *et al.,* 2010).

Nuclear factor kappa-light-chain-enhancer of activated B cells

NFκB is a transcription factor that plays important roles in the immune system (Ghosh *et al.,* 1998; Li & Verma, 2002; Bonizzi & Karin, 2004). Known inducers of NF-κB activity include reactive oxygen species (ROS), TNFα, IL-1β, bacterial lipopolysaccharides, isoproterenol, cocaine and ionizing radiation. ROS enhance the signal transduction pathways for NF-κB activation in the cytoplasm and translocation into the nucleus. NF-κB regulates the expression of cytokines, cyclooxgenase 2, growth factors, inhibitors of apoptosis and effector enzymes in response to ligation of many receptors involved in immunity including T-cell receptors, B-cell receptors and members of the Toll-like receptor/IL-1 receptor super family.

In mammals, the NF-κB family is composed of five related transcription factors: p50, p52, RelA (p65), c-Rel and RelB (Moynagh, 2005; Hoffmann *et al.,* 2006). The Rel/ NF-κB family of transcription factors are involved mainly in stress-induced, immune and inflammatory responses. In its inactive form, NF-κB is sequestered in the cytoplasm, bound by members of the IkB family of inhibitor proteins, which include IkBa, IkBb, IkBg, and IkBe. Thus, it exists in the cytosol

as a pre-formed trimeric complex. The various stimuli that activate NF-κB cause phosphorylation of IkB, which is followed by its ubiquitination and subsequent degradation. NF-κB is trapped in the cytoplasm in stimulated cells and translocates into the nucleus in response to several stimuli, including oxidative stress. There are two signaling pathways leading to the activation of NF-κB known as the canonical pathway (or classical) and the non-canonical pathway (or alternative pathway) (Karin, 1999; Gilmore, 2006; Scheidereit, 2006; Tergaonkar, 2006).

Functional NF-κB complexes are present in essentially all cell types in the nervous system, including neurons, astrocytes, microglia and oligodendrocytes (O'Neill & Kaltschmidt, 1997). Neurons and their neighboring cells employ the NF-κB pathway for distinctive functions as well, ranging from development to the coordination of cellular responses to injury of the nervous system and to brain specific processes such as the synaptic signaling that underlies learning and memory. NF-κB also plays a role in the development and the activity of a number of tissues including the CNS (Memet, 2006). NF-κB is also an important regulator in cell fate decisions, such as programmed cell death and proliferation control and is critical in tumorigenesis (Baldwin *et al.,* 1996). The most potent NF-κB activators are the proinflammatory cytokines IL-1 and TNFα. NF-κB is a ubiquitous transcription factor. It is activated during pathological conditions (Kaĭdashev, 2012).

Microglial cells can produce neurotoxic ROS and excitotoxins when activated. Cytokine mediated activation of microglia explains the ability of inhibitors of NF-κB to protect against cell damage in certain experimental paradigms that involve an inflammatory response (Qin *et al.,* 1998). Microglial activation is associated with a marked increase in expression of cyclooxygenase-2 (Cox), an oxyradical-generating enzyme and agents that inhibit NF-κB can

suppress lipo polysaccharide induced Cox-2 expression, suggesting an important role for NF-κB in microglial activation and oxyradical production.

Activation of NF-κB in astrocytes results in increased expression of NOS and increased nitric oxide production. A potent inducer of NF-κB activation in astrocytes is bradykinin, an inflammatory mediator produced in the brain in response to ischemia and trauma (Schwaninger *et al.,* 1999). Acting through an NF-κB mediated pathway, bradykinin induces production in astrocytes of IL-6, which stimulates production of several inflammation related cytokines. Immunohistochemical studies suggest that levels of NF-κB activity are increased in cholinergic neurons in the basal forebrains of Alzeihmer's Disease patients (Boissiere *et al.,* 1997).

Implicated in multiple biochemical pathways affected after SCI is the transcription factor NF-κB. In traumatic SCI in the rat, both NF-κB and the iNOS are activated in microglia and neurons within and surrounding the injury site (Bethea *et al.,* 1998). NF-κB signaling pathway operating in astrocytes is a major contributor to the pathological events occurring after SCI. Spinal cords of patients with Amyotrophic Lateral Sclerosis show increased NF-κB activation in astrocytes associated with degenerating motor neurons (Migheli *et al.,* 1997). SCI initiates a very robust inflammatory response, both within the spinal cord and systemically. The anti apoptotic role of NF-κB in developing neurons is seen in the mechanism whereby the protein synthesis inhibitor cycloheximide prevents neuronal apoptosis. Levels of cycloheximide that cause only a small impairment of protein synthesis can prevent apoptosis by inducing Bcl-2 and the antioxidant enzyme Mn-SOD (Manganese Superoxide Dismutase) (Furukawa *et al.,* 1997).

OXIDATIVE STRESS AND SPINAL CORD INJURY

Oxidative stress is considered as a hallmark of SCI (Jia *et al.,* 2012). The oxidative stress by induction of ROS initiates a cascade of oxidative events that

lead to cell death due to a combination of necrosis and apoptosis (Crowe *et al.*, 1997). Local and systemic inflammatory response, as well as neurodegenerative disease, is also associated with the production of ROS such as superoxide anion (O_2^-), Hydrogen peroxide (H_2O_2) and peroxynitrite (Cuzzocrea *et al.,* 2001). Oxygen free radical formation and lipid peroxidation enhance adverse mechanism of neuronal injury, such as spinal cord hypoperfusion, development of oedema, axonal conduction failure and breakdown of energy metabolism. Dusart & Schwab (1993) demonstrated that neutrophils and macrophages enter the spinal cord after SCI in an orchestrated temporal sequence. Neutrophils are able to release reactive oxygen and nitrosyl radicals as well as cytokines, chemokines and a variety of enzymes. Several studies have implicated the formation of ROS and reactive species of nitrogen in the secondary neuronal damage of SCI (Xu *et al.,* 2001). The importance of free radicals and peroxidation in SCI is supported by the large number of experimental and clinical studies demonstrating potential neuronal efficacy of agents with anti-oxidant proprieties (La Rosa *et al.,* 2004; Genovese *et al.,* 2005b; 2006a,b; Scott *et al.,* 2005). SCI initiates a sequence of events that lead to secondary neuronal cell damage. While the precise mechanisms responsible to damage in SCI remain undefined, several studies have implicated ROS in the secondary neuronal damage of SCI (Liu *et al.,* 1997; Xu *et al.,* 2001). ROS and peroxynitrite also cause DNA damage (Salgo *et al.,* 1995; Szabo *et al.,* 1997; Szabo *et al.,* 1998). Suppression of ROS renders a protective effect for injured spinal cord (Suzuki *et al.,* 2005). Thus, alleviating oxidative stress is an effective way of therapeutic intervention of SCI (Jia *et al.,* 2012). The reactive oxygen intermediates produced in mitochondria, peroxisomes and the cytosol are scavenged by cellular defending systems including enzymatic SOD, GPx, glutathione reductase, catalase and nonenzymatic antioxidants (ex. glutathione, thioredoxin, lipoic acid, ubiquinol, albumin, uric acid, flavonoids, vitamins A, C and E). Antioxidants are located in cell membranes, cytosol and in the blood plasma (Maritim *et al.,* 2003).

SUPER OXIDE DISMUTASE

SOD is an oxygen radical scavenger, which converts the superoxide anion radical present in the upper stream of reactive oxygen metabolism cascade and afford protection from cell damage. It is found in almost all organisms living in the presence of oxygen, including some anaerobic bacteria, supporting the notion that superoxide is a key and general component of oxidative stress. In aerobic cells, free radicals are constantly produced mostly as ROS. SOD catalyses the dismutation of the $O2^-$ into H_2O_2 (Michiels *et al.,* 1994). Imbalance between pro oxidant and anti- oxidant defenses in favour of pro oxidants results in oxidative stress. This results in damage to lipids, proteins and nucleic acids. Alone or in combination with primary factors, free radicals are involved in the cause of hundreds of diseases. There are two types of SOD: copper/zinc (Cu/Zn) SOD and manganese (Mn) SOD. Each type of SOD plays a different role in keeping cells healthy. Cu/Zn SOD protects the cell's cytoplasm and Mn SOD protects their mitochondria from free radical damage. Highly reactive oxygen-containing species form upon CNS injury and cause oxidative damage to important cellular components, thereby destroying cells. Removal of superoxide may be a realistic treatment strategy for reducing injury caused by free radicals (Liu *et al.,* 1998).

Free radical mediated mechanisms of cellular damage have been implicated in the early stages of SCI. Superoxide radicals contribute to the pathogenesis of SCI (Taoka *et al.,* 1995). Mn SOD is a potent scavenger of superoxide radicals and likely serves an important cytoprotective role in preventing cellular damage after SCI. SOD has been reported to promote functional recovery in ischemic SCI. SOD treatment, targeted to the early reperfusion period, reduced both motor dysfunction and incidence of spinal infarcts at 7 days after ischemia (Cuevas *et al.,* 1990).

GLUTATHIONE PEROXIDASE

GPx is a selenoprotein enzyme found in cytoplasmic and mitochondrial fractions of cells. The antioxidant enzyme catalyzes the reduction of H_2O_2 and hydroperoxides formed from fatty acids, thereby effectively removing toxic peroxides from living cells (Michiels *et al.,* 1994). It plays the important role of protecting cells from potential damage by free radicals, formed by peroxide decomposition (Mannervik *et al.,* 1985; Ursini *et al.,* 1985). The activity of GPx is coupled to glutathione reductase, which maintains reduced glutathione levels (Bompart *et al.,* 1990). Enzyme activity can be decreased by negative feedback from excess substrate or from damage by oxidative modification (Tabatabaie *et al.,* 1994). Decreased GPx activity has been reported in tissues where oxidative stress occurs. Therefore GPx activity is necessary for reducing the oxidative stress.

Tissue damage is induced by ROS in the primary and secondary process of SCI (Ikeda & Long 1990). GPx is involved in scavenging of free radicals. Absence of Gpx leads to neuronal apoptosis (Crack *et al.,* 2003). Neuronal apoptosis is attributed partly to diminished activation of Akt (Taylor *et al.,* 2006). Thus GPx indirectly affects neuronal cell survival. GPx protects cortical cells from oxidative injury (Ran *et al.,* 2006). Stimulation of GSH dependent antioxidative processes lead to reduced oxidative damage and greater locomotor function during the sub acute and chronic phases of injury (Richard *et al.,* 2006). Induction of oxidative stress results in decreased GPx, so decreasing oxidative stress by anti-oxidant agents play a key role in attenuating SCI (Ayromlou *et al.,* 2011).

NEURONAL SURVIVAL FACTORS IN SPINAL CORD INJURY

Neurogenesis is the life-long natural production and integration of new nerve cells in the brain. Neurons and glia are in close contact in the mature nervous systems of all animals. It has been suggested that all cells are

programmed to die unless they receive trophic support and that cell survival largely depends upon interactions between cells (Raff *et al.*, 1993). Both neurons and glia are overproduced in the normal nervous system and there is evidence indicating that one consequence of axon-glia contact is survival regulation in both cell types in the mature nervous system (Raff *et al.*, 1993). This ensures that axons are correctly myelinated, enabling normal neuronal function.

Neurotrophic factors (NTF) play an important role in the maintenance of structural integrity of the mature brain as well as the development of the CNS. Previous cell culture experiments have shown that several groups of growth factors exert trophic actions on CNS neurons by promoting survival and their morphological and biochemical differentiation. Among them are included the IGFs (Knusel *et al.,* 1990), GDNF (Lin *et al.,* 1993) and the neurotrophin family, BDNF. Neurotrophins regulate development, maintenance, and function of vertebrate nervous systems (Eide *et al.,* 1993; Korsching, 1993; Lewin & Barde, 1996; Segal & Greenberg, 1996; Reichardt & Fariñas, 1997; McAllister *et al.*, 1999; Sofroniew *et al.,* 2001). All neurotrophins have six conserved cysteine residues and share a 55% sequence identity at the amino acid level. Neurotrophins activate two different classes of receptors, the Trk family of receptor tyrosine kinases and p75NTR, a member of the TNF receptor superfamily. Through these, neurotrophins activate many signaling pathways, including those mediated by ras and members of the cdc-42/ras/rho G protein families, and the MAP kinase, PI-3 kinase, and Jun kinase cascades. During development, neurotrophins function as survival factors to ensure a match between the number of surviving neurons and the requirement for appropriate target innervation. They also regulate cell fate decisions, axon growth, dendrite pruning, the patterning of innervation and the expression of proteins crucial for normal neuronal function, such as neurotransmitters and ion channels. These proteins also regulate many aspects of neural function. In the mature nervous system, they control synaptic function and

synaptic plasticity, while continuing to modulate neuronal survival. Growth factors such as Nerve Growth Factor (NGF), BDNF, neurotrophin-3, ciliary neurotrophic factor, and GDNF all have been used to study their beneficial effects in spinal cord–injured animals (Lu & Tuszynski, 2008). IGF is activated by Akt and promotes neuronal survival. Cyclin D1 is a marker of proliferation and is in turn activated by IGF.

BRAIN DERIVED NEUROTROPHIC FACTOR

BDNF is a member of the neurotrophin family of growth factors that includes NGF, NT-3, and NT-4. It is a 13.6 kDa (or 27.2 kDa dimer) member of the neurotrophin family. The active form of recombinant human BDNF (27 kDa) is a dimer formed by two identical 119 amino acid subunits held together by strong hydrophobic interactions plays a key role in neuronal and axonal survival. BDNF has identical amino acid sequence in human, mouse and pig with full cross-reactivities. BDNF is regulated by another neurotrophin, NGF.

BDNF, a survival-promoting molecule, plays an important role in the growth, development, maintenance and function of several neuronal systems. Several populations of sensory neurons have been shown to synthesize BDNF (Brady *et al.,* 1999; Mannion *et al.,* 1999). Although some evidence has been presented suggesting that BDNF act in an autocrine or paracrine fashion to support DRG sensory neurons (Acheson *et al.,* 1995; Robinson *et al.,* 1996), in other instances it may be transported anterogradely and act trans-synaptically on targets of the central afferents of these neurons within the brain (von Bartheld *et al.,* 1996; Altar *et al.,* 1997; Fawcett *et al.,* 1998; Brady *et al.,* 1999).

BDNF is important in mature animals for regulating the mechanosensitivity of slowly adapting mechanoreceptors, myelinated fibers required for fine tactile discrimination (Carroll *et al.,* 1998). It is constitutively expressed by adult sensory neurons (Ernfors *et al.,* 1990; Apfel *et al.,* 1996; Cho

et al., 1997; Michael *et al.,* 1997). BDNF has been shown to enhance the survival and differentiation of several classes of neurons *in vitro*, including neural crest and placode derived sensory neurons, dopaminergic neurons in the substantia nigra, basal forebrain cholinergic neurons, hippocampal neurons, and retinal ganglial cells. It promotes the growth of GABAergic neurons of the striatum and ganglion cells of the retina and neural crest. It also protects motor neurons, cortical neurons and the hippocampus from various forms of toxic damage. It has a functional role in autoimmune demyelination by mediating axonal protection (Ralf *et al.,* 2010). It is involved in normal maturation of neuronal pathways and regulates neuronal plasticity. The versatility of BDNF is emphasized by its contribution to a range of adaptive neuronal responses including long-term potentiation, long-term depression, certain forms of short-term synaptic plasticity, as well as homeostatic regulation of intrinsic neuronal excitability (Egan *et al.,* 2003). Changes in the levels and activities of BDNF have been described in a number of neurodegenerative disorders, including Huntington's disease, Alzheimer's disease and Parkinson's disease.

In the lumbar DRG of the rat, the distribution of BDNF protein is not uniform but is localized to a restricted number of primary sensory neurons. The type of neurons that synthesize BDNF has been elucidated (Michael *et al.,* 1997). It has emerged that BDNF play an important neuro modulatory role in the dorsal horn of the spinal cord. It is markedly up regulated in inflammatory conditions in a NGF dependent fashion. Postsynaptic cells in this region express receptors for BDNF. The increase in BDNF mRNA reduced motor functional deficits in spinal cord transaction rats (Gao *et al.,* 2012; Jin *et al.,* 2012). BDNF results in enhanced connectivity of the peripheral motor bridge in a rodent model of SCI (Martin *et al.,* 2012). BDNF has a neuroprotective effect in SCI (Uchida *et al.,* 2012).

GLIAL DERIVED NEUROTROPHIC FACTOR

GDNF, a member of the TGFß superfamily (Lin *et al.,* 1993), is a NTF that promotes the survival of various neuronal populations in both the central and peripheral nervous systems during their development. It is the most potent motor NTF among NTFs found so far. GDNF binds to glial cell-derived neurotrophic factor receptor (GFR) α-1, a membrane-bound protein belonging to the GFRα family (Jing *et al.,* 1996; Treanor *et al.,* 1996). Cells known to express GDNF include sertoli cells, type1 astrocytes, schwann cells, neurons, pinealocytes and skeletal muscle cells. It maintains dopaminergic, noradrenergic and motor neurons of the CNS (Lin *et al.,* 1993; Henderson *et al.,* 1994; Arenas *et al.,* 1995; Oppenheim *et al.,* 1995; Tomac *et al.,* 1995; Yan *et al.,* 1995) as well as various sub populations of the peripheral sensory and sympathetic neurons (Henderson *et al.,* 1994; Buj-Bello *et al.,* 1995; Ebendal *et al.,* 1995; Trupp *et al.,* 1995).

The most prominent feature of GDNF is its ability to support the survival of dopaminergic and motorneurons. In addition, exogenously applied GDNF has been shown to rescue damaged facial motor neurons *in vivo*. GDNF play an important role in corneal regeneration and wound healing (You *et al.,* 2001). GDNF mRNA expression in schwann cells in sciatic nerves and in DRGs rises dramatically, a finding that implicates GDNF in peripheral nerve regeneration (Trupp *et al.,* 1995; Hammarberg *et al.,* 1996; Hoke *et al.,* 2000, 2002). GDNF mRNA expression is also increased in an experimental model of motor neuropathy in rats (Saita *et al.,* 1997), in various human neuropathies (Yamamoto *et al.,* 1997) and in traumatized human nerves (Ba¨r *et al.,* 1998). Members of the GDNF family of ligands do play essential early roles in development of one sympathetic ganglion, the superior cervical ganglion. There is a hope that GDNF might become useful in the treatment of neurodegenerative diseases and nerve injuries.

GDNF is one of the most powerful survival factors for spinal motor neurons (Chu *et al.,* 2012). The primary sensory neurons that respond to noxious stimulation and project to the spinal cord are known to fall into two distinct groups: one sensitive to NGF and the other sensitive to GDNF (Snider & McMahon, 1998). GDNF has been shown to protect cranial and spinal motoneurons. GDNF promote spinal repair and functional recovery (Li *et al.,* 2004). There is evidence that exogenous GDNF could reduce the hyperplasia of gIial cells and enhance the regeneration of injured corticospinal tract and restoring of the cytoskeleton in the injured neurons (Lu *et al.,* 2002). GDNF induced the growth of motor and sensory axons and remyelination in laboratory rats with partial and complete spinal cord transections (Blesch & Tuszynski, 2001; Zhou *et al.,* 2003).

Insulin like Growth Factor-1

IGFs are peptide hormones secreted from many different cells. IGF has been shown to play roles in the promotion of cell proliferation and the inhibition of cell death (apoptosis). There are two principle IGFs referred to as IGF-I and IGF-II. IGF-I is developmentally regulated such that peak levels coincide with neuronal proliferation and neurite outgrowth (D'Ercole *et al.,* 1996; Ye *et al.,* 1997; Dentremont *et al.,* 1999), whereas IGF-II is mainly expressed in cells of mesenchymal and neural crest origin (D'Ercole *et al.,* 1996). IGF-1 promotes cell survival (Segal & Greenberg, 1996; Stewart & Rotwein, 1996; Ataliotis & Mercola, 1997). The signaling of the IGF-1 receptor is activated through Akt in oligodendrocytes. IGF-1 signaling through Akt enhances oligodendrocyte progenitor cell survival after glutamate exposure (Ness & Wood, 2002; Ness *et al.,* 2002), growth factor deprivation (Cui *et al.,* 2005). IGF-I has an involvement in regulating neural development including neurogenesis, myelination, synaptogenesis and dendritic branching and neuroprotection after neuronal damage. It is physiologically important for the development of granule neurons

(Bondy *et al.,* 1991; Gao *et al.,* 1991; Calissano *et al.,* 1993; Ye *et al.,* 1996). It promotes the *in vitro* survival and neurite outgrowth of various sensory, sympathetic, cortical and motor neurons (Aizenman & de Vellis, 1987; Caroni & Grandes, 1990, Svrzic & Schubert, 1990; Bozyczko-Coyne *et al.,* 1993; Neff *et al.,* 1993).

Estrogen receptors and IGF-1 receptors interact in the promotion of neuronal survival and neuroprotection (Garcia-Segura *et al.,* 2000). IGF influences neuronal differentiation (Levi-Montalcini *et al.,* 1987; Barde, 1989; Ferrari *et al.,* 1989; Maisonpierre *et al.,* 1990). IGF-I mRNA is widely expressed in the vertebrate brain (Bartlett, 1991; Bondy, 1991). IGF-I and cAMP can protect cerebellar granule neurons from apoptosis in low K+. It is known that IGF-I is synthesized and secreted by cerebellar Purkinje cells (Andersson *et al.,* 1988; Bondy, 1991). Further, the IGF-I receptor is present in granule neurons (Lesniak *et al.,* 1988; Marks *et al.,* 1990; Bondy, 1991). IGF-I therefore serve as a survival factor for these neurons in vivo.

IGF-I has been shown to be a potent NTF that promotes the growth of projection neurons, dendritic arborization and synaptogenesis. Patients with SCI have been reported to have lower plasma IGF-I. IGF-1 protects motor neuron cells from ischemic SCI associated with differential regulation of Bcl-xL and Bax protein (Nakao *et al.,* 2001). Subcutaneous administration of IGF-I resulted in better locomotor recovery following SCI (Koopmans *et al.,* 2006). IGF-I attenuates caspase-9 cleavage, increases Bcl2, thus inhibiting apoptosis after SCI (Hung *et al.,* 2007). BDNF and IGF pre treatment is neuroprotective in SCI and that these NTF have the capacity to down regulate NOS expression following trauma to the spinal cord (Sharma *et al.,* 1998).

Akt

The serine–threonine kinase Akt (also known as protein kinase B) is a central convergence node in a broadly influential signaling network. Akt activation serves as a master switch of these cellular signaling pathways, generating a multitude of intracellular responses through a plethora of downstream targets and interacting partners.

Akt is expressed as three isoforms — AKT1/ PKBα, AKT2/ PKBβ and AKT3/ PKBγ, respectively (Vivanco & Sawyers, 2002). An amino terminal pleckstrin homology (PH) domain, a central serine–threonine catalytic domain, and a small carboxy-terminal regulatory domain characterize all the three isoforms. The PH domain binds to PIP2 and PIP3, products of PI3K. This binding causes Akt to locate to the plasma membrane, where it becomes phosphorylated by phosphoinositide-dependent kinase 1 on Thr308 in the activation loop of the catalytic domain. This phosphorylation leads to activation. Full activation requires phosphorylation at a second site (Ser473). Current evidence leads to the mTOR–rictor complex as the primary kinase for the second phosphorylation event, although other kinases like integrin linked kinase (Ilk) (Persad *et al.,* 2000), phosphoinositide-dependent kinase 1 (Balendran *et al.,* 1999), DNA-dependent protein kinase (DNA-PK) (Feng *et al.,* 2004), ataxia telangiectasia mutated (ATM) have also been identified (Dong & Liu, 2005).

Akt is a general mediator of growth factor induced survival and has been shown to suppress the apoptotic death of a number of cell types induced by a variety of stimuli, including growth factor withdrawal, cell-cycle discordance, loss of cell adhesion and DNA damage (Ahmed *et al.,* 1997; Dudek *et al.,* 1997; Kauffmann-Zeh *et al.,* 1997; Kennedy *et al.,* 1997; Khwaja *et al.,* 1997; Kulik *et al.,* 1997). Thus cellular processes regulated by AKT include cell proliferation and survival, cell size and response to nutrient availability, intermediary metabolism,

angiogenesis and tissue invasion. Growth factor receptor activation leads to the sequential activation of PI39K (Phosphatidylinositol-3-OH kinase)/Akt, which then, promotes cell factors such as IGF-1. Growth factor activation of the PI39K/Akt signaling pathway also culminates in the phosphorylation of the Bcl-2 family member Bad and caspase 9, thereby suppressing apoptosis and promoting cell survival (Sandeep *et al.,* 1997; Nakanishi *et al.,* 2005). Alternatively, inhibition of Akt promotes phosphorylation of the proapoptotic Bad, which favours the apoptotic process (Cardone *et al.,* 1998). It also affects the transcriptional response to apoptotic stimuli by acting on Forkhead factors and also influences the activity of the p53 family (Brunet *et al.,* 1999; Ogawara *et al.,* 2002; Karen *et al.,* 2008; Zhaohui, 2009). Akt phosphorylation has been considered a critical factor in the aggressiveness of cancer. Quercetin induced inactivation of Akt contributing to the promotion of apoptosis.

Akt is increasingly recognized as an important regulator of signal transduction pathways and play important roles in functional recovery after nervous system injury. A significant activation of the Akt/mTOR/p70S6K signaling pathway in the injured spinal cord produces beneficial effects on SCI induced motor function defects and repair potential (Hu *et al.,* 2010). Phospho-Akt and phospho-Bad were colocalized in motor neurons that survived SCI and inhibition of PI3K reduced expression of phospho-Akt and phospho-Bad (Yu *et al.,* 2005). Oxidative stress plays a role in modulating Akt/Bad signaling and subsequent motor neuron survival after SCI (Yu *et al.,* 2005). PI3 kinase/Akt pathway mediates oligodendrocyte progenitor cell survival and proliferation and survival of mature oligodendrocytes induced by numerous compounds (Canoll *et al.,* 1999; Ebner *et al.,* 2000; Flores *et al.,* 2000; Ness and Wood, 2002; Baron *et al.,* 2003; Jaillard *et al.,* 2005; Cui *et al.,* 2006; Pang *et al.,* 2007).

CYCLINS

Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. Cyclin forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. Together with its binding partners cyclin dependent kinase 4 and 6 (CDK4 and CDK6), cyclin D1 forms active complexes that promote cell cycle progression by phosphorylating and inactivating the retinoblastoma protein (RB) (Kato *et al.,* 1993; Weinberg, 1995; Lundberg & Weinberg, 1998). Cyclin D1 encodes the regulatory subunit of a holoenzyme that phosphorylates and inactivates the retinoblastoma protein. Amplification or overexpression of cyclin D1 plays pivotal roles in the development of a subset of human cancers including parathyroid adenoma, breast cancer, colon cancer, lymphoma, melanoma and prostate cancer. The cyclin D1 proto-oncogene is an important regulator of G1 to S phase progression in many different cell types.

Cyclin D1 associates with and regulates activity of transcription factors, coactivators and corepressors that govern histone acetylation and chromatin remodeling proteins. The recent findings that cyclin D1 regulates cellular metabolism, fat cell differentiation and cellular migration. Cyclin D1 rises rapidly after induction of proliferation (Tang *et al.,* 2001; Huang *et al.,* 2002). Frederick *et al.,* (2007). Studies have demonstrated that cyclin D1 also functions as transcriptional modulator by regulating the activity of several transcription factors and histone deacetylase (HDAC3) (Coqueret, 2002). This activity is independent of CDK4 activity. The cyclin D1 protein has been shown to be unstable with a short half-life (~24 min) (Diehl *et al.,* 1997; Diehl *et al.,* 1998) and is degraded mainly via the 26S proteasome in a ubiquitin-dependent manner (Diehl *et al.,* 1997). Early studies suggested that the Skp2 F-box protein might be involved in cyclin D1 degradation (Yu *et al.,* 1998). Recently, two further F-box proteins were

identified in separate studies as playing major roles in targeting the cyclin for degradation (Lin *et al.,* 2006; Okabe *et al.,* 2006).

Cyclin D1 plays a central role in the regulation of proliferation, linking the extracellular signaling environment to cell cycle progression (Sherr, 1995). The expression level of cyclin D1 is highly responsive to the action of proliferative signals including growth factor receptors, Ras and their downstream effectors. Regulation in expression level involves a variety of mechanisms including production, stability and utilization of cyclin D1 mRNA; as well as protein stability, localization and association. Its expression increases upon stimulation of quiescent cells to enter the cell cycle, while it has been proposed to shuttle in and out of the nucleus through the cell cycle of actively cycling cells (Aktas & Cooper, 1997). The evidence indicates that cyclin D1 levels not only regulate the initiation of cell cycle progression in quiescent cells, but that they play a critical role in the decision of a cell to continue proliferating. This decision is made during G2 phase when proliferative signaling induces an increase in cyclin D1 levels. The apparently automatic decline in cyclin D1 levels during the preceding S phase allows efficient DNA synthesis and ensures that proliferative conditions are conducive for continued growth at the time of commitment for continuing proliferation during G2 phase.

Cyclin D2 is a member of the family of D-type cyclins that is implicated in cell cycle regulation, differentiation and oncogenic transformation. cyclinD2 provides neuro protection (Cernak *et al.,* 2005; Di Giovanni *et al.,* 2005; Tian *et al.,* 2006). Forced maintenance of cyclin D2 favours proliferation at the expense of neuronal differentiation. Mice expressing only cyclin D3 lack normal cerebella while mice expressing only cyclin D2 present neurological abnormalities (Ciemerych *et al.,* 2002). Previous studies have also revealed the expression of cyclin D1 and D2 in post-mitotic cells of the developing nervous system (Tamaru *et al.,* 1993; De Falco *et al.,* 2004; Schmetsdorf *et al.,* 2005, 2006). High levels of

cyclin D2 mRNA were localized in S-phase cells. However, this would imply that in these neural progenitors Cyclin D2 has a function only in the daughter cells during the next cell cycle (Salles *et al.,* 2007). Cyclin D2 has also been linked to cancer, as abnormal expression of this gene also correlates with tumor development (Hanna *et al.,* 1993; Siciniski *et al.,* 1996). Cyclin D2 degradation has a role in many diseased conditions (Bill *et al.,* 2012). Overexpression of cyclin D2 protein efficiently inhibited cell cycle progression and DNA synthesis (Muthupalaniappan *et al.,* 1998).

Up regulation of cell cycle proteins occurs after CNS trauma (Di Giovanni *et al.,* 2003), and is associated with apoptotic cell death of post-mitotic cells such as neurons and proliferation of astrocytes and microglia (Becker & Bonni, 2004). Cell cycle proteins are normally down-regulated in post-mitotic neurons (Okano *et al.,* 1993) and re-entry into the cell cycle can cause apoptosis in such cells (Nguyen *et al.,* 2002; Becker & Bonni, 2004). Neurons are typically described as terminally differentiated, permanently held at the G0 phase of the cell cycle, but findings have demonstrated that post-mitotic neurons attempt to re-enter the cell cycle in pathological circumstances (Timsit *et al.,* 1999; Osuga *et al.,* 2000 Malik *et al.,* 2008). Up-regulation of cell cycle proteins is correlated with neuronal apoptosis after experimental SCI (Di Giovanni *et al.,* 2003) and brain injury (Natale *et al.,* 2003). Induction of cyclin D1 and Cdk4 may be implicated in programmed cell death change after transient spinal cord ischemia in rabbits (Sakurai *et al.,* 2000). Administration of cell cycle inhibitors such as flavopiridol, roscovitine or olomoucine can provide neuroprotection in various in vitro models, such as etoposide, kainic acid or colchicine induced injury (Jorda *et al.,* 2003; Cernak *et al.,* 2005; Di Giovanni *et al.,* 2005). Flavopiridol also blocks astrocyte and microglial proliferation in vitro (Cernak *et al.,* 2005; Di Giovanni *et al.,* 2005; Wu *et al.,* 2012).

CELL THERAPY IN SCI

Cell therapy is a highly hopeful method in clinical applications, raising so much optimism for the treatment of injured tissues with no self regeneration potential such as central and peripheral nervous system. A promising strategy in SCI repair being developed is stem cell or progenitor cell based therapy (Hwang *et al.,* 2011). It is hoped that these undifferentiated cells will provide an inexhaustible source of neurons and glia for therapies aimed at cell replacement or neuroprotection in disorders affecting the CNS. The transplantation of stem cells could promote functional recovery by reconstituting damaged nerve tracts, remyelinating axons and increasing plasticity or axon regeneration (Moraleda *et al.,* 2006). Indeed, several studies have reported partial functional improvements and fiber regeneration following spinal implantation of different types of cells (Garbuzova-Davis *et al.,* 2009). Locomotor improvements have been demonstrated in SCI mice and primates (Watanabe *et al.,* 2004; Cummings *et al.,* 2005; Iwanami *et al.,* 2005). However, no single therapeutic methodology has been proven effective enough for significant functional recovery because of difficulties such as post transplantation cellular lineage restriction (Reier, 2004), variable host responses to the engraftment (Ourednik & Ourednik, 2004 a,b), and unfavorable hostile environment for the implanted cells.

BONE MARROW CELLS

Transplantation of BMCs into the injured spinal cord has been found to improve neurologic functions in experimental animal studies. Cell transplantation technology has demonstrated that BMCs differentiate into mature neurons or glial cells under specific experimental conditions and that the transplantation of BMCs can promote functional improvements after SCI (Jung *et al.,* 2011). The hematopoietic or mesenchymal stem cells has a neuroprotective effect and increased neurite outgrowth (Ankeny *et al.,* 2004; Chen *et al.,* 2005). It was seen

that the mesenchymal stem cells are predisposed to differentiate into neuronal cells once the proper conditions are given. When transplanted into the CNS, they can develop into a variety of functional neural cell types, making them a potent resource for cell-based therapy. However, to date neural stem/progenitor cells transplantation has exhibited only limited success in the treatment of chronic SCI. BMCs can support the natural regeneration processes of the body by stimulating the repair of damaged tissues. BMCs differentiate into mature neurons or glial cells under specific experimental conditions (Sanchez *et al.,* 2000; Ha *et al.,* 2001; Munoz *et al.,* 2003). Transplanted BMCs improve neurological deficits in the CNS injury models by generating neural cells or myelin producing cells (Chopp *et al.,* 2000; Akiyama *et al.,* 2002). It has been reported that the bone marrow derived cells also have the potential to develop into neural lineages, such as neurons and astrocytes, both *in vivo* (Kopen *et al.,* 1999) and *in vitro* (Yamada *et al.,* 2001). BMCs which are adherent in the culture of bone marrow aspirates, have already been used for the treatment of the injured spinal cord (Chopp *et al.,* 2000) and brain. Studies suggest that transplantation of bone marrow stem cells into spinal cord lesions enhances axonal regeneration and promotes functional recovery in animal studies (Wright *et al.,* 2010). Thus modest improvements in neurological function have been reported following BMC administration in acute CNS injury (Mahmood *et al.,* 2004; Himes *et al.,* 2006).

Materials and Methods

CHEMICALS USED AND THEIR SOURCES

Biochemicals

Serotonin, GABA, pirenzepine, atropine, 4-deoxy acetyl methyl piperidine mustard (4-DAMP mustard), ethylene diamine tetra acetic acid (EDTA), [n′ (2-hydroxy ethyl)] piperazine-n′-[2-ethanesulfonic acid] (HEPES), Tris HCl, foetal calf serum (heat inactivated), bovine serum albumin fraction V, Brdu and paraformaldehyde were purchased from Sigma Chemical Co., St. Louis, MO. USA. Tissue freezing medium Jung was purchased from Leica Microsystems Nussloch GmbH, Germany. All other reagents were of analytical grade purchased locally from SRL, India.

Radiochemicals

Quinuclidinylbenzilate, L-[Benzilic-4,4'-^{3}H]-[4-^{3}H] (Sp. Activity 42 Ci/mmol) and 4-DAMP, [N-methyl-^{3}H] (Sp. Activity 83 Ci/mmol) was purchased from NEN life sciences products Inc., Boston, U.S.A. The [^{3}H]IP_3, [^{3}H]cGMP and [^{3}H]cAMP were purchased from American Radiolabelled Chemicals, USA.

Molecular biology chemicals

Tri-reagent kit was purchased from Sigma chemicals Co., St. Louis, MI, USA. ABI PRISM High capacity cDNA Archive kit, primers and Taqman probes for Real Time-PCR AChE (Rn 00596883_ m1), ChAT (Rn 01453446_m1), muscarinic M1 (Rn 00589936_s1), muscarinic M2 (Rn 02532311_s1), muscarinic M3 (Rn 00560986_s1), α7 nicotinic acetylcholine (Rn01644792_g1), CREB (Rn_ 00578826), PLC (Rn_01647142), Bax (Rn_01480160), caspase-8 (Rn_00574069),

SOD (Rn_01477289), GPx (Rn_00577994), TNF-α (Rn_99999017), NF-κB (Rn_01399583), BDNF (Rn_01484924), GDNF (Rn00569510), IGF-1 (Rn_99999087), Akt-1 (Rn00583646), cyclin D2 (Rn01492401_m1) and endogenous control (β-actin) were purchased from Applied Biosystems, Foster City, CA, USA.

Confocal dyes

Rat primary antibody for muscarinic M1 (M9808, Sigma Aldrich), M3 (M 9568 Sigma Aldrich), α7 nicotinic acetylcholine receptor (N 8158 Sigma Aldrich), Brdu (Cat. No. B8434, Sigma Aldrich), NeuN (ABN 78, Millipore), FITC coated secondary antibody (No. AP132 F, Millipore) and Alexa Fluor 594 (No: A11012, Invitrogen) were used for the immunohistochemistry studies using confocal microscope.

ANIMALS

Adult male Wistar rats of 220-270g body weight purchased from Amrita Institue of Medical Sciences, Cochin and Kerala Agriculture Unviersity, Mannuthy were used for all experiments. They were housed in separate cages under 12 hour light and 12 hour dark periods and were maintained on standard food pellets and water *ad libitum*. Adequate measures were also taken to minimize pain and discomfort of the animals. All animal care and procedures were taken in accordance with the Institutional, National Institute of Health and CPCSEA guidelines.

EXPERIMENTAL DESIGN

The experimental rats were divided into the following groups i) C (Control) ii) SCI (Spinal cord injury rats), iii) SCI + 5-HT (SCI treated with Serotonin iv) SCI +GABA (SCI treated with GABA) v) SCI + BMC (SCI treated with BMC vi) SCI+5-HT+BMC (SCI treated with Serotonin and BMC) vii)

SCI+GABA+BMC (SCI treated with GABA and BMC) viii) SCI+5-HT+GABA+BMC (SCI treated with Serotonin, GABA and BMC). Each group consisted of 4-6 animals.

Under all aseptic precautions and anesthesia, monoplegia was induced by shearing between the T12 and T13 vertebra of the experimental rats. The type of anaesthesia used was chloral hydrate at a dosage of 400mg/kg body weight i.p. A specially designed rubber chamber with silastic catheter (Paulose & Dakshinamurti, 1987) was inserted subcutaneously with the tip of silastic tube inserted to the injury site and fixed with sutures. Those rats that developed monoplegia were selected for further experiments. Proper postoperative care was provided till the animals recovered completely.

TREATMENT

Monoplegic SCI rats were given various treatments from day 1. 10µl of BMC (10^6 Cells/10 µl) suspension was given as a single dose on day 1 to the treatment rats that was administered with BMC individually and in combination. 5-HT (1 µg/ kg body weight) and GABA (1 µg/ kg body weight) were given through the chamber for a period of 21 days to the treatment rats that was administered with 5-HT and GABA individually and in combination (Paulose *et al*., 2009).

BMCs were drawn according to a modified protocol (Azizi *et al*., 1998). BMCs were collected from femurs with saline using a syringe with a no. 18 G needle. Cells were disaggregated by gentle pipetting several times. Cells were passed through a 30-µm nylon mesh to remove remaining clumps of tissue. Cells were washed by adding fresh saline and centrifuged for 10 min at 200×g, and the supernatant removed. The cell pellet was resuspended in 1 ml of saline. Cell

counting was done using a hemocytometer (Margaret *et al*., 1996; Majumdar *et al*., 1998).

SACRIFICE AND TISSUE PREPARATION

The animals were then sacrificed on 22nd day by decapitation. The spinal cord, cerebral cortex, cerebellum, brain stem and corpus striatum were dissected out quickly over ice according to the procedure of Glowinski and Iversen, (1966) and the pancreas was dissected quickly over ice.

BEHAVIOURAL STUDIES

Animals chosen for behavioural test was given training for 5 days 21 days of treatment period. On the 6th day the various behavioural tests were done and the readings were recorded.

Rotarod test

Rotarod has been used to evaluate motor coordination by testing the ability of rats to remain on revolving rod (Dunham & Miya, 1957). The apparatus has a horizontal rough metal rod of 3 cm diameter attached to a motor with variable speed. This 70 cm long rod was divided into four sections by wooden partitions. The rod was placed at a height of 50 cm to discourage the animals to jump from the rotating rod. The rate of rotation was adjusted in such a manner that it allowed the normal rats to stay on it for five minutes. Each rat was given five trials before the actual reading was taken. The readings were taken at 10, 15 and 25 rpm after 21 days of treatment in all groups of rats.

Grid walk test

Deficits in descending motor control were examined by assessing the ability to navigate across a 1 m long runway with irregularly assigned gaps (0.5–5

cm) between round metal bars. Crossing this runway requires that animals accurately place their limbs on the bars. In baseline training and postoperative testing, every animal had to cross the grid for at least three times. The number of footfalls (errors) was counted in each crossing for 3 minute and a mean error rate was calculated (Z'Graggen *.et al.*, 1998).

Narrow beam test

The narrow beam test was performed according to the descriptions of Haydn and Jasmine (1975). A rectangular 1.2-cm wide beam, 1.05m long and elevated 30 cm from the ground was used for the study. After training, normal rats were able to traverse the horizontal beams with less than three footfalls. When occasionally their feet slipped off the beam, they were retrieved and repositioned precisely. The time the rats could remain balanced on the beam was counted.

MUSCARINIC RECEPTOR BINDING STUDIES USING [^{3}H] RADIOLIGANDS

Binding studies in the spinal cord and brain regions

Total muscarinic, muscarinic M1 and M3 receptor binding studies

[^{3}H] QNB and [^{3}H] DAMP binding assay in spinal cord, cerebral cortex, cerebellum, brain stem and corpus striatum were done according to the modified procedure of Yamamura and Snyder (1981). Spinal cord and brain tissues were homogenised in a polytron homogeniser with 20 volumes of cold 50mM Tris-HCl buffer, containing 1mM EDTA, pH.7.4. The supernatant was then centrifuged at 30,000xg for 30 minutes and the pellets were resuspended in appropriate volume of Tris-HCl-EDTA buffer.

Total muscarinic and muscarinic M1 receptor binding parameter assays were done using [^{3}H] QNB (0.1-2.5nM) and M3 receptor using [^{3}H] DAMP (0.01-5nM) in the incubation buffer, pH 7.4 in a total incubation volume of 250µl

containing appropriate protein concentrations (200-250µg). The non-specific binding was determined using 100µM atropine for total muscarinic, pirenzepine for muscarinic M1 and 4-DAMP mustard for muscarinic M3 receptor. Total incubation volume of 250 µl contains 200-250µg protein concentrations. Tubes were incubated at 22°C for 60 minutes and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50mM Tris-HCl buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

PROTEIN DETERMINATION

Protein was measured by the method of Lowry *et al*., (1951) using bovine serum albumin as standard. The intensity of the purple blue colour formed was proportional to the amount of protein which was read in a spectrophotometer at 660nm.

ANALYSIS OF THE RECEPTOR BINDING DATA

Linear regression analysis for Scatchard plots

The data were analysed according to Scatchard (1949). The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant (K_d), were derived by linear regression analysis by plotting the specific binding of the radioligand on X-axis and bound/free on Y-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity.

GENE EXPRESSION STUDIES IN SPINAL CORD AND BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

Isolation of RNA

RNA was isolated from the spinal cord and brain regions of control and experimental rats using the Tri reagent from Sigma Chemicals Co., St. Louis, MO, U.S.A). 25-50 mg tissue homogenates were made in 0.5 ml Tri Reagent. The homogenate was kept in the room temperature for 5 minutes. 100 µl of chloroform was added to the homogenate, mixed vigorously for 15 seconds kept in the RT for 10-15 minutes and was centrifuged at 12,000xg for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh tube and 250µl of isopropanol was added and the tubes were allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000xg for 10 minutes at 4°C. RNA precipitated as a pellet on the sides and bottom of the tube. The supernatant was removed and the RNA pellet was washed with 500µl of 75% ethanol, vortexed and centrifuged at 12,000xg for 5 minutes at 4°C. The pellets were semi dried and dissolved in minimum volume of DEPC-treated water. 2 µl of RNA was made up to 1 ml and absorbance was measured at 260nm and 280nm. For pure RNA preparation the ratio of absorbance at 260/280 was ≥ 1.7. The concentration of RNA was calculated as one absorbance $_{260}$ = 42µg.

REAL-TIME POLYMERASE CHAIN REACTION

cDNA synthesis

Total cDNA synthesis was performed using ABI PRISM cDNA arhive kit in 0.2ml microfuge tubes. The reaction mixture of 20 µl contained 0.2µg total RNA, 10 X RT buffer, 25 X dNTP mixture, 10 X random primers, MultiScribe RT (50U/µl) and RNase free water. The cDNA synthesis reactions were carried out at 25 °C for 10 minutes and 37 °C for 2 hours using an Eppendorf Personal Cycler. The primers and probes were purchased from Applied Biosystems, Foster City, CA, USA designed using Primer Express software version (3.0).

Real-Time Pcr Assays

Real Time PCR assays were performed in 96-well plates in an ABI 7300 Real Time PCR instrument (Applied Biosystems). To detect gene expression, a TaqMan quantitative 2-step reverse transcriptase "polymerase chain reaction (RT-PCR) was used to quantify mRNA levels. First-strand cDNA was synthesized with use of a TaqMan RT reagent kit, as recommended by the manufacturer. PCR analyses were conducted with gene-specific primers and fluorescently labeled TaqMan probe, designed by Applied Biosystems. All reagents were purchased from Applied Biosystems. The probes for specific gene of interest were labeled with FAM at the 5' end and a quencher (Minor Groove Binding Protein - MGB) at the 3' end. The Real-Time data were analyzed with Sequence Detection Systems software version 1.7. All reactions were performed in duplicate.

The TaqMan reaction mixture of 20 µl contained 25 ng of total RNA-derived cDNAs, 200 nM each of the forward primer, reverse primer and PCR analyses were conducted with gene-specific primers and fluorescently labelled Taqman probes of ChAT, AChE, muscarinic M1, M2, M3, α7 nicotinic acetylcholine, CREB, PLC, Bax, Caspase-8, SOD, GPx, TNF-α NF-κB, BDNF, GDNF, IGF-1, Akt-1 and cyclin D2. Endogenous control (β-actin) was labeled with a reporter dye (VIC). 12.5 µl of TaqMan 2X Universal PCR Master Mix was taken and the volume was made up with RNAse free water. Each run contained both negative (no template) and positive controls.

The thermocycling profile conditions were as follows:

50ºC -- 2 minutes ---- Activation

95ºC -- 10 minutes ---- Initial Denaturation

95ºC -- 15 seconds ---- Denaturation 40 cycles

50ºC -- 30 seconds --- Annealing

60ºC -- 1 minutes --- Final Extension

Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The ΔΔCT method of relative quantification was used to determine the fold change in expression. This was done by first normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β- actin in the same samples ($\Delta CT = CT_{Target} - CT_{\beta\text{- actin}}$). It was further normalized with the control ($\Delta\Delta CT = \Delta CT - CT_{Control}$). The fold change in expression was then obtained ($2^{-\Delta\Delta}C\ T$).

IP_3 CONTENT IN THE SPINAL CORD AND BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS *IN VIVO*

The spinal cord and brain regions – cerebral cortex, cerebellum, brain stem and corpus striatum were homogenised in a polytron homogeniser in 50mM Tris-HCl buffer, pH 7.4, containing 1mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g for 15 minutes and the supernatant was transferred to fresh tubes for IP_3 assay using [^{3}H] IP_3 Biotrak Assay System kit.

Principle of the assay

The assay was based on competition between [^{3}H] IP_3 and unlabelled IP_3 in the standard or samples for binding to a binding protein prepared from bovine adrenal cortex. The bound IP_3 was then separated from the free IP_3 by centrifugation. The free IP_3 in the supernatant was then discarded by simple decantation, leaving the bound fraction adhering to the tube. Measurement of the radioactivity in the tube enables the amount of unlabelled IP_3 in the sample to be determined.

Assay Protocol

Standards, ranging from 0.19 to 25pmoles/tube, [^{3}H] IP_3 and binding protein were added together and the volume was made up to 100µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. The tubes were then vortexed and incubated on ice for 15min and they were centrifuged at 2000 x g for 10min at 4°C. The supernatant was aspirated out and the pellet was resuspended in water and incubated at room temperature for 10min. The tubes were then vortexed and decanted immediately into scintillation vials. The radioactivity in the suspension was determined using liquid scintillation counter.

A standard curve was plotted with %B/Bo on the Y-axis and IP_3 concentration (pmoles/tube) on the X-axis of a semi-log graph paper. $\%B/B_o$ was calculated as:

$$\frac{(\text{Standard or sample cpm} - \text{NSB cpm})}{(B_0 \text{ cpm} - \text{NSB cpm})} \times 100$$

NSB- non specific binding and B_0 - zero binding. IP_3 concentration in the samples was determined by interpolation from the plotted standard curve.

cGMP CONTENT IN THE SPINAL CORD AND BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS *IN VIVO*

The spinal cord and various brain regions – cerebral cortex, cerebellum, brain stem and corpus striatum were homogenised in a polytron homogeniser with cold 50mM Tris-HCl buffer, pH 7.4, containing 1mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g for 15min and the supernatant was transferred to fresh tubes for cGMP assay using [^{3}H] cGMP Biotrak Assay System kit.

Principle of the assay

The assay is based on the competition between unlabelled cGMP and a fixed quantity of the [^{3}H] cGMP for binding to an antiserum, which has a high specificity and affinity for cGMP. The amount of [^{3}H] cGMP bound to the antiserum is inversely related to the amount of cGMP present in the assay sample. Measurement of the antibody bound radioactivity enables the amount of unlabelled cGMP in the sample to be calculated. Separation of the antibody bound cGMP from the unbound nucleotide was done by ammonium sulphate precipitation, followed by centrifugation. The precipitate which contains the antibody bound complex was dissolved in water and its activity was determined by liquid scintillation counting. The concentration of unlabelled cGMP in the sample was determined from a linear standard curve.

Assay Protocol

Standards, ranging from 0.5 to 4.0pmoles/tube and [^{3}H] cGMP were added together and the volume was made up to 100µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. The antiserum was added to all the assay tubes and then vortexed. The tubes were incubated for 90min at 2 - 8°C. Ammonium sulphate was added to all tubes, mixed and allowed to stand for 5min in ice bath. The tubes were centrifuged at 12000 x g for 2min at room temperature. The supernatant was aspirated out and the pellet was dissolved in water and decanted immediately into scintillation vials. The radioactivity in the suspension was determined using liquid scintillation counter.

A standard curve was plotted with C_o/C_x on the Y-axis and cGMP concentration (pmoles/tube) on the X-axis of a linear graph paper. C_o- the cpm bound in the absence of unlabelled cGMP; C_x- the cpm bound in the presence of standard/unknown cGMP. cGMP concentration in the samples was determined by interpolation from the plotted standard curve.

cAMP CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS *IN VIVO*

The spinal cord and different brain regions – cerebral cortex, cerebellum, brain stem and corpus striatum were homogenised in a polytron homogeniser with cold 50mM Tris-HCl buffer, pH 7.4, containing 1mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g for 15min and the supernatant was transferred to fresh tubes for cAMP assay using [^{3}H] cAMP Biotrak Assay System kit.

Principle of the assay

cAMP assay kit was used. The assay is based on the competition between unlabelled cAMP and a fixed quantity of [^{3}H] cAMP for binding to a protein which has a high specificity and affinity for cAMP. The amount of labeled protein -cAMP complex formed is inversely related to the amount of unlabelled cAMP present in the assay sample. Measurement of the protein-bound radioactivity enables the amount of unlabelled cAMP in the sample to be calculated.

Free [^{3}H] cAMP Bound [^{3}H] cAMP-binding protein

\+ Binding protein = +

cAMP cAMP-binding protein

Separation of the protein bound cAMP from unbound nucleotide is achieved by adsorption of the free nucleotide on to a coated charcoal followed by centrifugation. An aliquot of the supernatant is then removed for liquid scintillation counting. The concentration of unlabelled cAMP in the sample is then determined from a linear standard curve.

Assay Protocol

The tubes were placed on a water bath at 0°C. The assay mixture consisted of different concentrations of standard, [^{3}H] cAMP and binding protein in case of standards; buffer, [^{3}H] cAMP and binding protein for zero blank and unknown samples, [^{3}H] cAMP and binding protein for determination of unknown samples. The mixture was incubated at 2°C for 2h. Cold charcoal reagent was added to the tubes and the tubes were immediately centrifuged at 12,000 x g for 2min at 2°C. Aliquots of the supernatant was immediately transferred to scintillation vials and mixed with cocktail-T and counted in a liquid scintillation counter (Wallac, 1409).

C_o/C_x is plotted on the Y-axis against picomoles of inactive cAMP on the X- axis of a linear graph paper, where C_o is the counts per minute bound in the absence of unlabelled cAMP and C_x is the counts per minute bound in the presence of standard or unknown unlabelled cAMP. From the C_o/C_x value for the sample, the number of picomoles of unknown cAMP was calculated.

IMMUNOHISTOCHEMISTRY OF MUSCARINIC M1, M3 AND α7 NICOTINIC ACETYLCHOLINE RECEPTOR IN THE SPINAL CORD AND BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

Anaesthetized animals with chloral hydrate were transcardially perfused with PBS, pH- 7.4, followed by 4% paraformaldehyde in PBS (Chen *et al*., 2007). After perfusion the spinal cords and brains were dissected and immersion fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in 0.1 M PBS, pH- 7.0. 10 µm sections were cut using Cryostat (Leica, CM1510 S). The sections were treated with PBST (PBS in 0.01% Triton X-100) for 20 min. Brain sections were blocked with 5% normal goat serum in PBS for 4 hours. Brain sections were then incubated overnight at 4°C with either rat primary antibody for

muscarinic M1, M3 and α7 nicotinic acetylcholine receptor, diluted in PBST at 1: 500 dilution in a 1X PBS solution containing 5% normal goat serum). After overnight incubation brain sections washed with PBS and then incubated for 1 hour with secondary antibody conjugated with FITC (1:1000 dilution in a 1X PBS solution containing 5% normal goat serum) in brain regions. Excess PBS was tapped off, the slides were mounted with cover glass with Prolong Gold anti-fade mounting media. The sections were observed and photographed using confocal imaging system (Leica SP 5). Expressions were analysed using pixel intensity method. The given mean pixel value is the net value which is deducted from the negative control pixel value.

BONE MARROW CELLS DIFFERENTIATION STUDIES USING BrdU AND NeuN

To study the differentiation of BMC, transplanted BMC's were labelled by BrdU and double stained with neuronal marker NeuN for identification of neurons. Forty-eight hours after the treatment with BMC, 5-HT and GABA individually and in combinations, the rats were injected intraperitoneally with BrdU, at a dose of 100 mg/kg body weight. This dose was administered twice weekly until the rats were sacrificed. Tissue fixation was done by transcardial perfusion with Phosphate buffered saline (PBS), pH 7.4, followed by 4% PFA in PBS (Chen *et al*., 2007). After perfusion the brains were dissected and immersion fixed in 4% PFA for 1 hr and then equilibrated with 30% sucrose solution in 0.1M PBS, pH- 7.0. 10µm sections were cut using Cryostat (Leica, CM1510 S). The sections were treated with PBST (PBS in 0.01% Triton X-100) for 20 min. To block unspecific binding the sections were incubated for 1 hour at room temperature with 5% normal goat serum in 1X PBS. Spinal cord sections were incubated overnight at 4°C with rat primary antibody for BrdU diluted in PBST at 1:500 dilution. After overnight incubation, the spinal cord sections were rinsed with PBST and then incubated with Alexa Fluor 594 secondary antibody (1:1000)

for 2hours. At the end of incubation period, the spinal cord sections were rinsed thrice with PBST and incubated overnight with rat primary antibody for NeuN (1:1000). The slides were washed with PBST after the incubation time and secondary antibody Alexa Fluor 488 was added and incubated for 2 hours at room temperature. The brain slices were thoroughly washed, mounted, observed and photographed using confocal imaging system (Leica SP 5).

STATISTICS

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression Scatchard plots were made using Sigma Plot software (version 2.0, Jandel GmbH, Erkrath, Germany). Competitive binding data were analysed using non-linear regression curve-fitting procedure (GraphPad PRISM^{TM}, San Diego, USA). Relative Quantification Software was used for analyzing Real-Time PCR results.

Results

Body weight of control and experimental rats

SCI rats showed a showed a significant ($p<0.001$) decrease in body weight after 21 days compared to control rats. SCI+5-HT, SCI+GABA and SCI+BMC treated rats showed no significant reversal in the body weight when compared to SCI. Meanwhile, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats showed significant ($p<0.001$) reversal in the body weight when compared to control rats. SCI+5-HT+GABA+BMC rats showed prominent reversal in the weight when compared to other group of rats (Table-1).

Behavioural studies

Rotarod performance of control and experimental rats

Rotarod experiment showed a significant ($p<0.001$) decrease in the retention time on the rotating rod in the SCI rats at 10, 15 and 25 rpm when compared to control. SCI+5-HT, SCI+GABA and SCI+BMC treated rats showed no significant reversal in the retention time when compared to SCI rats. But, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats significantly ($p<0.001$) reversed the retention time to near control when compared with SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal when compared to the other groups of rats (Figure-1).

Behavioural response of control and experimental rats on grid walk test

There was significant ($p<0.001$) increase in the foot falls in SCI rats compared to control. SCI+5-HT, SCI+GABA and SCI+BMC treated rats showed no significant reversal in foot falls when compared to SCI rats. SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats showed significant

($p<0.001$) reversal in foot falls when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal when compared to the other combination treatment groups of rats (Figure-2).

Behavioural response of control and experimental rats on narrow beam test

There was significant ($p<0.001$) decrease in the retention of balance on the narrow beam ($p<0.001$) in SCI rats compared to control. SCI+5-HT, SCI+GABA and SCI+BMC treated rats showed no significant reversal in the retention of balance on the narrow beam when compared to SCI rats. Balance on the narrow beam significantly ($p<0.001$) reversed in SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal when compared to all the other combination treatment groups of rats (Figure-3).

Spinal cord

Total muscarinic receptor analysis

Scatchard analysis of [^{3}H] QNB binding against atropine to total muscarinic receptor in the spinal cord of control and experimental rats

The total muscarinic receptor status was assayed using the specific ligand, [^{3}H] QNB and muscarinic general antagonist atropine. The Scatchard analysis showed that the B_{max} and K_d decreased significantly ($p<0.001$) in SCI rats compared to control group. SCI+5-HT, SCI+GABA and SCI+BMC treated rats showed a significant ($p<0.001$) reversal in B_{max} when compared to SCI rats. K_d reversed significantly in SCI+5-HT ($p<0.001$) and SCI+GABA ($p<0.001$) rats but not in SCI+BMC rats when compared to SCI rats. The combination treatment group of rats, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats showed significant ($p<0.001$) reversal in B_{max} and K_d when compared to SCI group. SCI+5-HT+GABA+BMC rats showed prominent reversal in B_{max} to near control when compared to all the other treatment groups (Figure 4&5; Table 2&3).

Muscarinic M1 receptor analysis

Scatchard analysis of [^{3}H] QNB binding against pirenzepine to muscarinic M1 receptor in the spinal cord of control and experimental rats

Binding analysis of muscarinic M1 receptor was done using [^{3}H] QNB and M1 subtype specific antagonist pirenzepine. The B_{max} and K_d decreased significantly ($p<0.001$) in SCI group when compared to control group. SCI+5-HT, SCI+GABA and SCI+BMC treated rats showed a significant reversal ($p<0.001$) in B_{max} when compared to SCI rats. K_d reversed significantly ($p<0.001$) in SCI+5-HT and SCI+GABA rats but not in SCI+BMC rats when compared to SCI rats. The combination treatment group of rats, SCI+5-HT+BMC, SCI+GABA+BMC and

SCI+5-HT+GABA+BMC rats showed significant reversal ($p<0.001$) in B_{max} when compared to SCI group. K_d was reversed significantly ($p<0.001$ in SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal in B_{max} to near control when compared to all the other treatment groups (Figure 6&7; Table 4&5).

Muscarinic M3 receptor analysis

Scatchard analysis of [^{3}H] DAMP binding against 4-DAMP mustard to muscarinic M3 receptor in the spinal cord of control and experimental rats

Binding analysis of muscarinic M3 receptors was done using [^{3}H] DAMP and M3 subtype specific antagonist 4-DAMP mustard. The B_{max} was decreased significantly ($p<0.001$) in SCI group with no significant change in K_d when compared to control. SCI+5-HT, SCI+GABA, SCI+BMC, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats showed significant reversal ($p<0.001$) in B_{max} when compared to SCI group. K_d showed significant increase in SCI+GABA ($p<0.001$) but not in other treatment groups of rats when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal in B_{max} to near control when compared to all the other treatment groups (Figure 8&9; Table 6&7).

REAL TIME-PCR ANALYSIS

Real Time PCR analysis of acetylcholine esterase in the spinal cord of control and experimental rats

Gene expression of AChE mRNA showed significant ($p<0.001$) up regulation in the spinal cord of SCI rats when compared to control. SCI+5-HT and SCI+GABA treated rats showed a significant ($p<0.01$) reversal when compared to SCI rats. SCI+BMC did not show significant reversal when compared to SCI rats. The combination treatment group of rats, SCI+5-HT+BMC, SCI+GABA+BMC

and SCI+5-HT+GABA+BMC rats significantly ($p<0.001$) reversed the altered expression when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal when compared to all the other treatment groups (Figure-10, Table-8).

Real Time-PCR analysis of choline acetyl transferase in the spinal cord of control and experimental rats

Gene expression of ChAT mRNA showed significant ($p<0.001$) down regulation in the spinal cord of SCI rats compared to control. SCI+5-HT and SCI+GABA treated rats showed a significant ($p<0.001$) reversal when compared to SCI rats. SCI+BMC did not show significant reversal when compared to SCI rats. The combination treatment group of rats, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats significantly ($p<0.001$) reversed and up regulated the gene expression of ChAT mRNA when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal up regulation compared to all the other treatment groups (Figure-11, Table-9).

Real Time-PCR analysis of muscarinic M1 receptor in the spinal cord of control and experimental rats

Real Time-PCR analysis showed that the muscarinic M1 receptor gene expression was decreased significantly ($p<0.001$) in the spinal cord of SCI rats when compared to control. SCI+5-HT, SCI+GABA, SCI+BMC, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats significantly ($p<0.001$) reversed these changes when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal to near control compared to all the other treatment groups (Figure-12, Table-10).

Real Time-PCR analysis of muscarinic M2 receptor in the spinal cord of control and experimental rats

Real Time-PCR analysis showed that the muscarinic M2 receptor gene expression was down regulated significantly ($p<0.001$) in the spinal cord of SCI rats when compared to control. SCI+5-HT, SCI+GABA, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats significantly ($p<0.001$) reversed the down regulation when compared to SCI rats. SCI+BMC rats showed no significant reversal in gene expression compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal to near control compared to all the other treatment groups (Figure-13, Table-11).

Real Time-PCR analysis of muscarinic M3 receptor in the spinal cord of control and experimental rats

Real Time-PCR analysis showed that the muscarinic M3 receptor gene expression was decreased significantly ($p<0.001$) in the spinal cord of SCI rats when compared to control. SCI+5-HT, SCI+GABA, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats significantly ($p<0.001$) reversed these changes when compared to SCI rats. SCI+BMC rats showed no significant reversal in gene expression compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal to near control compared to all the other treatment groups (Figure-14, Table-12).

Real Time-PCR analysis of α7 nicotinic acetylcholine receptor in the spinal cord of control and experimental rats

Real Time-PCR analysis showed that α7 nicotinic acetylcholine receptor gene expression showed significant ($p<0.001$) down regulation in the spinal cord of SCI rats when compared to control. SCI+5-HT and SCI+GABA treated rats showed significant ($p<0.01$) reversal when compared to SCI rats. It was significantly ($p<0.001$) reversed and up regulated in SCI+5-HT+BMC,

SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats. SCI+BMC rats showed no significant reversal in gene expression compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent up regulation compared to all the other treatment groups (Figure-15, Table-13).

Real Time-PCR analysis of phospholipase C receptor in the spinal cord of control and experimental rats

Real Time-PCR analysis showed that the PLC gene expression was down regulated significantly ($p<0.001$) in the spinal cord SCI rats when compared to control. It was significantly ($p<0.001$) reversed and up regulated in SCI+5-HT, SCI+GABA, SCI+BMC, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent up regulation than all the other group of rats (Figure-16, Table-14).

Real Time-PCR analysis of CREB in the spinal cord of control and experimental rats

Gene expression of CREB mRNA showed significant ($p<0.001$) down regulation in the spinal cord of SCI rats compared to control. SCI+5-HT, SCI+GABA, SCI+5-HT+BMC and SCI+GABA+BMC rats significantly ($p<0.001$) reversed these changes when compared to SCI rats. SCI+BMC showed no significant reversal in gene expression of CREB when compared to SCI rats. SCI+5-HT+GABA+BMC treatment showed significant ($p<0.001$) reversal and up regulation when compared to SCI rats (Figure-17, Table-15).

Real Time-PCR analysis of Bax in the spinal cord of control and experimental rats

Gene expression of Bax mRNA showed significant ($p<0.001$) up regulation in the spinal cord of SCI rats compared to control. SCI+5-HT,

SCI+GABA, SCI+5-HT+BMC and SCI+GABA+BMC rats significantly ($p<0.001$) reversed these changes when compared to SCI rats. SCI+BMC showed no significant reversal in gene expression of Bax when compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed significant ($p<0.001$) reversal and down regulation when compared to SCI rats (Figure-18, Table-16).

Real Time-PCR analysis of caspase-8 in the spinal cord of control and experimental rats

Gene expression of caspase-8 mRNA showed significant ($p<0.001$) up regulation in the spinal cord of SCI rats compared to control. SCI+5-HT, SCI+GABA, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats significantly ($p<0.001$) reversed these changes when compared to SCI rats. SCI+BMC showed no significant reversal in gene expression of caspase-8 when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal than all the other groups (Figure-19, Table-17).

Real Time-PCR analysis of superoxide dismutase in the spinal cord of control and experimental rats

Gene expression of superoxide dismutase mRNA showed significant ($p<0.001$) down regulation in the spinal cord of SCI rats compared to control. SCI+5-HT, SCI+GABA, SCI+5-HT+BMC and SCI+GABA+BMC rats significantly ($p<0.001$) reversed these changes when compared to SCI rats. SCI+BMC rats showed no significant reversal in gene expression compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed significant ($p<0.001$) reversal and up regulation when compared to SCI rats (Figure-20, Table-18).

Real Time-PCR analysis of glutathione peroxidase in the spinal cord of control and experimental rats

Gene expression of glutathione peroxidase mRNA showed significant ($p<0.001$) down regulation in the spinal cord of SCI rats compared to control. SCI+5-HT, SCI+GABA, SCI+5-HT+BMC and SCI+GABA+BMC rats significantly ($p<0.001$) reversed these changes when compared to SCI rats. SCI+BMC group showed no significant reversal in gene expression compared to SCI rats. SCI+5-HT+GABA+BMC rats showed significant ($p<0.001$) reversal and up regulation when compared to SCI group (Figure-21, Table-19).

Real Time-PCR analysis of TNFα in the spinal cord of control and experimental rats

Gene expression of TNFα mRNA showed significant ($p<0.001$) up regulation in the spinal cord of SCI rats compared to control. SCI+5-HT, SCI+GABA, SCI+BMC, SCI+5-HT+BMC and SCI+GABA+BMC rats significantly ($p<0.001$) reversed these changes when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal compared to all the other treatment groups (Figure-22, Table-20).

Real Time-PCR analysis of NF-ĸB in the spinal cord of control and experimental rats

Gene expression of NF-ĸB mRNA showed significant ($p<0.001$) up regulation in the spinal cord of SCI rats compared to control. SCI+5-HT, SCI+GABA and SCI+BMC rats significantly ($p<0.001$) reversed these changes when compared to SCI rats. SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats showed significant ($p<0.001$) reversal and down regulation when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent

reversal and down regulation when compared to control and treatment groups (Figure-23, Table-21).

Real Time-PCR analysis of BDNF in the spinal cord of control and experimental rats

Gene expression of BDNF mRNA showed significant ($p<0.001$) up regulation in the spinal cord of SCI rats compared to control. SCI+5-HT, SCI+GABA, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC groups showed further significant ($p<0.001$) up regulation when compared to SCI rats. SCI+BMC group showed no significant change when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent up regulation when compared to control (Figure-24, Table-22).

Real Time-PCR analysis of GDNF in the spinal cord of control and experimental rats

Gene expression of GDNF mRNA showed significant ($p<0.001$) down regulation in the spinal cord of SCI and rats compared to control. SCI+5-HT, SCI+GABA, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC group showed significant ($p<0.001$) reversal and up regulation when compared to SCI rats. SCI+BMC group showed no significant change when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent up regulation when compared to control and all treatment groups (Figure-25, Table-23).

Real Time-PCR analysis of IGF-1 in the spinal cord of control and experimental rats

Gene expression of IGF-1 mRNA showed significant ($p<0.001$) up regulation in the spinal cord of SCI rats compared to control. SCI+5-HT, SCI+GABA, SCI+BMC, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC groups showed further significant ($p<0.001$) up regulation

when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent up regulation when compared to control and all treatment groups (Figure-26, Table-24).

Real Time-PCR analysis of Akt-1 in the spinal cord of control and experimental rats

Gene expression of Akt-1 mRNA showed significant ($p<0.001$) up regulation in the spinal cord of SCI rats compared to control. SCI+5-HT, SCI+GABA, SCI+BMC, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC groups showed further significant ($p<0.001$) up regulation when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent up regulation when compared to control and all treatment groups (Figure-27, Table-25).

Real Time-PCR analysis of cyclin D2 in the spinal cord of control and experimental rats

Gene expression of cyclin D2 mRNA showed significant ($p<0.001$) up regulation in the spinal cord of SCI rats compared to control. SCI+5-HT, SCI+GABA and SCI+BMC treated rats showed a significant ($p<0.001$) reversal when compared to SCI rats. The combination treatment group of rats, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats showed significant reversal and down regulation ($p<0.001$) in the gene expression of cyclin D2 mRNA when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal and down regulation compared to control and treatment groups (Figure-28, Table-26).

IP3 content in the spinal cord of control and experimental rats

IP3 content in the spinal cord was significantly ($p<0.001$) decreased in SCI rats when compared to control. SCI+5-HT ($p<0.01$), SCI+GABA ($p<0.01$),

SCI+GABA+BMC ($p<0.001$) and SCI+5-HT+GABA+BMC ($p<0.001$) treatment groups showed significant reversal in the IP3 content when compared to SCI rats. SCI+BMC rats showed no significant change when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal and increase in IP3 content compared to control and all treatment groups (Figure-29, Table-27).

cAMP content in the spinal cord of control and experimental rats

cAMP content in the spinal cord was significantly ($p<0.001$) decreased in SCI rats when compared to control. SCI+5-HT ($p<0.01$), SCI+GABA ($p<0.01$), SCI+5-HT+BMC ($p<0.01$), SCI+GABA+BMC ($p<0.01$) and SCI+5-HT+GABA+BMC ($p<0.001$) treatment groups significantly reversed the cAMP content when compared to SCI rats. SCI+BMC showed no significant reversal when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal compared to SCI and other treatment groups of rats (Figure-30, Table-28).

cGMP content in the spinal cord of control and experimental rats

cGMP content in the spinal cord was significantly ($p<0.001$) decreased in SCI rats when compared to control. SCI+5-HT, SCI+GABA and SCI+BMC showed no significant reversal when compared to SCI rats. SCI+5-HT+BMC ($p<0.05$), SCI+GABA+BMC ($p<0.05$) and SCI+5-HT+GABA+BMC ($p<0.001$) significantly reversed the cGMP content when compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed prominent reversal compared to SCI and other treatment groups of rats (Figure-31, Table-29).

CONFOCAL STUDIES

Muscarinic M1 receptor antibody staining in the spinal cord of control and experimental rats

Muscarinic M1 receptor subunit antibody staining in the spinal cord showed a significant ($p<0.001$) decrease in the mean pixel value in SCI rats compared to

control. SCI+5-HT ($p<0.01$), SCI+GABA ($p<0.01$), SCI+5-HT+BMC ($p<0.001$), SCI+GABA+BMC ($p<0.001$) and SCI+5-HT+GABA+BMC ($p<0.001$) rats significantly reversed the muscarinic M1 receptor expression in the spinal cord when compared to SCI rats. SCI+BMC showed no significant reversal when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal compared to SCI and other treatment groups of rats (Figure-32 a&b, Table-30).

Muscarinic M3 receptor antibody staining in the spinal cord of control and experimental rats

Muscarinic M3 receptor subunit antibody staining in the spinal cord showed a significant ($p<0.001$) decrease in the mean pixel value of SCI rats compared to control. SCI+5-HT, SCI+GABA, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats significantly ($p<0.001$) reversed the muscarinic M3 receptor expression in the spinal cord when compared to SCI rats. SCI+BMC showed no significant reversal when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal SCI and other treatment groups of rats (Figure-33 a&b, Table-31).

α7 nicotinic acetylcholine receptor antibody staining in the spinal cord of control and experimental rats

α7 nicotinic acetylcholine receptor subunit antibody staining in the spinal cord showed a significant ($p<0.001$) decrease in the mean pixel value of SCI rats compared to control. SCI+5-HT, SCI+GABA, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats significantly ($p<0.001$) reversed the α7 nicotinic acetylcholine receptor expression in the spinal cord when compared to SCI rats. SCI+BMC showed no significant reversal when compared to SCI rats. SCI+5-HT+GABA+BMC rats showed prominent reversal SCI and other treatment groups of rats (Figure-34 a&b, Table-32).

Brdu-NeuN co-labelling studies in the spinal cord of control and experimental rats

Brdu positive BMC was visualized in red while the NeuN labeled neurons were visualized in green colour. Brdu-NeuN co-labelled cells appeared yellow in colour. Our results proved that BMC differentiate to neuronal cells once the accurate conditions are given. When autologous BMC treatment was given to spinal cord, they differentiated to neurons. Proliferating BMC were tagged by Brdu which expressed neuronal marker NeuN when administered along with 5-Ht and GABA. 5-HT and GABA along with BMC expressed maximum number of co-labelled cells (Figure-35 a,b,c&d, Table-33).

Cerebral Cortex

Total muscarinic receptor analysis

Scatchard analysis of [^{3}H] QNB binding against atropine to total muscarinic receptor in the cerebral cortex of control and experimental rats

The total muscarinic receptor was assayed using the specific ligand, [^{3}H] QNB and muscarinic general antagonist atropine. The Scatchard analysis showed that the B_{max} and K_d decreased significantly ($p<0.001$) in SCI rats compared to control group. SCI+5-HT+GABA+BMC rats significantly ($p<0.001$) reversed B_{max} and K_d when compared to SCI group (Figure-36, Table-34).

Muscarinic M1 receptor analysis

Scatchard analysis of [^{3}H] QNB binding against pirenzepine to muscarinic M1 receptor in the cerebral cortex of control and experimental rats

Binding analysis of muscarinic M1 receptor was done using [^{3}H] QNB and M1 subtype specific antagonist pirenzepine. The B_{max} decreased and K_d increased significantly ($p<0.001$) in SCI group when compared to control group. In SCI+5-HT+GABA+BMC rats, B_{max} ($p<0.05$) and K_d ($p<0.001$) significantly reversed when compared to SCI group (Figure-37, Table-35).

Muscarinic M3 receptor analysis

Scatchard analysis of [^{3}H] DAMP binding against 4-DAMP mustard to muscarinic M3 receptor in the cerebral cortex of control and experimental rats

Binding analysis of muscarinic M3 receptors was done using [^{3}H] DAMP and M3 subtype specific antagonist 4-DAMP mustard. The B_{max} and K_d were

increased significantly ($p<0.001$) in SCI group when compared to control. In SCI+5-HT+GABA+BMC rats, B_{max} and K_d were significantly ($p<0.001$) reversed when compared to SCI group (Figure-38, Table-36).

REAL TIME-PCR ANALYSIS

Real Time PCR analysis of acetylcholine esterase in the cerebral cortex of control and experimental rats

Gene expression of acetylcholine esterase mRNA showed significant ($p<0.001$) up regulation in the cerebral cortex of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment significantly ($p<0.001$) reversed the up regulated expression when compared to SCI rats (Figure-39, Table-37).

Real Time-PCR analysis of choline acetyl transferase in the cerebral cortex of control and experimental rats

Gene expression of choline acetyl transferase mRNA showed significant ($p<0.001$) up regulation in the cerebral cortex of SCI rats compared to control. SCI+5-HT+GABA+BMC rats showed further significant ($p<0.001$) up regulation when compared to control and SCI rats (Figure-40, Table-38).

Real Time-PCR analysis of muscarinic M1 receptor in the cerebral cortex of control and experimental rats

Real Time-PCR analysis showed that the muscarinic M1 receptor gene expression was down regulated significantly ($p<0.001$) in SCI compared to control rats and it was reversed and up regulated significantly ($p<0.001$) in SCI+5-HT+GABA+BMC rats (Figure-41, Table-39).

Real Time-PCR analysis of muscarinic M2 receptor in the cerebral cortex of control and experimental rats

Real Time-PCR analysis showed that the muscarinic M2 receptor gene expression was down regulated significantly ($p<0.001$) in SCI rats compared to control and it was reversed and up regulated significantly ($p<0.001$) in SCI+5-HT+GABA+BMC rats (Figure-42, Table-40).

Real Time-PCR analysis of muscarinic M3 receptor in the cerebral cortex of control and experimental rats

Real Time-PCR analysis showed that the muscarinic M3 receptor gene expression was up regulated significantly ($p<0.001$) in SCI rats compared to control and it was reversed significantly ($p<0.001$) in SCI+5-HT+GABA+BMC rats (Figure-43, Table-41).

Real Time-PCR analysis of α7 nicotinic acetylcholine receptor in the cerebral cortex of control and experimental rats

Real Time-PCR analysis showed that α7 nicotinic acetylcholine receptor gene expression did not show significant change in SCI rats when compared to control. It was also significantly ($p<0.001$) up regulated in SCI+5-HT+GABA+BMC rats when compared to control and SCI rats (Figure-44, Table-42).

Real Time-PCR analysis of phospholipase C receptor in the cerebral cortex of control and experimental rats

Real Time-PCR analysis showed that the phospholipase C receptor gene expression was up regulated significantly ($p<0.001$) in SCI rats when compared to control. It was further significantly ($p<0.001$) up regulated in SCI+5-HT+GABA+BMC rats when compared to control and SCI rats (Figure-45, Table-43).

Real Time-PCR analysis of CREB in the cerebral cortex of control and experimental rats

Gene expression of CREB mRNA showed significant ($p<0.001$) down regulation in the cerebral cortex of SCI rats compared to control. SCI+5-HT+GABA+BMC rats showed significant ($p<0.001$) reversal and up regulation when compared to control (Figure-46, Table-44).

Real Time-PCR analysis of Bax in the cerebral cortex of control and experimental rats

Gene expression of Bax mRNA showed significant ($p<0.001$) up regulation in the cerebral cortex of SCI rats compared to control. SCI+5-HT+GABA+BMC rats showed significant ($p<0.001$) reversal and down regulation when compared to control (Figure-47, Table-45).

Real Time-PCR analysis of caspase-8 in the cerebral cortex of control and experimental rats

Gene expression of caspase-8 mRNA showed significant ($p<0.001$) up regulation in the cerebral cortex of SCI rats compared to control. SCI+5-HT+GABA+BMC rats showed significant ($p<0.001$) reversal and down regulation when compared to control (Figure-48, Table-46).

Real Time-PCR analysis of superoxide dismutase in the cerebral cortex of control and experimental rats

Gene expression of superoxide dismutase mRNA showed significant ($p<0.001$) up regulation in the cerebral cortex of SCI rats compared to control. SCI+5-HT+GABA+BMC rats showed further significant ($p<0.001$) up regulation when compared to control and SCI rats (Figure-49, Table-47).

Real Time-PCR analysis of glutathione peroxidase in the cerebral cortex of control and experimental rats

Gene expression of glutathione peroxidase mRNA showed significant ($p<0.001$) up regulation in the cerebral cortex of SCI rats compared to control. SCI+5-HT+GABA+BMC rats showed further significant ($p<0.001$) up regulation when compared to control and SCI rats (Figure-50, Table-48).

IP3 content in the cerebral cortex of control and experimental rats

IP3 content in the cerebral cortex was significantly ($p<0.001$) decreased in SCI rats. It was significantly ($p<0.001$) reversed in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats (Figure-51, Table-49).

cAMP content in the cerebral cortex of control and experimental rats

cAMP content in the cerebral cortex was significantly ($p<0.001$) decreased in SCI rats compared to control. It was significantly ($p<0.001$) reversed in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats (Figure-52, Table-50).

cGMP content in the cerebral cortex of control and experimental rats

cGMP content in the cerebral cortex was significantly ($p<0.001$) decreased in SCI rats compared to control. It was significantly ($p<0.001$) reversed in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats (Figure-53, Table-51).

CONFOCAL STUDIES

Muscarinic M1 receptor antibody staining in the cerebral cortex of control and experimental rats

Muscarinic M1 receptor subunit antibody staining in the cerebral cortex showed a significant ($p<0.001$) decrease in the mean pixel value in SCI rats compared to control. SCI+5-HT+GABA+BMC rats significantly ($p<0.001$) reversed the

muscarinic M1 receptor expression in the cerebral cortex when compared to SCI rats (Figure-54, Table-52).

Muscarinic M3 receptor antibody staining in the cerebral cortex of control and experimental rats

Muscarinic M3 receptor subunit antibody staining in the cerebral cortex showed a significant ($p<0.001$) increase in the mean pixel value in SCI rats compared to control. SCI+5-HT+GABA+BMC rats significantly ($p<0.001$) reversed the muscarinic M3 receptor expression in the cerebral cortex when compared to SCI rats (Figure-55, Table-53).

α7 nicotinic acetylcholine receptor antibody staining in the cerebral cortex of control and experimental rats

α7 nicotinic acetylcholine receptor subunit antibody staining in the cerebral cortex showed a significant ($p<0.001$) decrease in the mean pixel value in SCI rats compared to control. SCI+5-HT+GABA+BMC rats showed a significant ($p<0.001$) reversal in the α7 nicotinic acetylcholine expression in the cerebral cortex when compared to SCI rats (Figure-56, Table-54).

Cerebellum

Total muscarinic receptor analysis

Scatchard analysis of [^{3}H] QNB binding against atropine to total muscarinic receptor in the cerebellum of control and experimental rats

The total muscarinic receptor was assayed using the specific ligand, [^{3}H] QNB and muscarinic general antagonist atropine. The Scatchard analysis showed that both B_{max} and K_d decreased significantly ($p<0.01$) in SCI rats compared to control group. In SCI+5-HT+GABA+BMC rats B_{max} was reversed significantly ($p<0.001$) when compared to SCI group. There was no significant change in K_d when compared to SCI group (Figure-57, Table-55).

Muscarinic M1 receptor analysis

Scatchard analysis of [^{3}H] QNB binding against pirenzepine to muscarinic M1 receptor in the cerebellum of control and experimental rats

Binding analysis of muscarinic M1 receptors was done using [^{3}H] QNB and M1 subtype specific antagonist pirenzepine. The B_{max} and K_d decreased significantly ($p<0.001$) in SCI group when compared to control. In SCI+5-HT+GABA+BMC treatment group B_{max} was reversed significantly ($p<0.01$) when compared to SCI rats. K_d did not show any significant change when compared to SCI rats (Figure-58, Table-56).

Muscarinic M3 receptor analysis

Scatchard analysis of [^{3}H] DAMP binding against muscarinic M3 receptor antagonist, 4-DAMP mustard in the cerebellum of control and experimental rats.

Binding analysis of muscarinic M3 receptors was done using [^{3}H] DAMP and M3 subtype specific antagonist 4-DAMP mustard. The B_{max} and K_d was increased significantly ($p<0.001$) in SCI group when compared to control. In SCI+5-HT+GABA+BMC treatment group, B_{max} and K_d significantly ($p<0.001$) reversed when compared to SCI rats (Figure-59, Table-57).

REAL TIME-PCR ANALYSIS

Real Time-PCR analysis of acetylcholine esterase in the cerebellum of control and experimental rats

Gene expression of acetylcholine esterase mRNA showed significant ($p<0.001$) up regulation in the cerebellum of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group significantly ($p<0.001$) reversed the altered expression when compared to SCI rats (Figure-60, Table-58).

Real Time-PCR analysis of choline acetyltransferase in the cerebellum of control and experimental rats

Gene expression of choline acetyltransferase mRNA showed significant ($p<0.001$) down regulation in the cerebellum of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group significantly ($p<0.001$) reversed the changes when compared to SCI rats (Figure-61, Table-59).

Real Time-PCR analysis of muscarinic M1 receptor in the cerebellum of control and experimental rats

Real Time-PCR analysis showed that the muscarinic M1 receptor gene expression was down regulated significantly ($p<0.001$) in SCI rats compared to

control and it was reversed significantly ($p<0.001$) in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats (Figure-62, Table-60).

Real Time-PCR analysis of muscarinic M2 receptor in the cerebellum of control and experimental rats

Real Time-PCR analysis showed that the muscarinic M2 receptor gene expression was down regulated significantly ($p<0.001$) in SCI rats compared to control and it reversed significantly ($p<0.001$) in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats (Figure-63, Table-61).

Real Time-PCR analysis of muscarinic M3 receptor in the cerebellum of control and experimental rats

Real Time-PCR analysis showed that the muscarinic M3 receptor gene expression was up regulated significantly ($p<0.001$) in SCI group compared to control and it was reversed significantly ($p<0.001$) in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats (Figure-64, Table-62).

Real Time-PCR analysis of α7 nicotinic acetylcholine receptor in the cerebellum of control and experimental rats

Real Time-PCR analysis showed that α7 nicotinic acetylcholine receptor gene expression was down regulated significantly ($p<0.001$) in SCI rats when compared to control and it was reversed significantly ($p<0.001$) in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats (Figure-65, Table-63).

Real Time-PCR analysis of phospholipase C in the cerebellum of control and experimental rats

Gene expression of phospholipase C mRNA showed significant ($p<0.001$) down regulation in the cerebellum of SCI rats compared to control. SCI+5-

HT+GABA+BMC treatment group showed significant ($p<0.001$) reversal and up regulation when compared to control rats (Figure-66, Table-64).

Real Time-PCR analysis of CREB in the cerebellum of control and experimental rats

Gene expression of CREB mRNA showed significant ($p<0.001$) down regulation in the cerebellum of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group showed significant ($p<0.001$) reversal when compared to SCI rats **(**Figure-67, Table-65**).**

Real Time-PCR analysis of Bax in the cerebellum of control and experimental rats

Gene expression of Bax mRNA showed significant ($p<0.001$) up regulation in the cerebellum of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group showed significant ($p<0.001$) reversal and down regulation when compared to control and SCI rats (Figure-68, Table-66).

Real Time-PCR analysis of caspase-8 in the cerebellum of control and experimental rats

Gene expression of caspase-8 mRNA showed significant ($p<0.001$) up regulation in the cerebellum of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group showed significant ($p<0.001$) reversal and down regulation when compared to SCI rats **(**Figure-69, Table-67**)**.

Real Time-PCR analysis of superoxide dismutase in the cerebellum of control and experimental rats

Gene expression of superoxide dismutase mRNA showed significant ($p<0.001$) down regulation in the cerebellum of SCI rats compared to control.

SCI+5-HT+GABA+BMC treatment group showed significant ($p<0.001$) reversal and up regulation when compared to control and SCI rats **(**Figure-70, Table-68**).**

Real Time-PCR analysis of Glutathione peroxidase in the cerebellum of control and experimental rats

Gene expression of glutathione peroxidase mRNA showed significant ($p<0.001$) down regulation in the cerebellum of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group showed significant ($p<0.001$) reversal and up regulation when compared to control and SCI rats **(**Figure-71, Table-69**).**

IP3 content in the cerebellum of control and experimental rats

IP3 content in the cerebellum was significantly ($p<0.001$) decreased in SCI rats compared to control. It was significantly ($p<0.001$) reversed and increased in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats (Figure-72, Table-70).

cAMP content in the cerebellum of control and experimental rats

cAMP content in the cerebellum was significantly ($p<0.001$) decreased in SCI rats compared to control. It was significantly ($p<0.001$) reversed and increased in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats (Figure-73, Table-71).

cGMP content in the cerebellum of control and experimental rats

cGMP content in the cerebellum was significantly ($p<0.001$) decreased in SCI rats compared to control. It was significantly ($p<0.001$) reversed and increased in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats (Figure-74, Table-72).

CONFOCAL STUDIES

Muscarinic M1 receptor antibody staining in the cerebellum of control and experimental rats

Muscarinic M1 receptor subunit antibody staining in the cerebellum showed a significant ($p<0.001$) decrease in the mean pixel value of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group significantly ($p<0.001$) reversed the muscarinic M1 receptor expression in the cerebellum when compared to SCI rats (Figure-75, Table-73).

Muscarinic M3 receptor antibody staining in the cerebellum of control and experimental rats

Muscarinic M3 receptor subunit antibody staining in the cerebellum showed a significant ($p<0.001$) increase in the mean pixel value of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group significantly ($p<0.001$) reversed the muscarinic M3 receptor expression in the cerebellum when compared to SCI rats (Figure -76, Table -74).

α7 nicotinic acetylcholine receptor antibody staining in the cerebellum of control and experimental rats

α7 nicotinic acetylcholine receptor subunit antibody staining in the cerebellum showed a significant ($p<0.001$) decrease in the mean pixel value of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group significantly ($p<0.001$) reversed the α7 nicotinic acetylcholine receptor expression in the cerebellum when compared to SCI rats (Figure -77, Table -75).

Brain Stem

Total muscarinic receptor analysis

Scatchard analysis of [^{3}H] QNB binding atropine to against total muscarinic receptor in the brain stem of control and experimental rats

The total muscarinic receptor status was assayed using the specific ligand, [^{3}H] QNB and muscarinic general antagonist, atropine. The scatchard analysis showed that the B_{max} and K_d decreased significantly ($p<0.001$) in SCI rats compared to control. SCI+5-HT+GABA+BMC treated rats significantly reversed the B_{max} ($p<0.001$) and K_d ($p<0.01$) when compared to SCI group **(**Figure-78, Table-76**)**.

Muscarinic M1 receptor analysis

Scatchard analysis of [^{3}H] QNB binding against pirenzepine to muscarinic M1 receptor in the brain stem of control and experimental rats

Binding analysis of muscarinic M1 receptors was done using [^{3}H] QNB and M1 subtype specific antagonist pirenzepine. The B_{max} and K_d decreased significantly ($p<0.001$) in SCI rats when compared to control. In SCI+5-HT+GABA+BMC rats, B_{max} and K_d significantly ($p<0.001$) reversed when compared to SCI group **(**Figure-79, Table-77**)**.

Muscarinic M3 receptor analysis

Scatchard analysis of [^{3}H] DAMP binding against 4-DAMP mustard to muscarinic M3 receptor in the brain stem of control and experimental rats.

Binding analysis of muscarinic M3 receptors was done using [^{3}H] DAMP and M3 subtype specific antagonist 4-DAMP mustard. The B_{max} and K_d decreased significantly ($p<0.001$) in SCI group when compared to control group. B_{max} and K_d of SCI+5-HT+GABA+BMC rats significantly ($p<0.001$) reversed when compared to SCI group **(**Figure-80, Table-78**)**.

REAL TIME-PCR ANALYSIS

Real Time-PCR analysis of acetylcholine esterase in the brain stem of control and experimental rats

Gene expression of acetylcholine esterase mRNA showed significant ($p<0.001$) up regulation in the brain stem of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group significantly ($p<0.001$) reversed the expression when compared to SCI rats **(**Figure-81, Table-79**)**.

Real Time-PCR analysis of choline acetyl transferase in the brain stem of control and experimental rats

Gene expression of choline acetyltransferase mRNA showed significant ($p<0.001$) up regulation in the brain stem of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group further showed further significant ($p<0.001$) up regulation when compared to control and SCI rats **(**Figure-82, Table-80**)**.

Real Time-PCR analysis of muscarinic M1 receptor in the brain stem of control and experimental rats

Real Time-PCR analysis showed that the muscarinic M1 receptor gene expression was down regulated significantly ($p<0.001$) in SCI rats compared to control and it was significantly ($p<0.001$) reversed and up regulated in SCI+5-HT+GABA+BMC treatment group when compared to control and SCI rats **(**Figure-83, Table-81**)**.

Real Time-PCR analysis of muscarinic M2 receptor in the brain stem of control and experimental rats

Real Time-PCR analysis showed that the muscarinic M2 receptor gene expression was down regulated significantly ($p<0.001$) in SCI rats compared to control and it was significantly ($p<0.001$) reversed and up regulated in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats **(**Figure-84, Table-82**)**.

Real Time-PCR analysis of muscarinic M3 receptor in the brain stem of control and experimental rats

Real Time-PCR analysis showed that the muscarinic M3 receptor gene expression was down regulated significantly ($p<0.001$) in SCI rats compared to control and it was significantly ($p<0.001$) reversed in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats **(**Figure-85, Table-83**)**.

Real Time-PCR analysis of α7 nicotinic acetylcholine receptor in the brain stem of control and experimental rats

Real Time-PCR analysis showed that α7 nicotinic acetylcholine receptor gene expression down regulated significantly ($p<0.001$) in SCI rats compared to control and it was significantly ($p<0.001$) reversed in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats **(**Figure-86, Table-84**)**.

Real Time-PCR analysis of phospholipase C in the brain stem of control and experimental rats

Gene expression of phospholipase C mRNA showed significant ($p<0.001$) down regulation in the brain stem of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group showed significant ($p<0.001$) reversal and up regulation when compared to control and SCI rats **(**Figure-87, Table-85**)**.

Real Time-PCR analysis of CREB in the brain stem of control and experimental rats

Gene expression of CREB mRNA showed significant ($p<0.001$) down regulation in the brain stem of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group showed significant ($p<0.001$) reversal and up regulation when compared to SCI group **(**Figure-88, Table-86**)**.

Real Time-PCR analysis of Bax in the brain stem of control and experimental rats

Real Time-PCR analysis showed that the Bax gene expression up regulated significantly ($p<0.001$) in SCI rats compared to control and in SCI+5-HT+GABA+BMC treatment group, it was reversed and down regulated significantly ($p<0.001$) when compared to control and SCI rats **(**Figure-89, Table-87**)**.

Real Time-PCR analysis of caspase-8 in the brain stem of control and experimental rats

Real Time-PCR analysis showed that the caspase-8 gene expression up regulated significantly ($p<0.001$) in SCI group compared to control and it was significantly ($p<0.001$) reversed and down regulated in SCI+5-HT+GABA+BMC treatment group when compared to control and SCI rats **(**Figure-90, Table-88**)**.

Real Time-PCR analysis of superoxide dismutase in the brain stem of control and experimental rats

Gene expression of superoxide dismutase mRNA showed significant ($p<0.001$) down regulation in the brain stem of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group showed significant ($p<0.001$) reversal and up regulation when compared to control and SCI rats **(**Figure-91, Table-89**)**.

Real Time-PCR analysis of glutathione peroxidase in the brain stem of control and experimental rats

Gene expression of glutathione peroxidase mRNA showed significant ($p<0.05$) up regulation in the brain stem of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group showed further significant ($p<0.001$) up regulation when compared to both control and SCI rats **(**Figure-92, Table-90**)**.

IP3 content in the brain stem of control and experimental rats

IP3 content in the brain stem was significantly ($p<0.001$) decreased in SCI rats compared to control. It was significantly ($p<0.001$) reversed in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats (Figure-93, Table-91).

cAMP content in the brain stem of control and experimental rats

cAMP content in the brain stem was significantly ($p<0.001$) decreased in SCI rats compared to control. It was significantly ($p<0.001$) reversed in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats (Figure-94, Table-92).

cGMP content in the brain stem of control and experimental rats

cGMP content in the brain stem was significantly ($p<0.001$) decreased in SCI rats compared to control. It was significantly ($p<0.001$) reversed and increased in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats (Figure-95, Table-93).

CONFOCAL STUDIES

Muscarinic M1 receptor antibody staining in the brain stem of control and experimental rats

Muscarinic M1 receptor subunit antibody staining in the brainstem showed a significant ($p<0.001$) decrease in the mean pixel value in SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group significantly ($p<0.001$) reversed the muscarinic M1 receptor expression in the brain stem when compared to SCI rats (Figure-96, Table-94).

Muscarinic M3 receptor antibody staining in the brain stem of control and experimental rats

Muscarinic M3 receptor subunit antibody staining in the brainstem showed a significant ($p<0.001$) decrease in the mean pixel value in SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group significantly ($p<0.001$) reversed the muscarinic M3 receptor expression in the brain stem when compared to SCI rats (Figure-97, Table-95).

α7 nicotinic acetylcholine receptor antibody staining in the brain stem of control and experimental rats

α7 nicotinic acetylcholine receptor antibody staining in the brainstem showed a significant ($p<0.001$) decrease in the mean pixel value in SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group significantly ($p<0.001$) reversed the α7 nicotinic acetylcholine receptor expression in the brain stem when compared to SCI rats (Figure-98, Table-96).

Corpus Striatum

Total muscarinic receptor analysis

Scatchard analysis of [^{3}H] QNB binding against total muscarinic receptor antagonist, atropine in the corpus striatum of control and experimental rats

The total muscarinic receptor was assayed using the specific ligand, [^{3}H] QNB and muscarinic general antagonist, atropine. The scatchard analysis showed that the B_{max} decreased significantly ($p<0.01$) and K_d increased significantly ($p<0.001$) in SCI rats when compared to control. In SCI+5-HT+GABA+BMC treatment group, B_{max} was reversed and increased significantly ($p<0.001$) and K_d was significantly ($p<0.001$) increased compared to both SCI and control rats **(**Figure-99, Table-97**)**.

Muscarinic M1 receptor analysis

Scatchard analysis of [^{3}H] QNB binding against muscarinic M1 receptor antagonist, pirenzepine in the corpus striatum of control and experimental rats

Binding analysis of muscarinic M1 receptors was done using [^{3}H] QNB and M1 subtype specific antagonist pirenzepine. The B_{max} was decreased significantly ($p<0.001$) and K_d was increased significantly ($p<0.001$) in SCI group when compared to control group. In SCI+5-HT+GABA+BMC treatment group, B_{max} was reversed and increased significantly ($p<0.001$) and K_d was significantly ($p<0.001$) increased compared to both SCI and control rats **(**Figure-100, Table-98**)**.

Muscarinic M3 receptor analysis

Scatchard analysis of [^{3}H] DAMP binding against muscarinic M3 receptor antagonist, 4-DAMP mustard in the corpus striatum of control and experimental rats.

Binding analysis of muscarinic M3 receptors was done using [^{3}H] DAMP and M3 subtype specific antagonist, 4-DAMP mustard. The B_{max} was significantly ($p<0.001$) increased and K_d was significantly ($p<0.01$) decreased in SCI group when compared to control group. In SCI+5-HT+GABA+BMC treatment group, both B_{max} ($p<0.001$) and K_d ($p<0.01$) were significantly reversed when compared to SCI rats **(**Figure-101, Table-99**)**.

REAL TIME-PCR ANALYSIS

Real Time-PCR analysis of acetylcholine esterase in the corpus striatum of control and experimental rats

Gene expression of acetylcholine esterase mRNA showed significant ($p<0.001$) up regulation in the corpus striatum of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group significantly ($p<0.001$) reversed the changes when compared to SCI rats **(**Figure-102, Table-100**)**.

Real Time-PCR analysis of choline acetyl transferase in the corpus striatum of control and experimental rats

Gene expression of choline acetyl transferase mRNA showed significant ($p<0.001$) up regulation in the corpus striatum of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group showed further significant up regulation ($p<0.001$) when compared to SCI rats **(**Figure-103, Table-101**)**.

Real Time-PCR analysis of muscarinic M1 receptor in the corpus striatum of control and experimental rats

Real Time-PCR analysis showed that the muscarinic M1 receptor gene expression was significantly ($p<0.001$) down regulated in SCI rats compared to control and it was reversed and significantly up regulated ($p<0.001$) in SCI+5-HT+GABA+BMC treatment group when compared to control and SCI rats **(**Figure-104, Table-102**)**.

Real Time-PCR analysis of muscarinic M2 receptor in the corpus striatum of control and experimental rats

Real Time-PCR analysis showed that the muscarinic M2 receptor gene expression was significantly ($p<0.001$) down regulated in SCI rats compared to control and it was reversed and significantly up regulated ($p<0.001$) in SCI+5-HT+GABA+BMC treatment group when compared to control and SCI rats **(**Figure-105, Table-103**)**.

Real Time-PCR analysis of muscarinic M3 receptor in the corpus striatum of control and experimental rats

Real Time-PCR analysis showed that the muscarinic M3 receptor gene expression up regulated significantly ($p<0.001$) in SCI rats when compared to control. SCI+5-HT+GABA+BMC treatment group significantly ($p<0.001$) reversed the change when compared to SCI rats **(**Figure-106, Table-104**)**.

Real Time-PCR analysis of α7 nicotinic acetylcholine receptor in the corpus striatum of control and experimental rats

Real Time-PCR analysis showed that α7 nicotinic acetylcholine receptor gene expression was down regulated significantly ($p<0.001$) in SCI rats when compared to control. SCI+5-HT+GABA+BMC rats showed significant ($p<0.001$) reversal when compared to control and SCI group **(**Figure-107, Table-105**)**.

Real Time-PCR analysis of phospholipase C in the corpus striatum of control and experimental rats

Gene expression of phospholipase C mRNA showed significant ($p<0.001$) up regulation in the corpus striatum of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group showed further significant ($p<0.001$) up regulation when compared to SCI rats **(**Figure-108, Table-106**)**.

Real Time-PCR analysis of CREB in the corpus striatum of control and experimental rats

Gene expression of CREB mRNA showed significant ($p<0.001$) down regulation in the corpus striatum of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group significantly ($p<0.001$) reversed and up regulated CREB mRNA expression when compared to control and SCI rats **(**Figure-109, Table-107**)**.

Real Time-PCR analysis of Bax in the corpus striatum of control and experimental rats

Real Time-PCR analysis showed that Bax gene expression was up regulated significantly ($p<0.001$) in SCI rats compared to control and it was reversed significantly ($p<0.001$) in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats **(**Figure-110, Table-108**)**.

Real Time-PCR analysis of caspase-8 in the corpus striatum of control and experimental rats

Real Time-PCR analysis showed that caspase-8 gene expression up regulated significantly ($p<0.001$) in SCI rats when compared to control. SCI+5-HT+GABA+BMC treatment group showed significant ($p<0.001$) reversal when compared to SCI rats **(**Figure-111, Table-109**)**.

Real Time-PCR analysis of superoxide dismutase in the corpus striatum of control and experimental rats

Gene expression of superoxide dismutase mRNA showed significant ($p<0.001$) up regulation in the corpus striatum of SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group showed further significant ($p<0.001$) up regulation when compared to control and SCI rats **(**Figure-112, Table-110**)**.

Real Time-PCR analysis of glutathione peroxidase in the corpus striatum of control and experimental rats

Real Time-PCR analysis showed that glutathione peroxidase gene expression up regulated significantly ($p<0.001$) in SCI rats compared to control. SCI+5-HT+GABA+BMC treatment group showed further significant ($p<0.001$) up regulation when compared to control and SCI rats **(**Figure-113, Table-111**)**.

IP3 content in the corpus striatum of control and experimental rats

IP3 content in the corpus striatum was significantly ($p<0.001$) decreased in SCI rats compared to control. It was significantly ($p<0.001$) reversed in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats (Figure-114, Table-112).

cAMP content in the corpus striatum of control and experimental rats

cAMP content in the corpus striatum was significantly ($p<0.001$) decreased in SCI rats compared to control. It was significantly ($p<0.001$) reversed and increased in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats (Figure-115, Table-113).

cGMP content in the corpus striatum of control and experimental rats

cGMP content in the corpus striatum was significantly ($p<0.001$) decreased in SCI rats compared to control. It was significantly ($p<0.001$)

reversed and increased in SCI+5-HT+GABA+BMC treatment group when compared to SCI rats (Figure-116, Table-114).

CONFOCAL STUDIES

Muscarinic M1 receptor antibody staining in the corpus striatum of control and experimental rats

Muscarinic M1 receptor subunit antibody staining in the corpus striatum showed a significant ($p<0.001$) decrease in the mean pixel value in SCI rats compared to control. SCI+5-HT+GABA+BMC rats significantly ($p<0.001$) reversed and increased the muscarinic M1 receptor expression in the corpus striatum when compared to control and SCI rats (Figure-117, Table-115).

Muscarinic M3 receptor antibody staining in the corpus striatum of control and experimental rats

Muscarinic M3 receptor subunit antibody staining in the corpus striatum showed a significant ($p<0.001$) increase in the mean pixel value of SCI rats compared to control. SCI+5-HT+GABA+BMC rats significantly ($p<0.001$) reversed the muscarinic M3 receptor expression in the corpus striatum when compared to SCI rats (Figure-118, Table-116).

α7 nicotinic acetylcholine receptor antibody staining in the corpus striatum of control and experimental rats

α7 nicotinic acetylcholine receptor subunit antibody staining in the corpus striatum showed a significant ($p<0.001$) decrease in the mean pixel value of SCI rats compared to control. SCI+5-HT+GABA+BMC rats further significantly ($p<0.001$) reversed the α7 nicotinic acetylcholine receptor expression in the corpus striatum when compared to SCI rats (Figure-119, Table-117).

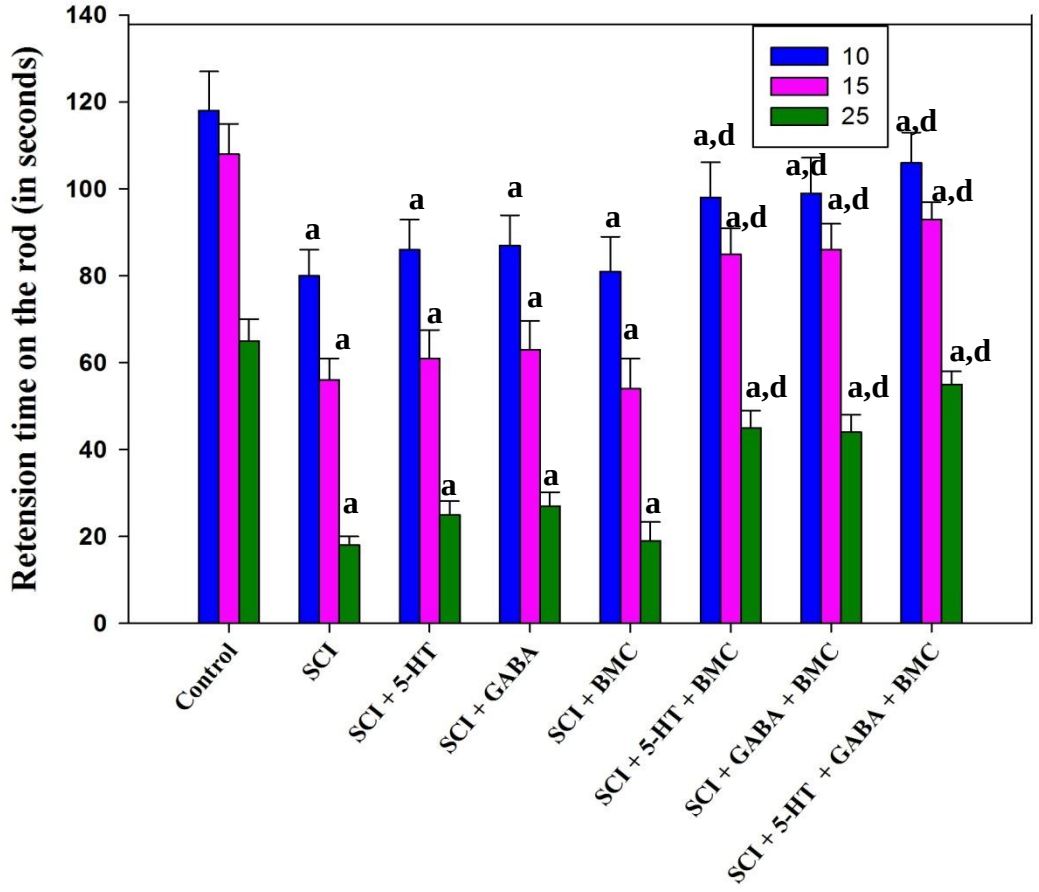
Table-1
Body weight of control and experimental rats

Experimental groups	Body weight in grams	
	Day 1	Day 21
Control	**241.2 ± 3.5**	**265.2 ± 5.3**
SCI	**225.9 ± 4.4[a]**	**193.5 ± 4.5[a]**
SCI+5-HT	**227.1 ± 4.1[a]**	**198.7 ±2.1[a]**
SCI+GABA	**228.4 ± 4.0[a]**	**197.8 ± 3.2[a]**
SCI+BMC	**221.6 ± 3.9[a]**	**192.1 ± 3.9[a]**
SCI+5-HT+BMC	**223.4 ± 3.3[a]**	**210.6 ± 5.6[a,d]**
SCI+GABA+BMC	**228.2 ± 4.4[a]**	**204.3 ± 1.2[a,d]**
SCI+5-HT+GABA+BMC	**230.7 ± 3.2 [a]**	**214.2 ± 3.8[a, d]**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

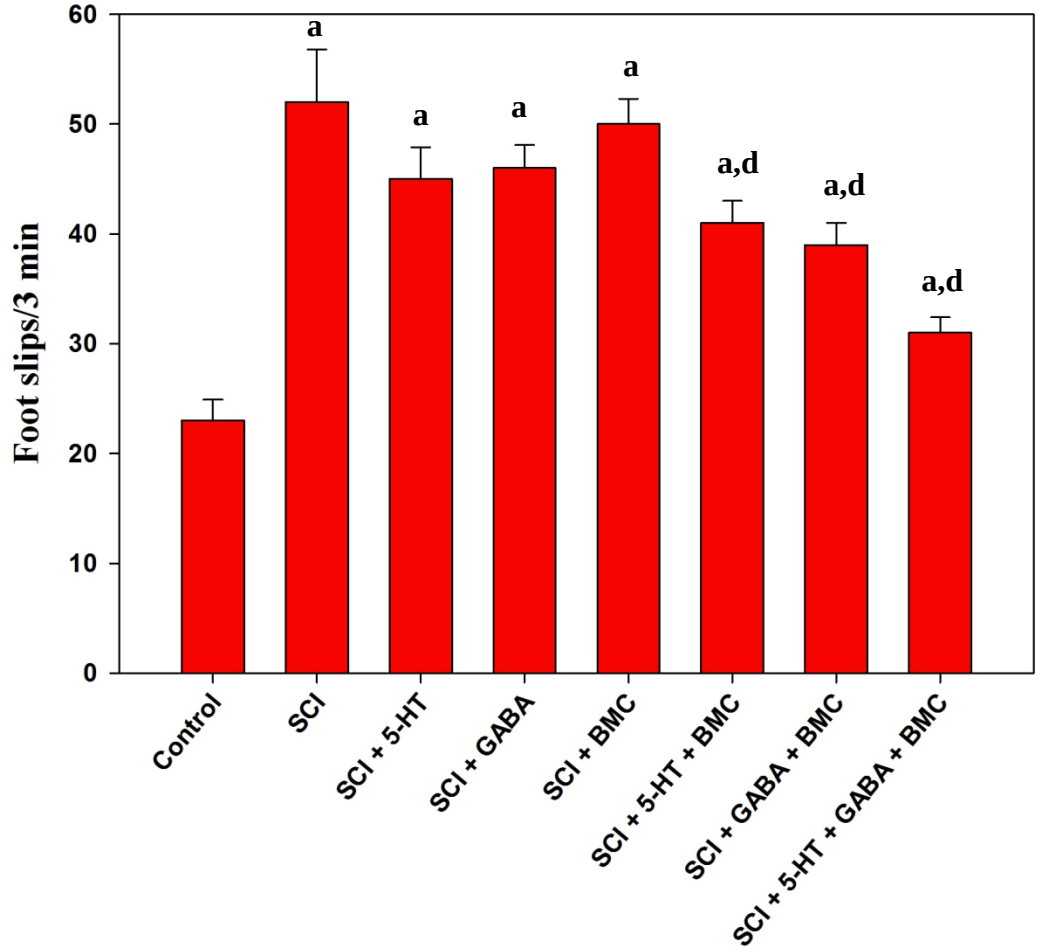
Figure-1

Time spent on metallic rod of control and experimental rats in rotarod experiment



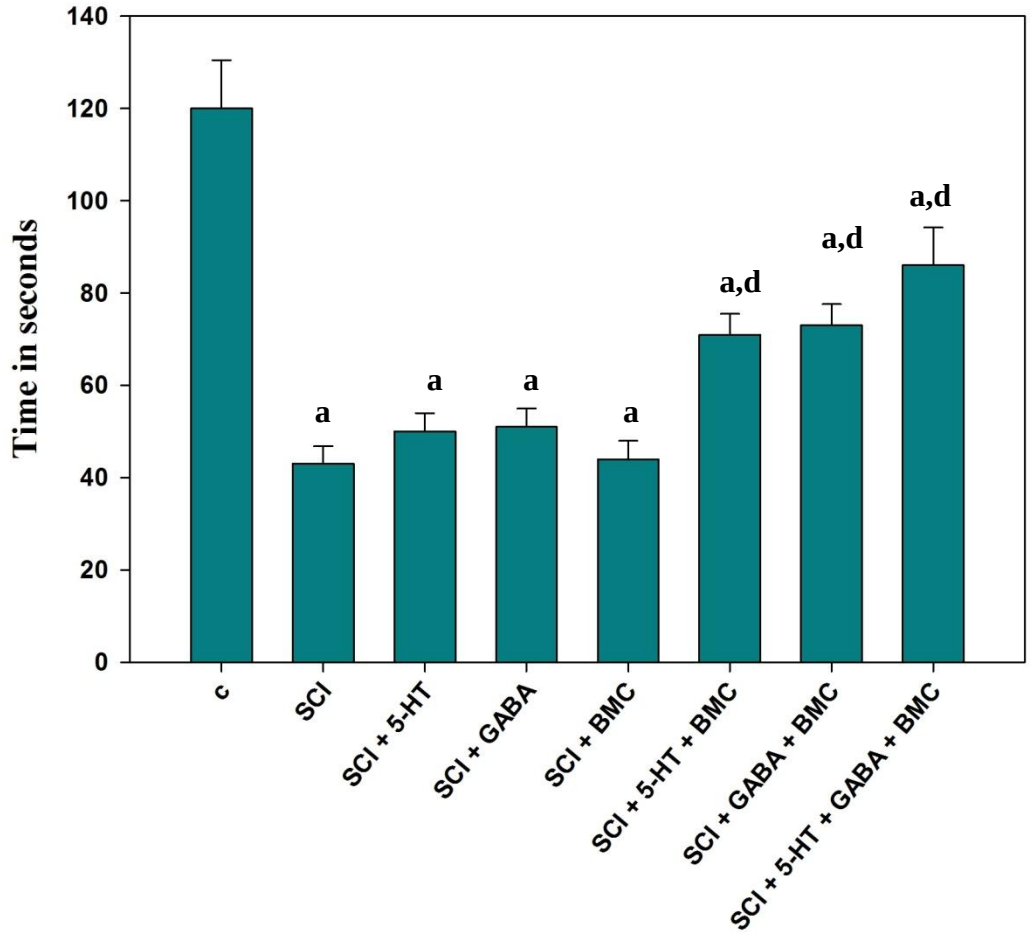
Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]p<0.001 when compared to Control. [d]p<0.001 when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-2
Behavioural response of control and experimental rats on grid walk test



Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]p<0.001 when compared to Control. [d]p<0.001 when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-3
Behavioural response of control and experimental rats on narrow beam test



Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]p<0.001 when compared to Control. [d]p<0.001 when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-4

Scatchard analysis of [^{3}H] QNB binding against atropine to total muscarinic receptor in the spinal cord of control and experimental rats

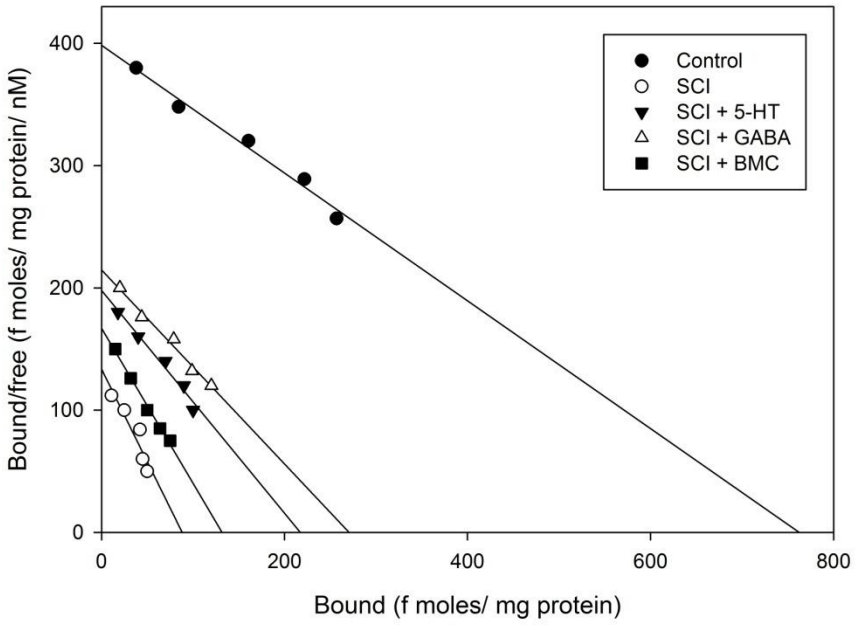


Table-2

Scatchard analysis of [^{3}H] QNB binding against atropine to total muscarinic receptor in the spinal cord of control and experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	**768.63 ± 13.44**	**1.92 ± 0. 08**
SCI	**94.64 ± 8.72** [a]	**0.72 ± 0.03** [a]
SCI + 5-HT	**219.95 ± 10.69**[a,d]	**1.11 ± 0.09**[a,d]
SCI + GABA	**274.18 ± 13.44**[a,d]	**1.27 ± 0.08**[a,d]
SCI + BMC	**131.87 ± 9.23**[a,d]	**0.79 ± 0.06** [a]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC.

Figure-5

Scatchard analysis of [^{3}H] QNB binding against atropine to total muscarinic receptor in the spinal cord of control and experimental rats

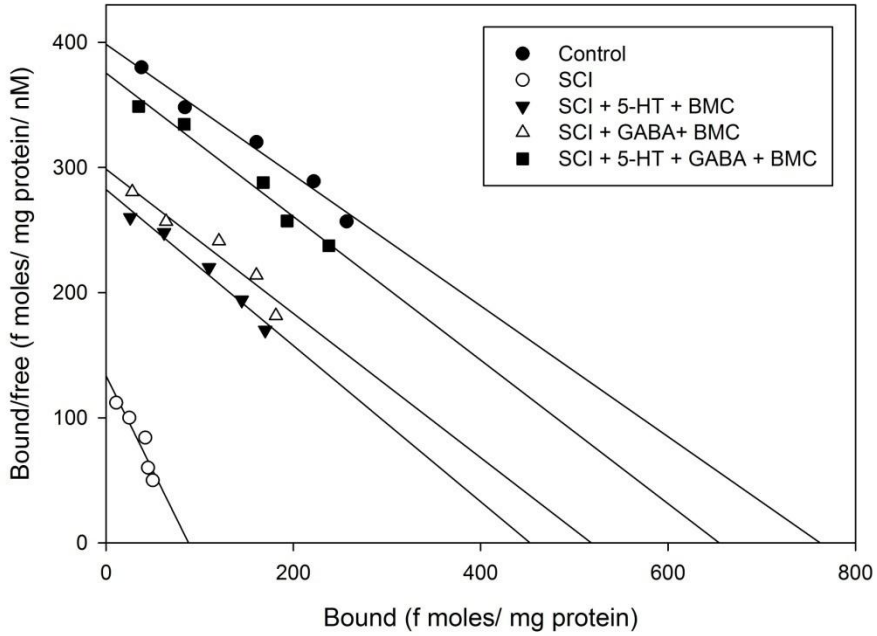


Table-3

Scatchard analysis of [^{3}H] QNB binding against atropine to total muscarinic receptor in the spinal cord of control and experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	**768.63 ± 13.44**	**1.92 ± 0. 08**
SCI	**94.64 ± 8.72**[a]	**0.72 ± 0.03**[a]
SCI + 5-HT + BMC	**453.56 ± 18.34**[a,d]	**1.63 ± 0.08**[a,d]
SCI + GABA + BMC	**524. 77 ± 15.34**[a,d]	**1.75 ± 0.07**[b,d]
SCI + 5-HT + GABA + BMC	**653.36 ± 21.27**[a,d]	**1.67 ±0.07**[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$,[b]$p<0.01$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT+BMC – SCI treated with Serotonin and BMC, SCI+GABA+BMC – SCI treated with GABA and BMC, SCI+5-HT+GABA+BMC– SCI treated with Serotonin, GABA and BMC.

Figure-6

Scatchard analysis of [^{3}H] QNB binding against pirenzepine to muscarinic M1 receptor in the spinal cord of control and experimental rats

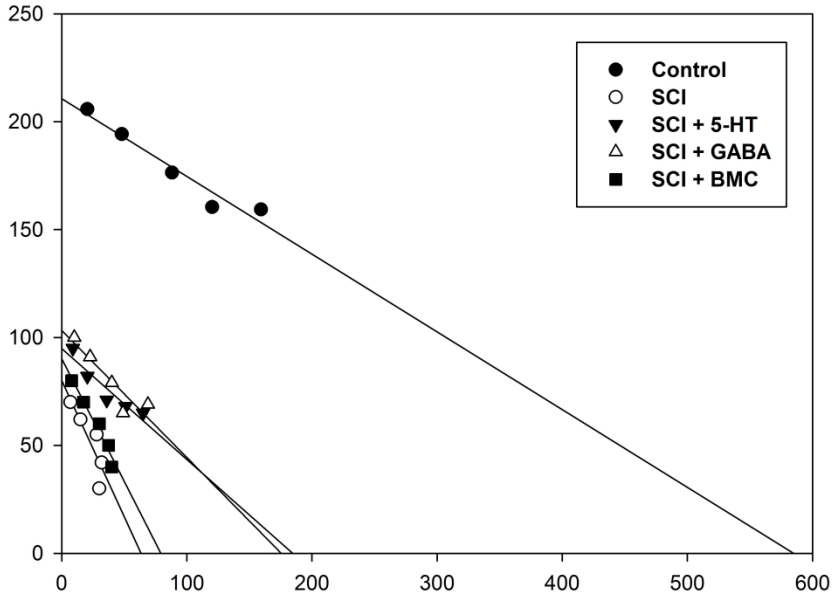


Table-4

Scatchard analysis of [^{3}H] QNB binding against pirenzepine to muscarinic M1 receptor in the spinal cord of control and experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	**584. 02 ± 15.65**	**2.78 ± 0.17**
SCI	**68.40 ± 2.13** [a]	**0.95 ± 0.06** [a]
SCI + 5-HT	**185. 20 ± 8.35** [a,d]	**2.03 ± 0. 16** [a,d]
SCI + GABA	**175.08 ± 6.57** [a,d]	**1.78 ±0.12** [a,d]
SCI + BMC	**83. 64 ± 3.56** [a,d]	**0.94 ± 0.05** [a]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC.

Figure-7

Scatchard analysis of [^{3}H] QNB binding against pirenzepine to muscarinic M1 receptor in the spinal cord of control and experimental rats

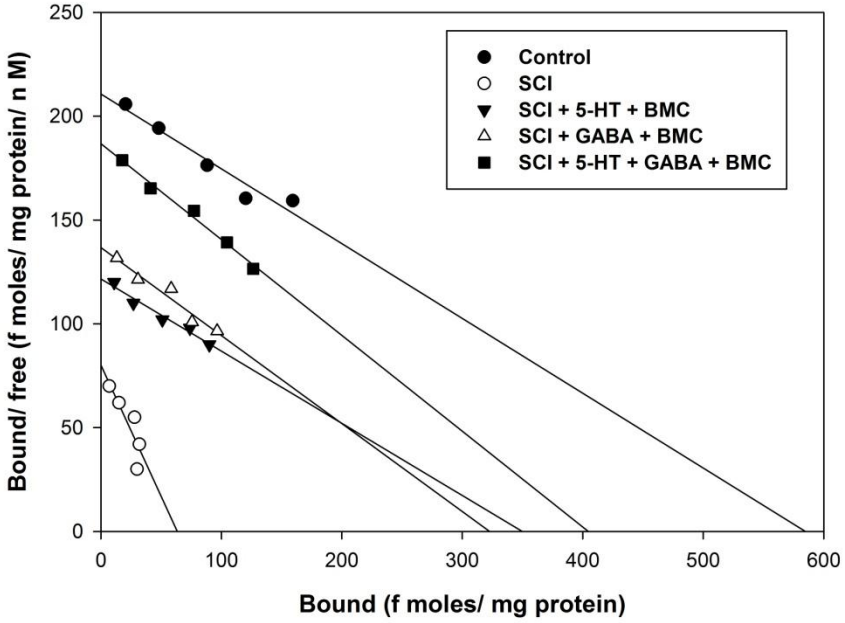


Table-5

Scatchard analysis of [^{3}H] QNB binding against pirenzepine to muscarinic M1 receptor in the spinal cord of control and experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	**584. 02 ± 15.65**	**2.78 ± 0.17**
SCI	**68.40 ± 2.13** [a]	**0.95 ± 0.06** [a]
SCI + 5-HT + BMC	**347. 34 ± 10.67**[a,d]	**2.89 ± 0.19**[d]
SCI + GABA + BMC	**322. 12± 8.87**[a,d]	**2.46 ±0.17**[b,d]
SCI + 5-HT + GABA + BMC	**408. 25 ± 13.21**[a,d]	**2.18 ± 0.15**[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$, [b]$p<0.01$ when compared to Control. [d]$p<0.001$ when compared to SCI group
C – Control, SCI – Spinal cord injured rats, SCI+5-HT+BMC – SCI treated with Serotonin and BMC, SCI+GABA+BMC – SCI treated with GABA and BMC, SCI+5-HT+GABA+BMC– SCI treated with Serotonin, GABA and BMC.

Figure-8

Scatchard analysis of [^{3}H] DAMP binding against 4-DAMP mustard to muscarinic M3 receptor in the spinal cord of control and experimental rats

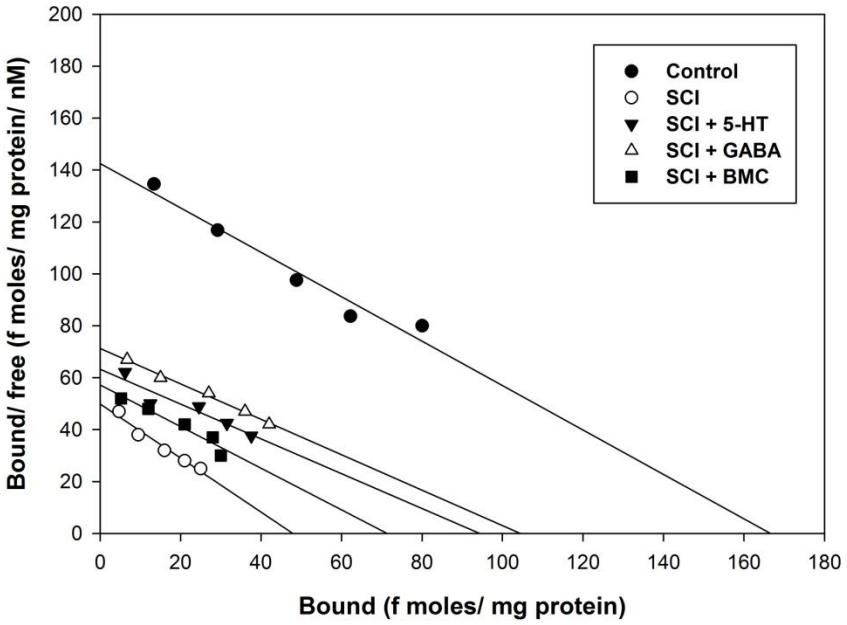


Table-6

Scatchard analysis of [^{3}H] DAMP binding against 4-DAMP mustard to muscarinic M3 receptor in the spinal cord of control and experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	**167.61 ± 5.47**	**1.18 ± 0.08**
SCI	**49.48 ± 3.84**[a]	**1.23 ± 0.06**
SCI + 5-HT	**94.44 ± 7.31**[a,d]	**1.31 ± 0.07**[c]
SCI + GABA	**102.82 ± 8.36**[a,d]	**1.43 ± 0.11**[a,d]
SCI + BMC	**72.35 ± 2.81**[a,d]	**1.28 ± 0.09**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$, [c]$p<0.05$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC.

Figure-9

Scatchard analysis of [^{3}H] DAMP binding against 4-DAMP mustard to muscarinic M3 receptor in the spinal cord of control and experimental rats

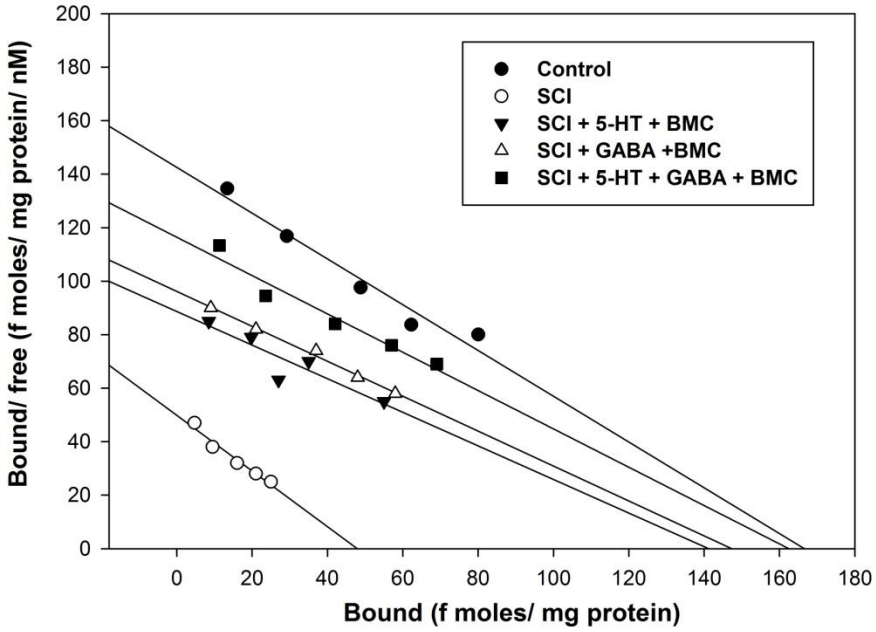


Table-7

Scatchard analysis of [^{3}H] DAMP binding against 4-DAMP mustard to muscarinic M3 receptor in the spinal cord of control and experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	**167.61 ± 5.47**	**1.18 ± 0.08**
SCI	**49.48 ± 3.84** [a]	**1. 23 ± 0.06**
SCI + 5-HT + BMC	**140.76 ± 11.34** [c,d]	**1.36 ± 0.11** [c]
SCI + GABA + BMC	**147.58 ± 10.97** [c,d]	**1.37 ± 0. 10** [c]
SCI + 5-HT + GABA + BMC	**162. 67 ± 13.23** [d]	**1.23 ± 0.07**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$, [c]$p<0.05$ when compared to Control [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT+BMC – SCI treated with Serotonin and BMC, SCI+GABA+BMC – SCI treated with GABA and BMC, SCI+5-HT+GABA+BMC– SCI treated with Serotonin, GABA and BMC.

Figure-10

Real Time PCR amplification of acetylcholine esterase mRNA in the spinal cord of control and experimental rats

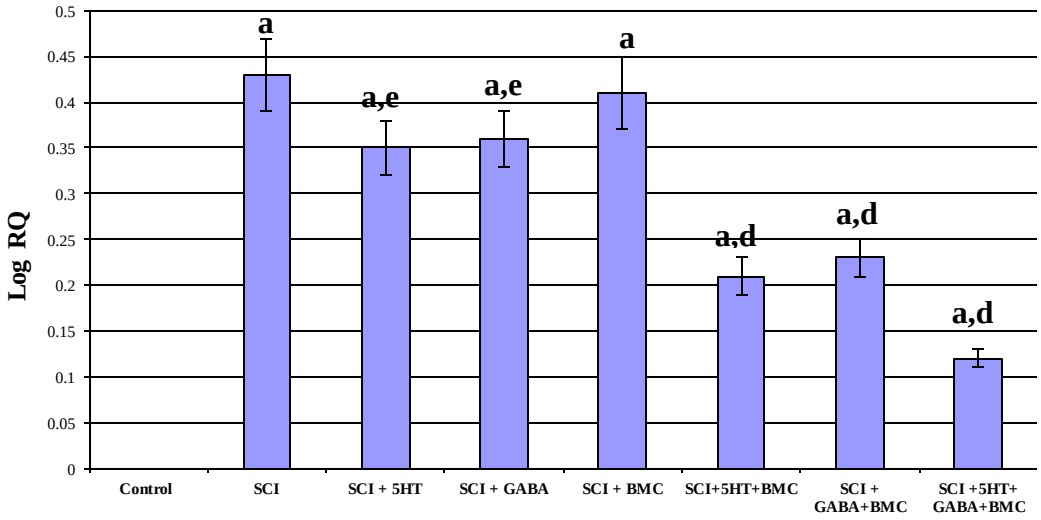


Table-8

Real Time PCR amplification of acetylcholine esterase mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**0.43 ± 0.03** [a]
SCI +5-HT	**0.35 ± 0.02** [a,e]
SCI +GABA	**0.36 ± 0.03** [a,e]
SCI +BMC	**0.41 ± 0.03** [a]
SCI +5-HT+BMC	**0.21 ± 0.02** [a,d]
SCI+GABA+BMC	**0.23 ± 0.01** [a,d]
SCI +5-HT+GABA+BMC	**0.12 ± 0.01** [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$, [e]$p<0.01$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-11

Real Time PCR amplification of choline acetyl transferase mRNA in the spinal cord of control and experimental rats

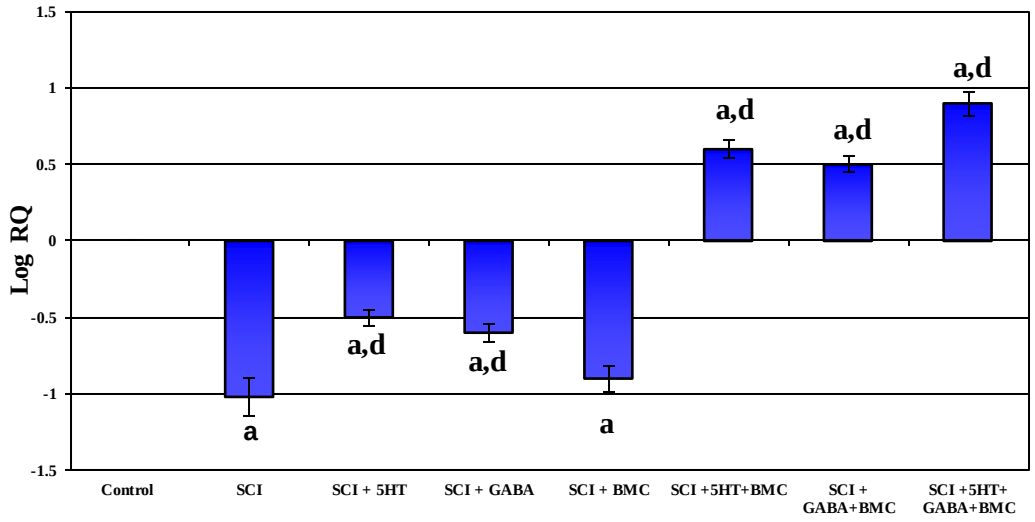


Table-9

Real Time PCR amplification of choline acetyl transferase mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**-1.02 ± 0. 09[a]**
SCI +5-HT	**-0.50± 0.04[a,d]**
SCI +GABA	**-0.61± 0.05[a,d]**
SCI +BMC	**-0.91± 0.08[a]**
SCI +5-HT+BMC	**0.62± 0.05[a,d]**
SCI+GABA+BMC	**0.50± 0.03[a,d]**
SCI +5-HT+GABA+BMC	**0.91± 0.07[a,d]**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]p<0.001 when compared to Control. [d]p<0.001 when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-12

Real Time PCR amplification of muscarinic M1 receptor mRNA in the spinal cord of control and experimental rats

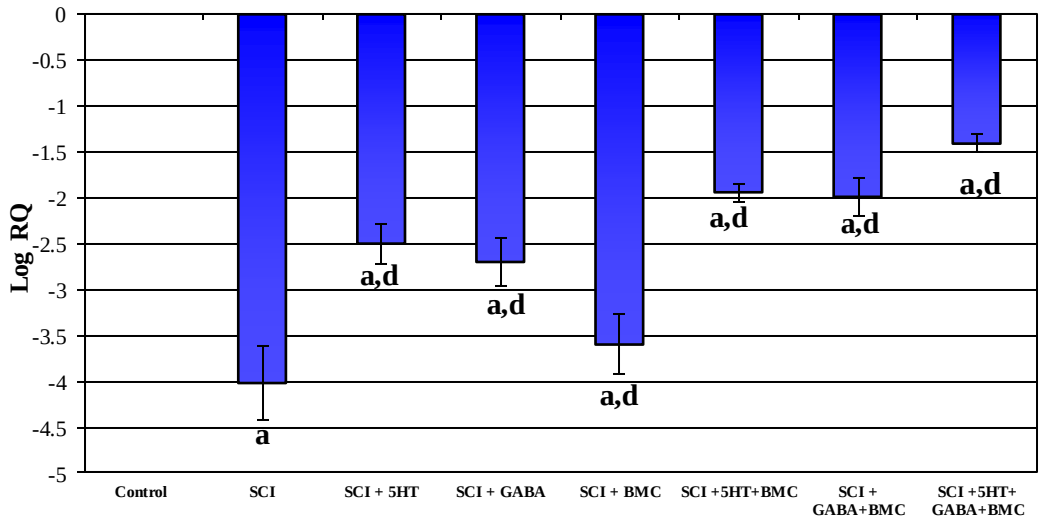


Table-10

Real Time PCR amplification of muscarinic M1 receptor mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**-4.02± 0.03** [a]
SCI +5-HT	**-2.52 ± 0.02** [a,d]
SCI +GABA	**-2.73 ± 0.02** [a,d]
SCI +BMC	**-3.66 ± 0.03** [a,d]
SCI +5-HT+BMC	**-1.94± 0.01** [a,d]
SCI+GABA+BMC	**-1.99± 0.01** [a,d]
SCI +5-HT+GABA+BMC	**-1.41± 0.01** [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-13

Real Time PCR amplification of muscarinic M2 receptor mRNA in the spinal cord of control and experimental rats

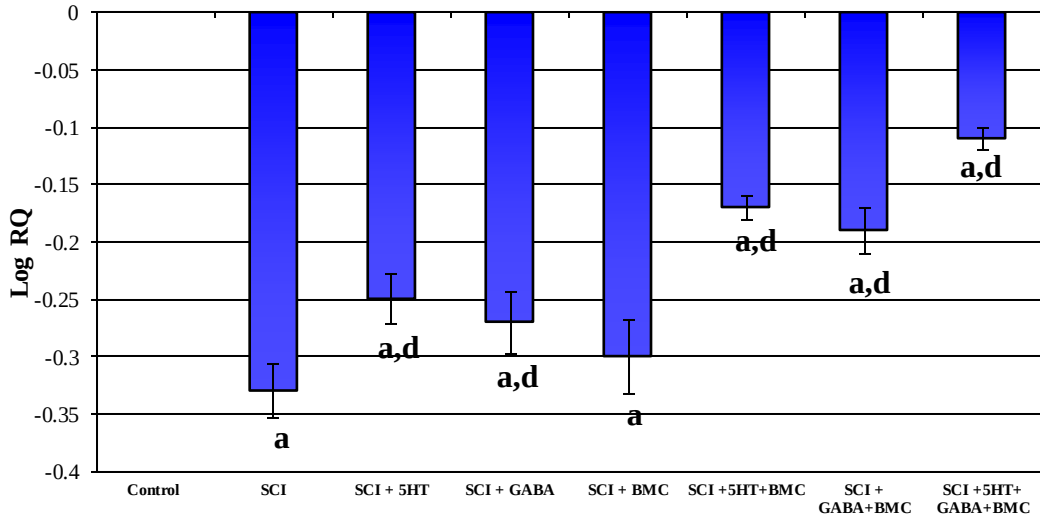


Table-11

Real Time PCR amplification of muscarinic M2 receptor mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**-0.33 ± 0.03** [a]
SCI +5-HT	**-0.25 ± 0.02** [a,d]
SCI +GABA	**-0.27 ± 0.02** [a,d]
SCI +BMC	**-0.30 ± 0.02** [a]
SCI +5-HT+BMC	**-0.17 ± 0.01** [a,d]
SCI+GABA+BMC	**-0.19 ± 0.01** [a,d]
SCI +5-HT+GABA+BMC	**-0.11 ± 0.01** [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-14

Real Time PCR amplification of muscarinic M3 receptor mRNA in the spinal cord of control and experimental rats

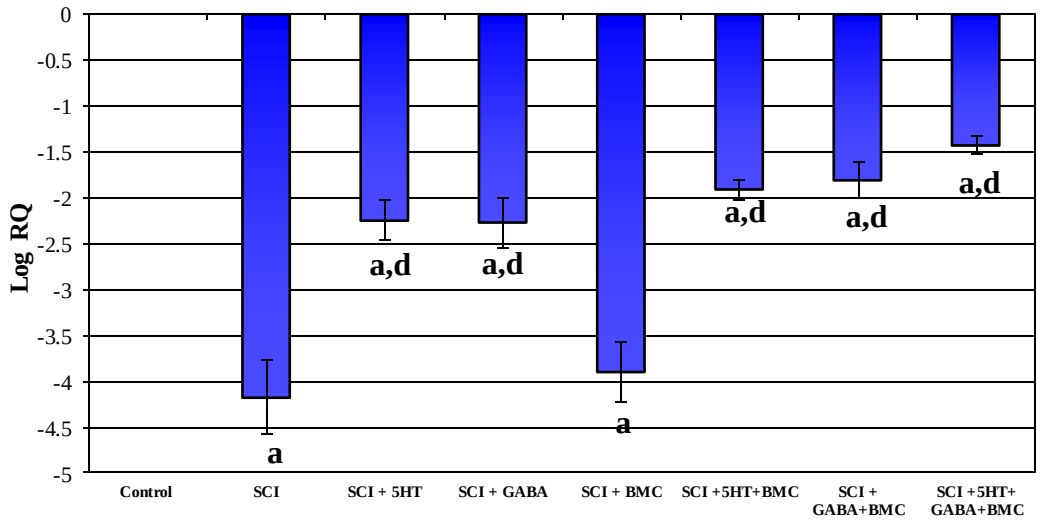


Table-12

Real Time PCR amplification of muscarinic M3 receptor mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**-4.18 ± 0.04** [a]
SCI +5-HT	**-2.25 ± 0.02** [a,d]
SCI +GABA	**-2.27 ± 0.02** [a,d]
SCI +BMC	**-3.9 ± 0.03** [a]
SCI +5-HT+BMC	**-1.91 ± 0.01** [a,d]
SCI+GABA+BMC	**-1.81 ± 0.01** [a,d]
SCI +5-HT+GABA+BMC	**-1.43 ± 0.01** [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-15

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the spinal cord of control and experimental rats

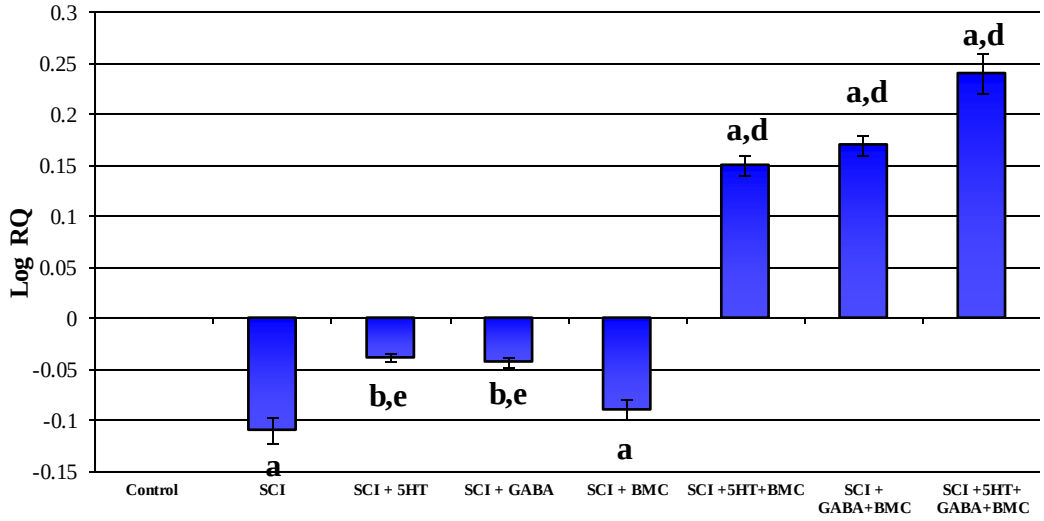


Table-13

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**-0.11 ± 0.01[a]**
SCI +5-HT	**-0.04 ± 0.03[b,e]**
SCI +GABA	**-0.04 ± 0.04[b,e]**
SCI +BMC	**-0.09 ± 0.01[a]**
SCI +5-HT+BMC	**0.15 ± 0.01[a,d]**
SCI+GABA+BMC	**0.17 ± 0.01[a,d]**
SCI +5-HT+GABA+BMC	**0.24 ± 0.02[a,d]**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]p<0.001, [b]p<0.01 when compared to Control. [d]p<0.001, [e]p<0.01 when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-16

Real Time PCR amplification of phospholipase C mRNA in the spinal cord of control and experimental rats

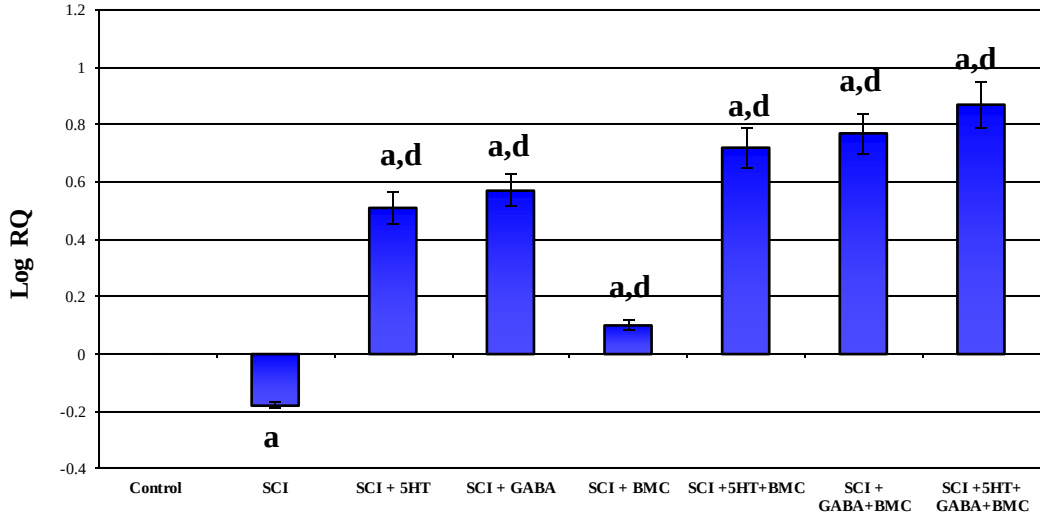


Table-14

Real Time PCR amplification of phospholipase C mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**-0.18 ± 0.1[a]**
SCI +5-HT	**0.51 ± 0.05[a,d]**
SCI +GABA	**0.57 ± 0.04[a,d]**
SCI +BMC	**0.10 ± 0.01[a,d]**
SCI +5-HT+BMC	**0.72 ± 0.07[a,d]**
SCI+GABA+BMC	**0.77 ± 0.06[a,d]**
SCI +5-HT+GABA+BMC	**0.87 ± 0.08[a,d]**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-17

Real Time PCR amplification of CREB mRNA in the spinal cord of control and experimental rats

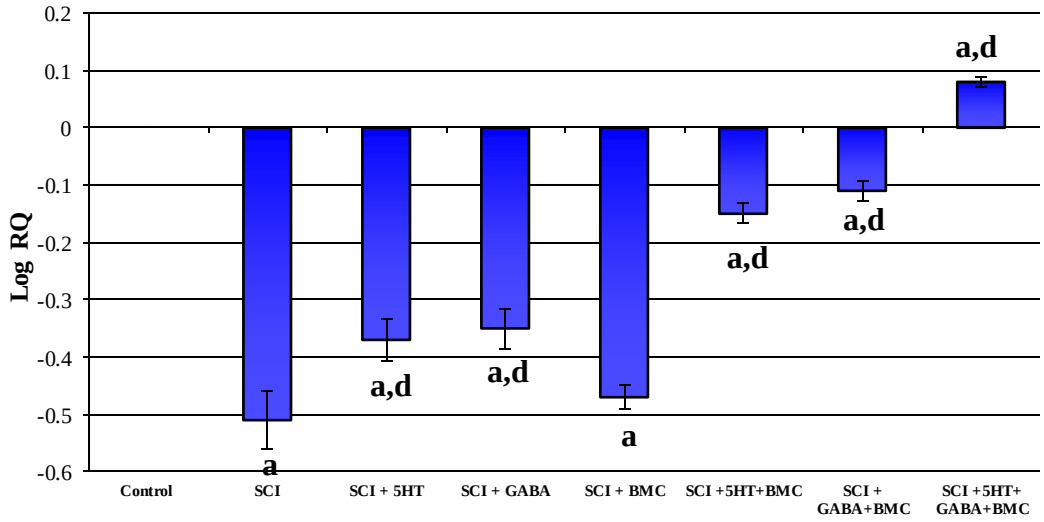


Table-15

Real Time PCR amplification of CREB mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**-0.51 ± 0.05** [a]
SCI +5-HT	**-0.37 ± 0.03** [a,d]
SCI +GABA	**-0.35 ± 0.03** [a,d]
SCI +BMC	**-0.47 ± 0.04** [a]
SCI +5-HT+BMC	**-0.15 ± 0.01** [a,d]
SCI+GABA+BMC	**-0.11 ± 0.01** [a,d]
SCI +5-HT+GABA+BMC	**0.08 ± 0.007** [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-18

Real Time PCR amplification of Bax mRNA in the spinal cord of control and experimental rats

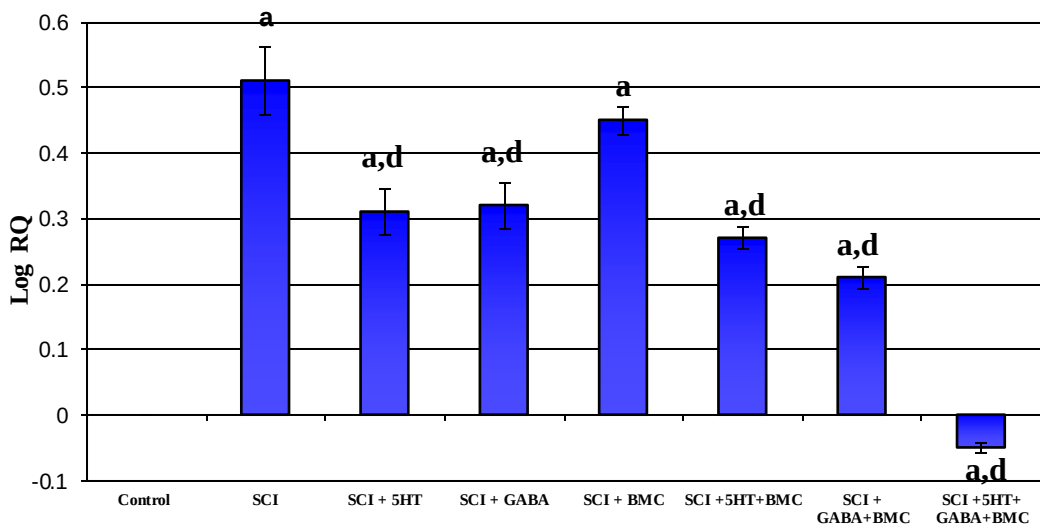


Table-16

Real Time PCR amplification of Bax mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**0.51 ± 0.05** [a]
SCI +5-HT	**0.31 ± 0.03** [a,d]
SCI +GABA	**0.32 ± 0.03** [a,d]
SCI +BMC	**0.45 ± 0.04** [a]
SCI +5-HT+BMC	**0.27 ± 0.02** [a,d]
SCI+GABA+BMC	**0.21± 0.02** [a,d]
SCI +5-HT+GABA+BMC	**-0.05 ± 0.05** [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-19

Real Time PCR amplification of caspase-8 mRNA in the spinal cord of control and experimental rats

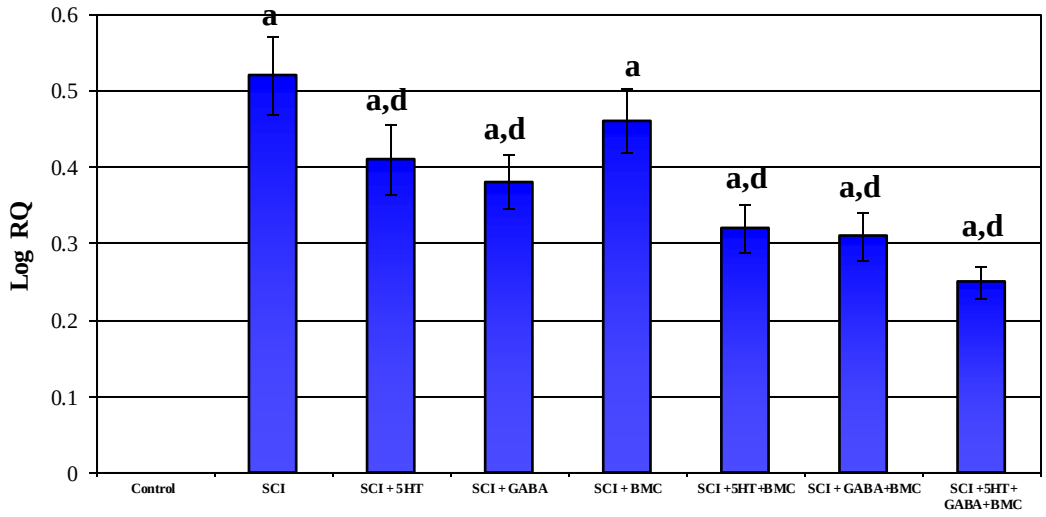


Table-17

Real Time PCR amplification of caspase-8 mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**0.52 ± 0.05** [a]
SCI +5-HT	**0.41 ± 0.04** [a,d]
SCI +GABA	**0.38 ± 0.03** [a,d]
SCI +BMC	**0.46 ± 0.04** [a]
SCI +5-HT+BMC	**0.32 ± 0.03** [a,d]
SCI+GABA+BMC	**0.31 ± 0.03** [a,d]
SCI +5-HT+GABA+BMC	**0.25 ± 0.02** [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-20

Real Time PCR amplification of superoxide dismutase mRNA in the spinal cord of control and experimental rats

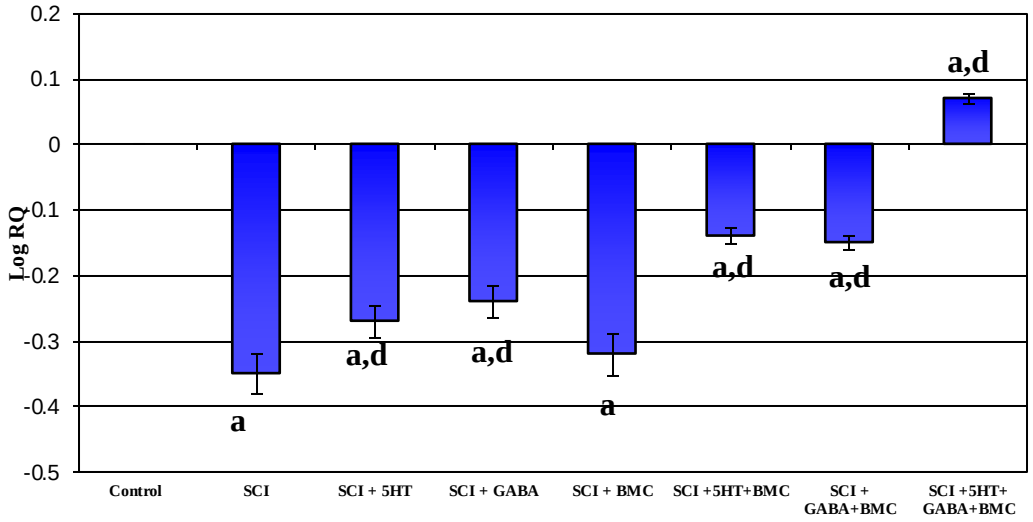


Table-18

Real Time PCR amplification of superoxide dismutase mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control±	**0**
SCI	**-0.35 ± 0.03 [a]**
SCI +5-HT	**-0.27 ± 0.02 [a,d]**
SCI +GABA	**-0.24 ± 0.02 [a,d]**
SCI +BMC	**-0.32 ± 0.03 [a]**
SCI +5-HT+BMC	**-0.14 ± 0.01 [a,d]**
SCI+GABA+BMC	**-0.15 ± 0.01 [a,d]**
SCI +5-HT+GABA+BMC	**0.07 ± 0.006 [a,d]**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-21

Real Time PCR amplification of glutathione peroxidase mRNA in the spinal cord of control and experimental rats

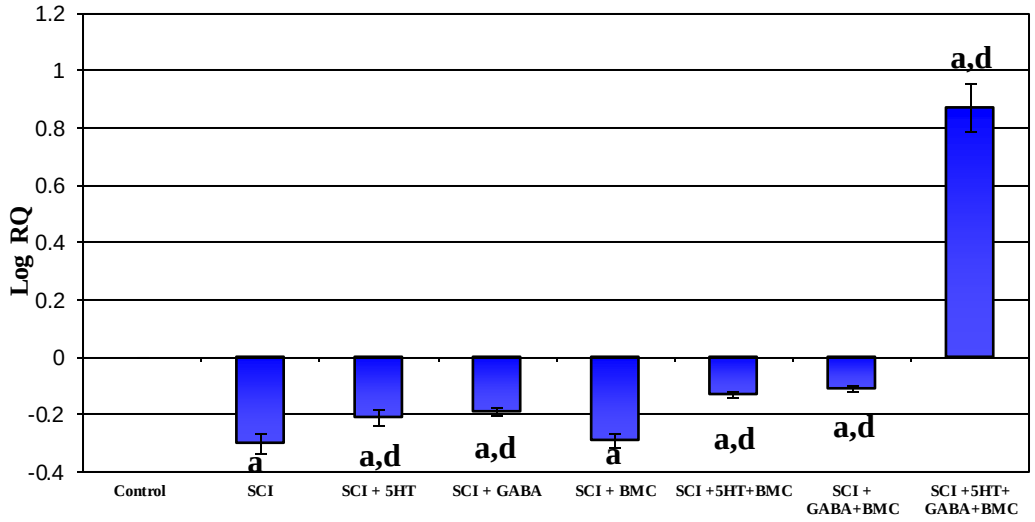


Table-19

Real Time PCR amplification of glutathione peroxidase mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**-0.30± 0.03[a]**
SCI +5-HT	**-0.21 ± 0.02[a,d]**
SCI +GABA	**-0.19 ± 0.01[a,d]**
SCI +BMC	**-0.29 ± 0.02[a]**
SCI +5-HT+BMC	**-0.13 ± 0.01[a,d]**
SCI+GABA+BMC	**-0.11 ± 0.01[a,d]**
SCI +5-HT+GABA+BMC	**0.87 ± 0.07[a,d]**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-22

Real Time PCR amplification of TNFα mRNA in the spinal cord of control and experimental rats

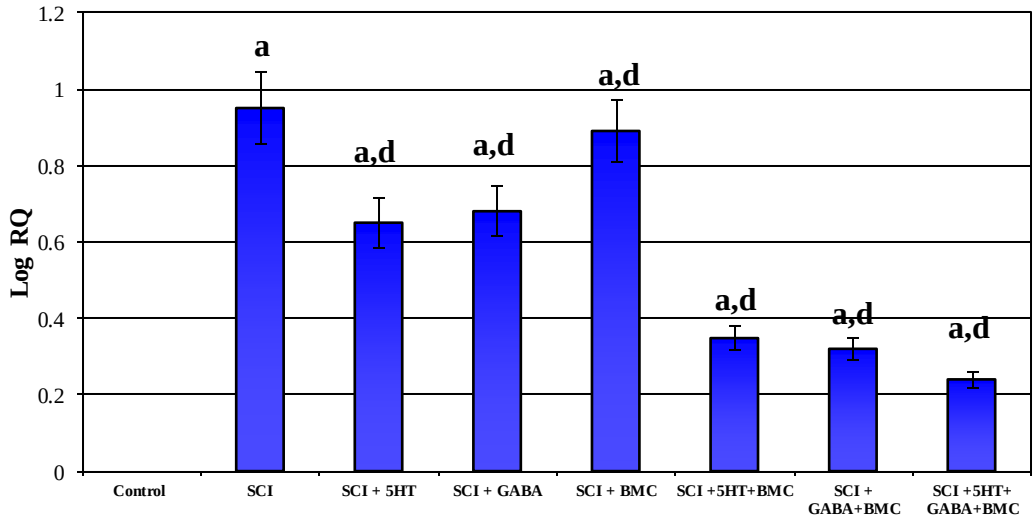


Table-20

Real Time PCR amplification of TNFα mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**0.95 ± 0.09** [a]
SCI +5-HT	**0.65 ± 0.06** [a,d]
SCI +GABA	**0.68 ± 0.05** [a,d]
SCI +BMC	**0.89 ± 0.08** [a,d]
SCI +5-HT+BMC	**0.35 ± 0. 02** [a,d]
SCI+GABA+BMC	**0.32 ± 0.03** [a,d]
SCI +5-HT+GABA+BMC	**0.24 ± 0.02** [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]p<0.001 when compared to Control. [d]p<0.001 when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-23

Real Time PCR amplification of NF-κB mRNA in the spinal cord of control and experimental rats

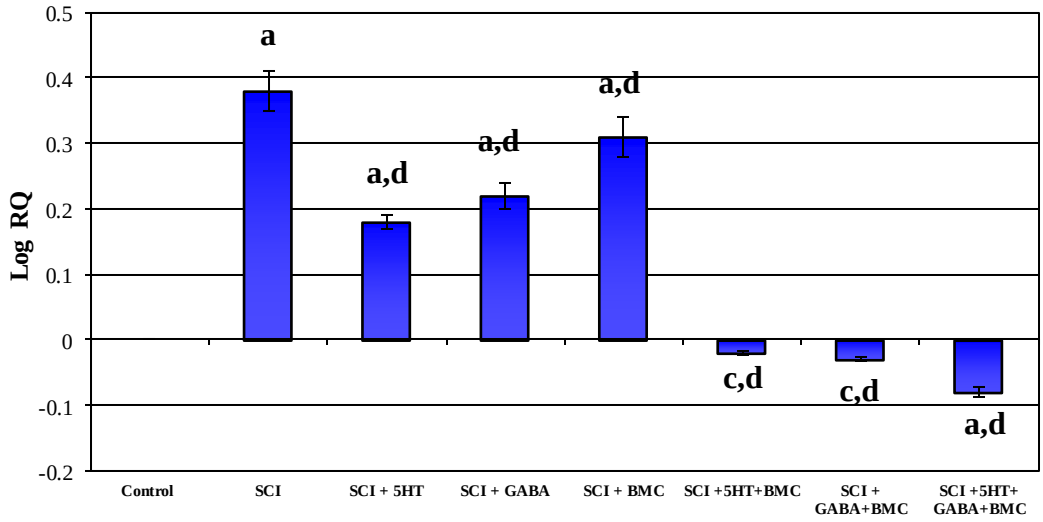


Table-21

Real Time PCR amplification of NF-κB mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**0.38 ± 0.030** [a]
SCI +5-HT	**0.18 ± 0.180** [a,d]
SCI +GABA	**0.22 ± 0.020** [a,d]
SCI +BMC	**0.31 ± 0.030** [a,d]
SCI +5-HT+BMC	**-0.02 ± 0.002** [c,d]
SCI+GABA+BMC	**-0.03 ± 0.003** [c,d]
SCI +5-HT+GABA+BMC	**-0.08 ± 0.008** [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ [c]$p<0.05$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-24

Real Time PCR amplification of BDNF mRNA in the spinal cord of control and experimental rats

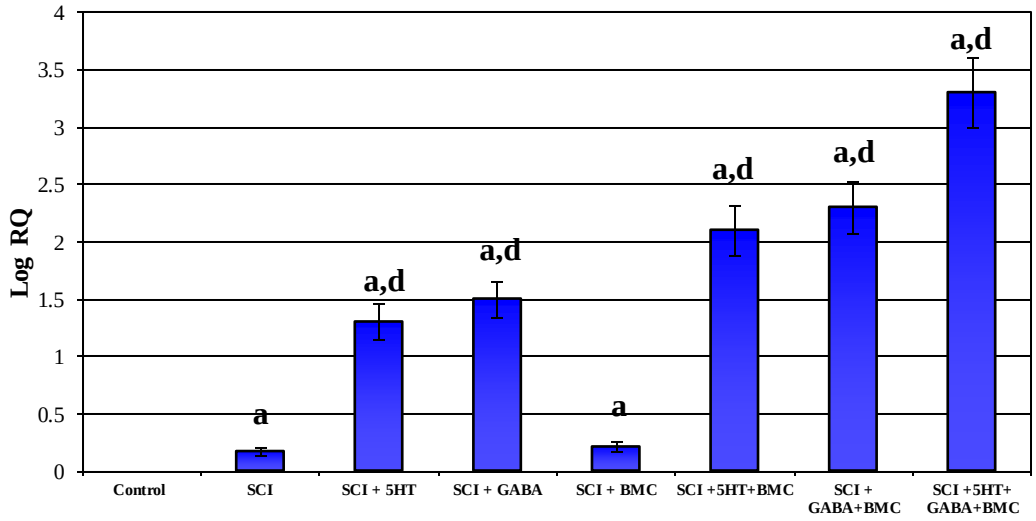


Table-22

Real Time PCR amplification of BDNF mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**0.17 ± 0.01** [a]
SCI +5-HT	**1.31 ± 0.09** [a,d]
SCI +GABA	**1.52 ± 0.01** [a,d]
SCI +BMC	**0.21 ± 0.02** [a]
SCI +5-HT+BMC	**2.11 ± 0.20** [a,d]
SCI+GABA+BMC	**2.33 ± 0.22** [a,d]
SCI +5-HT+GABA+BMC	**3.32 ± 0.29** [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-25

Real Time PCR amplification of GDNF mRNA in the spinal cord of control and experimental rats

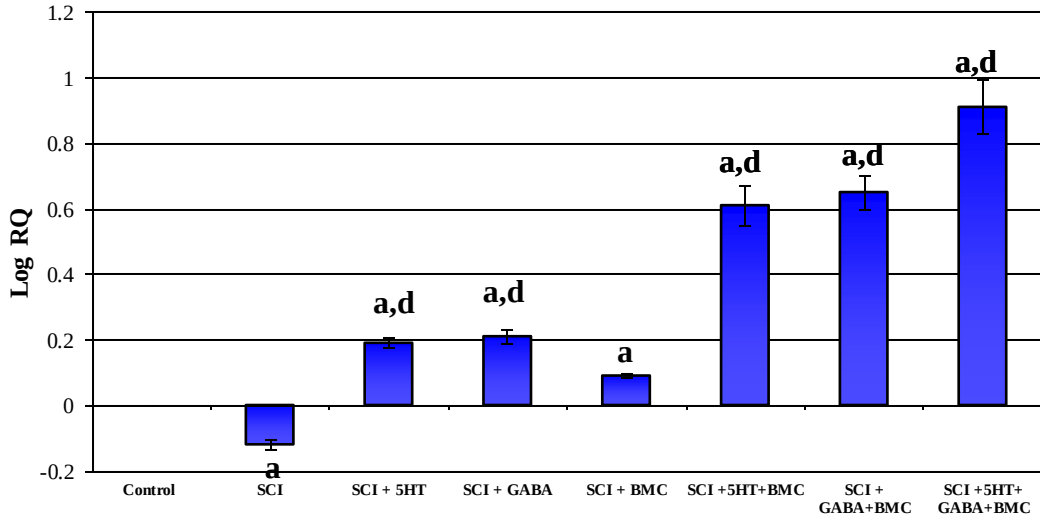


Table-23

Real Time PCR amplification of GDNF mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**-0.12 ± 0.01** [a]
SCI +5-HT	**0.19 ± 0.01** [a,d]
SCI +GABA	**0.21 ± 0.02** [a,d]
SCI +BMC	**0.09 ± 0.008** [a]
SCI +5-HT+BMC	**0.61 ± 0.06** [a,d]
SCI+GABA+BMC	**0.65 ± 0.05** [a,d]
SCI +5-HT+GABA+BMC	**0.91 ± 0.08** [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-26

Real Time PCR amplification of IGF-1 mRNA in the spinal cord of control and experimental rats

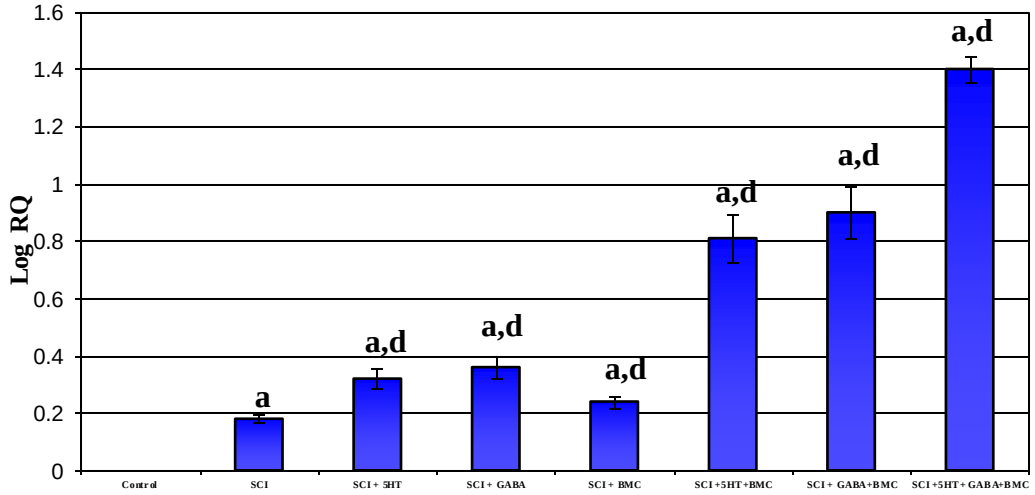


Table-24

Real Time PCR amplification of IGF-1 mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**0.18 ± 0.01[a]**
SCI +5-HT	**0.32 ± 0.03[a,d]**
SCI +GABA	**0.36 ± 0.02[a,d]**
SCI +BMC	**0.24 ± 0.01[a,d]**
SCI +5-HT+BMC	**0.81 ± 0.07[a,d]**
SCI+GABA+BMC	**0.91 ± 0.08[a,d]**
SCI +5-HT+GABA+BMC	**1.40 ± 0.11[a,d]**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-27

Real Time PCR amplification of Akt-1 mRNA in the spinal cord of control and experimental rats

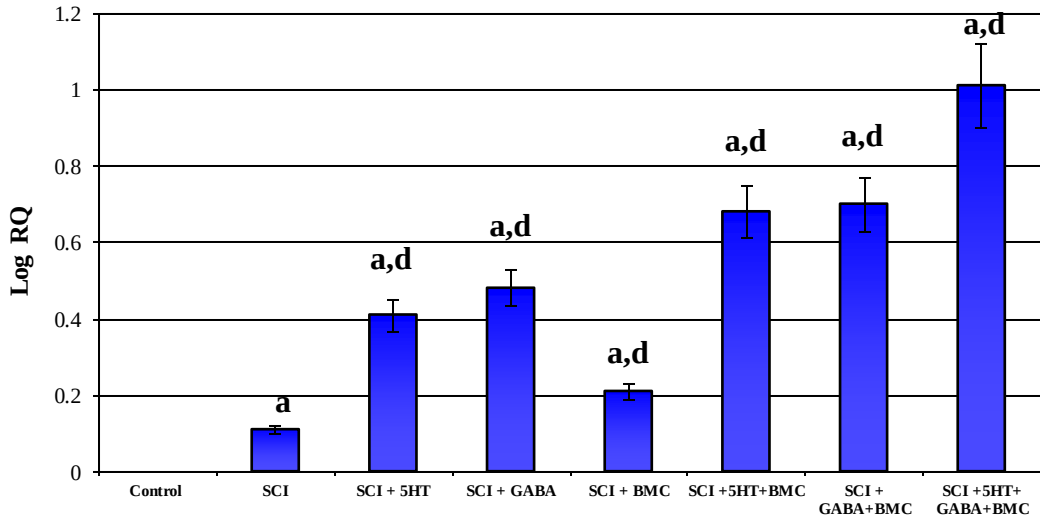


Table-25

Real Time PCR amplification of Akt-1 mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**0.11 ± 0.01[a]**
SCI +5-HT	**0.41 ± 0.03[a,d]**
SCI +GABA	**0.48 ± 0.04[a,d]**
SCI +BMC	**0.21 ± 0.02[a,d]**
SCI +5-HT+BMC	**0.68 ± 0.06[a,d]**
SCI+GABA+BMC	**0.70 ± 0.07[a,d]**
SCI +5-HT+GABA+BMC	**1.01 ± 0.09[a,d]**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-28

Real Time PCR amplification of cyclin D2 mRNA in the spinal cord of control and experimental rats

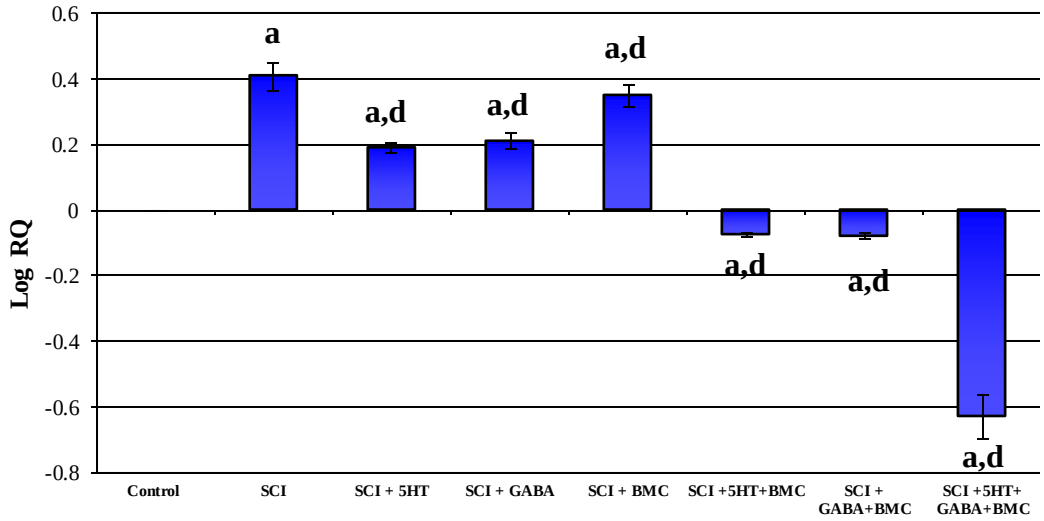


Table-26

Real Time PCR amplification of cyclin D2 mRNA in the spinal cord of control and experimental rats

Experimental groups	Log RQ
Control	**0**
SCI	**0.41 ± 0.041** [a]
SCI +5-HT	**0.19 ± 0.012** [a,d]
SCI +GABA	**0.21 ± 0.026** [a,d]
SCI +BMC	**0.35 ± 0.035** [a,d]
SCI +5-HT+BMC	**-0.07 ± 0.006** [a,d]
SCI+GABA+BMC	**-0.08 ± 0.007** [a,d]
SCI +5-HT+GABA+BMC	**-0.63 ± 0.051** [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-29

IP3 content in the spinal cord of control and experimental rats

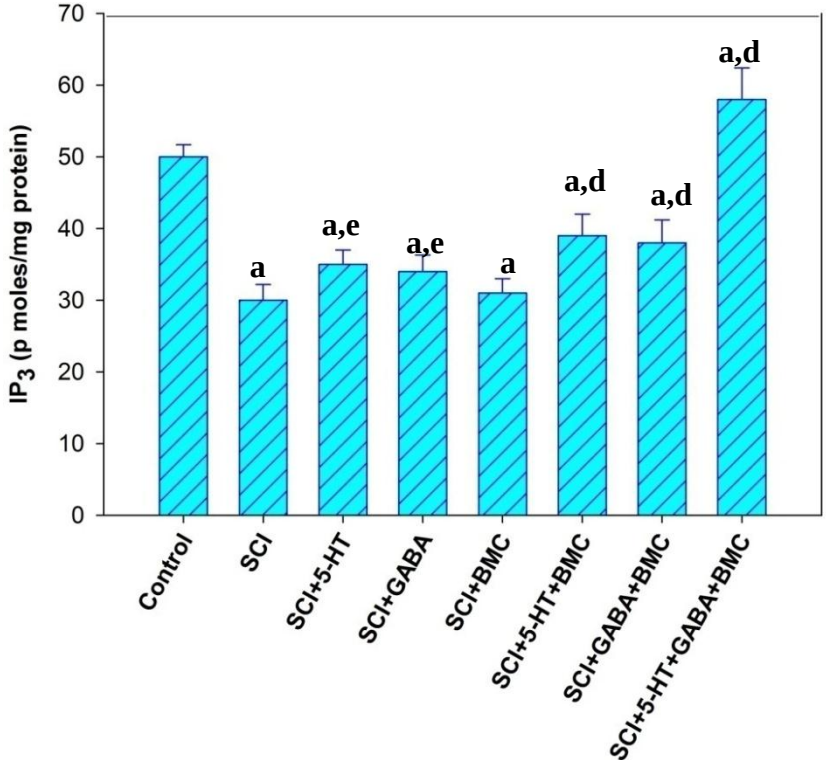


Table-27

IP3 content in the spinal cord of control and experimental rats

Experimental groups	IP3 Content (p moles/mg protein)
Control	**50.05 ± 1.67**
SCI	**30.78 ±2.18[a]**
SCI +5-HT	**35.24 ±2.09[a,e]**
SCI +GABA	**34.67±2.31[a,e]**
SCI +BMC	**31.25±2.67[a]**
SCI +5-HT+BMC	**39.56±3.01[a,d]**
SCI+GABA+BMC	**38.14±3.21[a,d]**
SCI +5-HT+GABA+BMC	**58.34±4.37[a,d]**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ [e]$p<0.01$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC-SCI treated with Serotonin, GABA and BMC.

Figure-30
cAMP content in the spinal cord of control and experimental rats

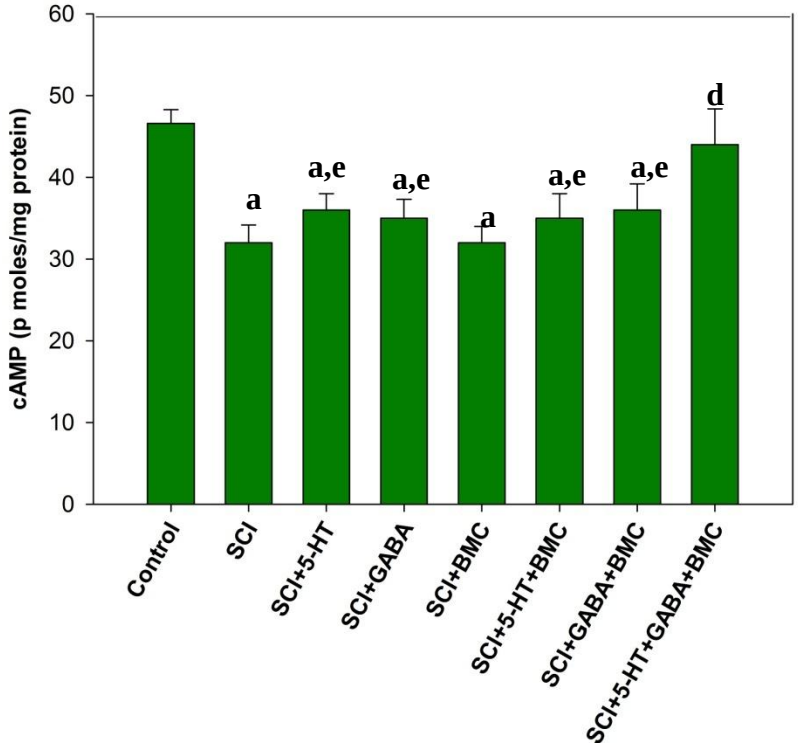


Table-28
cAMP content in the spinal cord of control and experimental rats

Experimental groups	cAMP Content (p moles/mg protein)
Control	**46.89± 1.32**
SCI	**32.12±1.97[a]**
SCI +5-HT	**36.75±2.02[a,e]**
SCI +GABA	**35.78 ± 2.31[a,e]**
SCI +BMC	**32.67 ±2.45[a]**
SCI +5-HT+BMC	**35.78 ±2.98[a,e]**
SCI+GABA+BMC	**36.87 ±2.43[a,e]**
SCI +5-HT+GABA+BMC	**44.09 ±3.39[d]**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]p<0.001 when compared to Control. [d]p<0.001[e]p<0.01 when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC-SCI treated with Serotonin, GABA and BMC.

Figure-31
cGMP content in the spinal cord of control and experimental rats

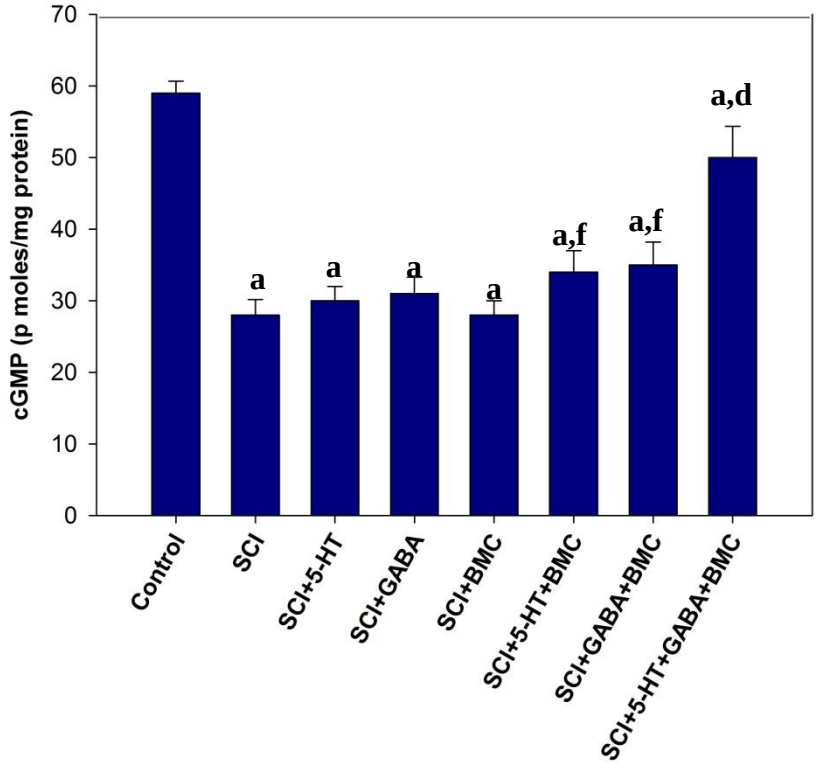
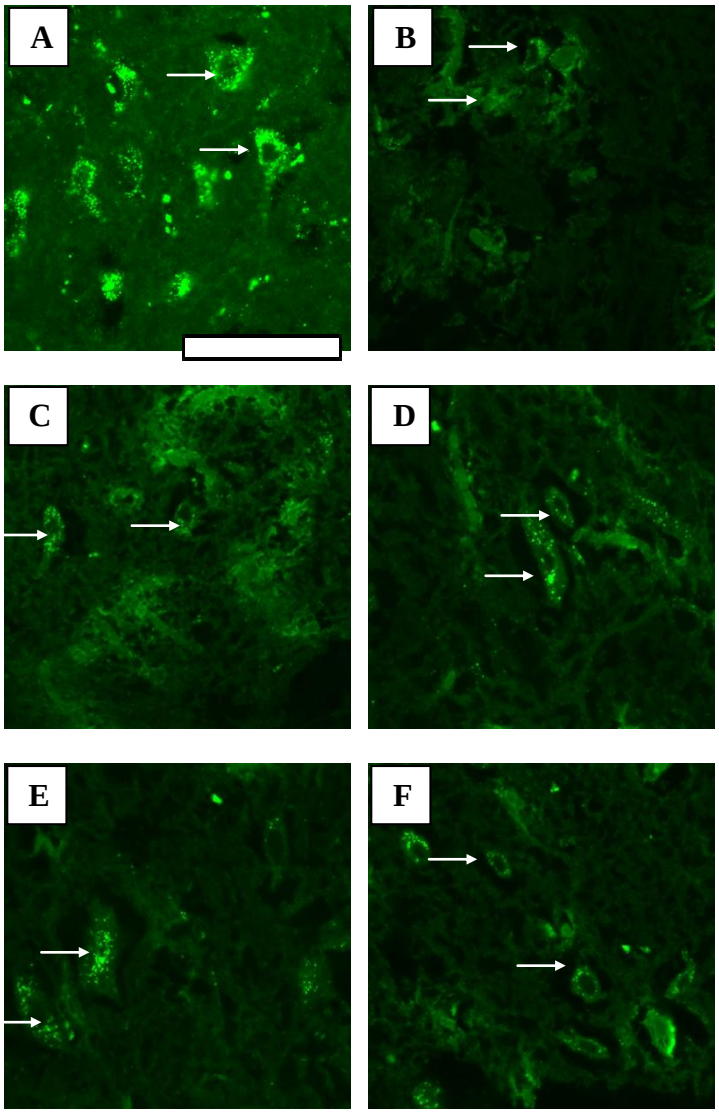


Table-29
cGMP content in the spinal cord of control and experimental rats

Experimental groups	cGMP Content (p moles/mg protein)
Control	**59.78 ± 1.84**
SCI	**28.45± 2.22[a]**
SCI +5-HT	**30.12 ± 2.62[a]**
SCI +GABA	**31.33 ± 2.79 [a]**
SCI +BMC	**28.56 ± 2.15[a]**
SCI +5-HT+BMC	**34.45 ± 2.87[a,f]**
SCI+GABA+BMC	**35.53 ± 2.92[a,f]**
SCI +5-HT+GABA+BMC	**50.02 ± 3.97[a,d]**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ [f]$p<0.05$ when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC-SCI treated with Serotonin, GABA and BMC.

Figure-32 (a)
Muscarinic M1 receptor expression in the spinal cord of control and experimental rats



A - Control, B – Spinal cord injured rats, C – SCI treated with Serotonin, D – SCI treated with GABA, E– SCI treated with BMC, F- SCI treated with Serotonin and BMC.
Scale bars represent 75 µm. → in white shows muscarinic M1 receptor

Figure– 32 (b)
Muscarinic M1 receptor expression in the spinal cord of control and experimental rats

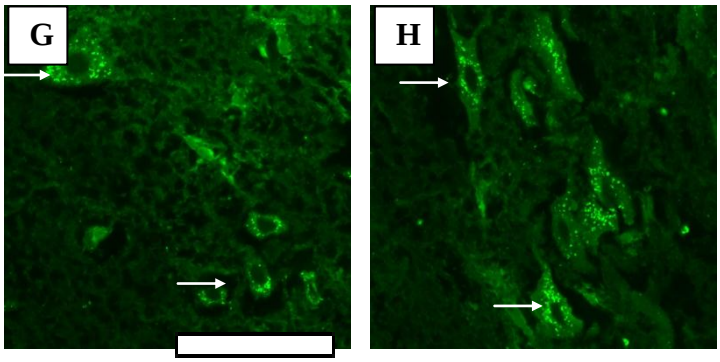
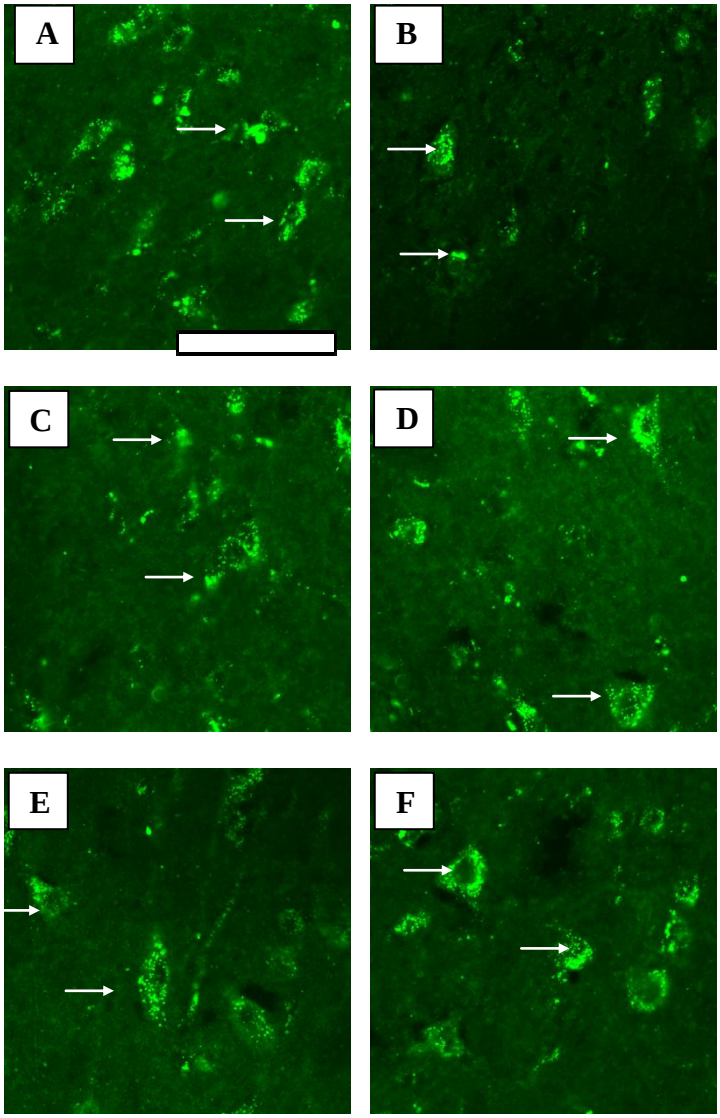


Table-30
Muscarinic M1 receptor expression in the spinal cord of control and experimental rats

Experimental groups		Mean Pixel Intensity
Control	(A)	66.41 ± 3.97
SCI	(B)	28.14 ± 1.11 [a]
SCI + 5-HT	(C)	34.67 ± 2.45 [a,e]
SCI + GABA	(D)	35.55 ± 3.01 [a,e]
SCI + BMC	(E)	31.82 ± 2.22 [a]
SCI +5-HT+BMC	(F)	40.41 ± 3.66 [a,d]
SCI +GABA+BMC	(G)	42.25 ± 3.89 [a,d]
SCI +5-HT+GABA+BMC	(H)	50.65 ± 4.19 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$, [e]$p<0.01$ when compared to SCI group.
G- SCI treated with GABA and BMC, H -SCI treated with Serotonin, GABA and BMC.
Scale bars represent 75 µm. → in white shows muscarinic M1 receptor

Figure-33 (a)
Muscarinic M3 receptor expression in the spinal cord of control and experimental rats



A - Control, B – Spinal cord injured rats, C – SCI treated with Serotonin, D – SCI treated with GABA, E– SCI treated with BMC, F- SCI treated with Serotonin and BMC.
Scale bars represent 75 µm. → in white shows muscarinic M3 receptor.

Figure-33 (b)
Muscarinic M3 receptor expression in the spinal cord of control and experimental rats

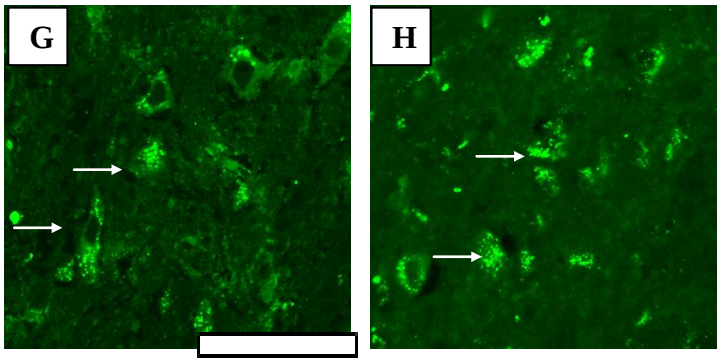
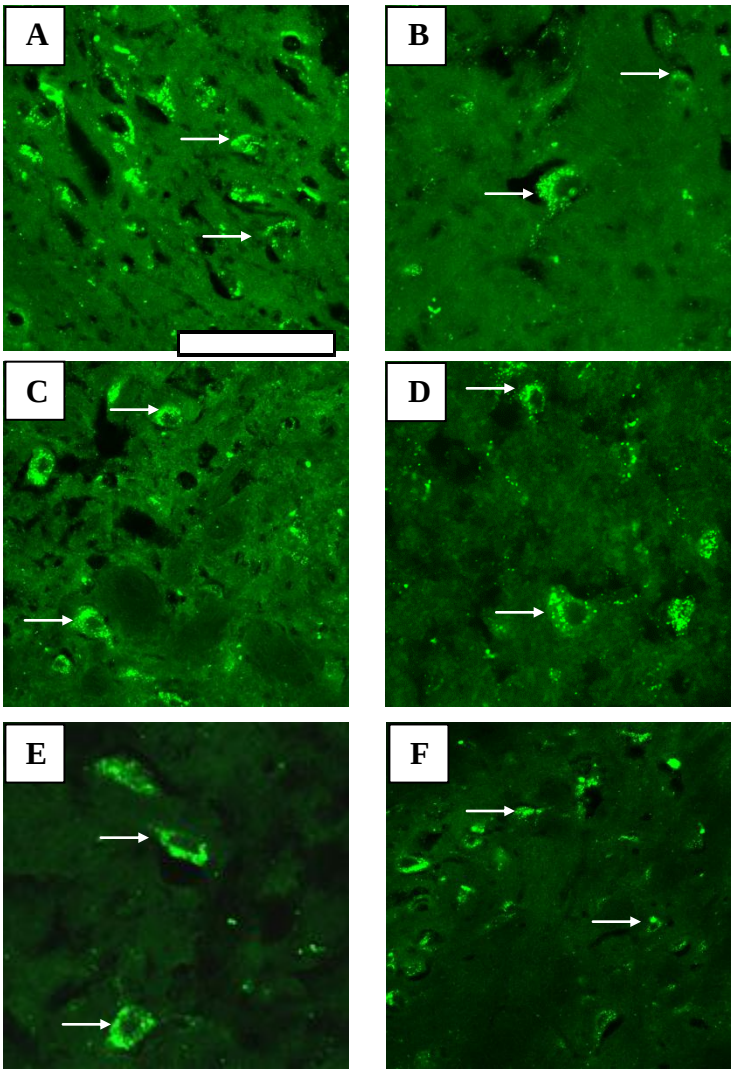


Table-31
Muscarinic M3 receptor expression in the spinal cord of control and experimental rats

Experimental groups		Mean Pixel Intensity
Control	(A)	63.98 ± 5.23
SCI	(B)	36.93 ± 2.98 [a]
SCI + 5-HT	(C)	45.32 ± 3.75 [a,d]
SCI + GABA	(D)	48.39 ± 3.32 [a,d]
SCI + BMC	(E)	40.03 ± 3.03 [a]
SCI +5-HT+BMC	(F)	58.1 ± 4.12 [a,d]
SCI +GABA+BMC	(G)	55.53 ± 4.61 [a,d]
SCI +5-HT+GABA+BMC	(H)	62.03 ± 5.32 [d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
G- SCI treated with GABA and BMC, H -SCI treated with Serotonin, GABA and BMC.
Scale bars represent 75 µm. → in white shows muscarinic M3 receptor

Figure-34 (a)
α7 nicotinic acetylcholine receptor expression in the spinal cord of control and experimental rat



A - Control, B – Spinal cord injured rats, C – SCI treated with Serotonin, D – SCI treated with GABA, E– SCI treated with BMC, F- SCI treated with Serotonin and BMC. Scale bars represent 75 µm. → in white shows α7 nicotinic acetylcholine receptor.

Figure-34 (b)
α7 nicotinic acetylcholine receptor expression in the spinal cord of control and experimental rats

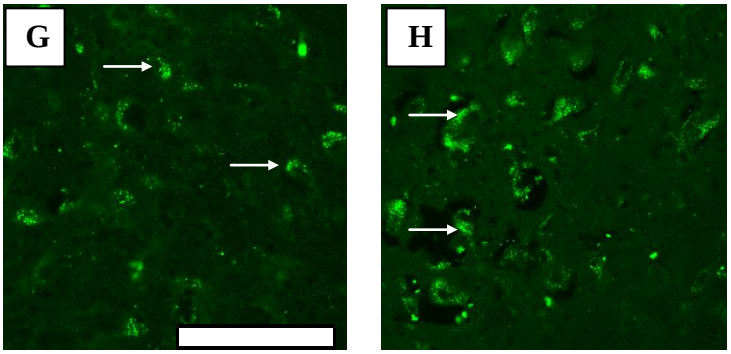
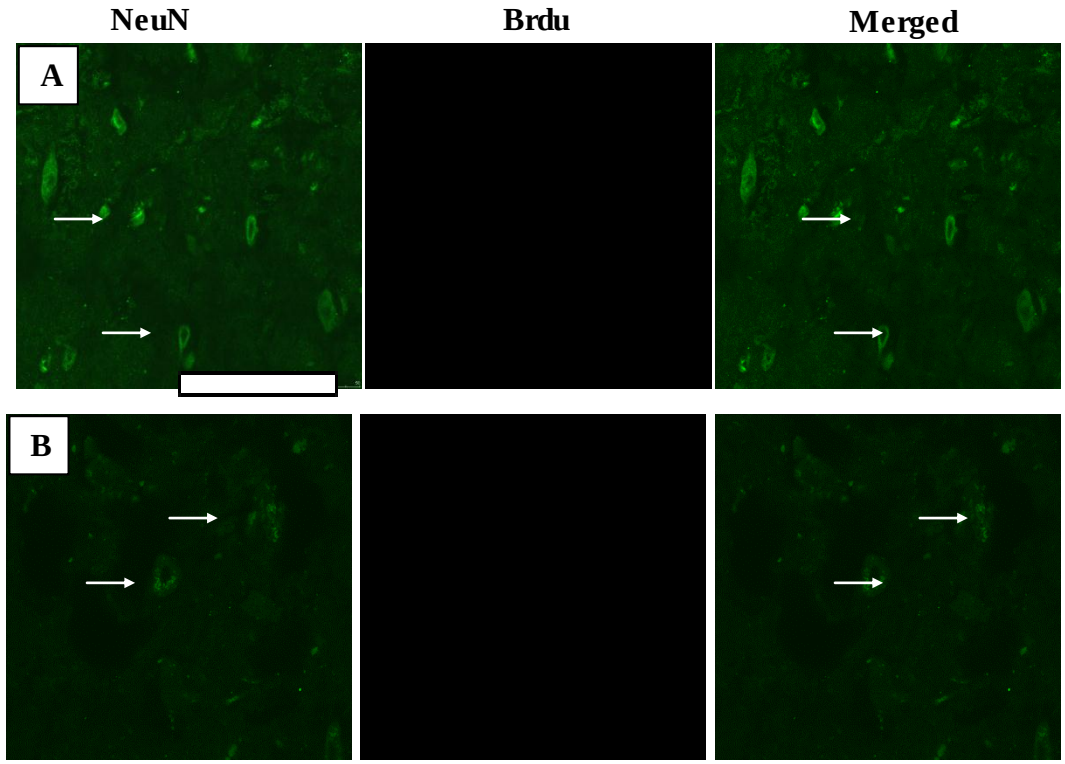


Table-32
α7 nicotinic acetylcholine receptor expression in the spinal cord of control and experimental rats

Experimental groups		Mean Pixel Intensity
Control	(A)	69.45 ± 4.98
SCI	(B)	18.97 ± 1.76 [a]
SCI + 5-HT	(C)	31.47 ± 2.87[a,d]
SCI + GABA	(D)	33.78 ± 2.56[a,d]
SCI + BMC	(E)	23.12 ± 1.45[a]
SCI +5-HT+BMC	(F)	42.89 ± 3.36[a,d]
SCI +GABA+BMC	(G)	40.64 ± 3.12[a,d]
SCI +5-HT+GABA+BMC	(H)	55.31 ± 3.97[a,d]

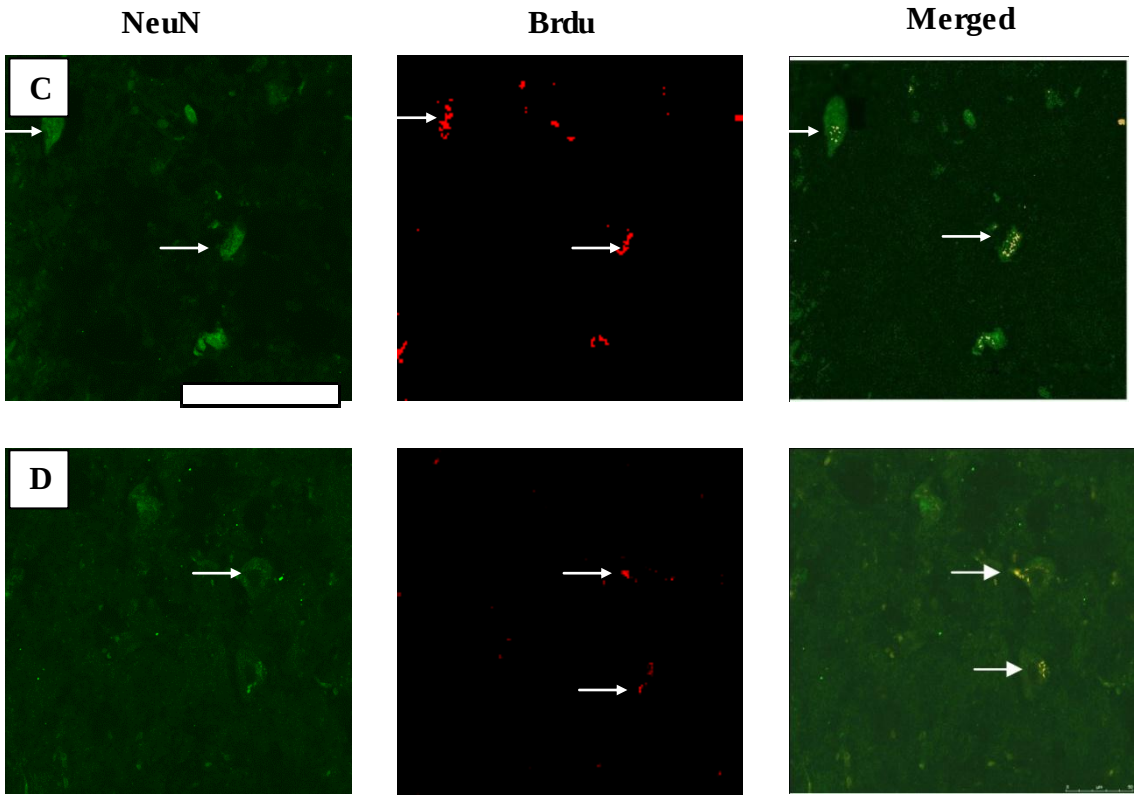
Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
G- SCI treated with GABA and BMC, H -SCI treated with Serotonin, GABA and BMC.
Scale bars represent 75 μm. → in white shows α7 nicotinic acetylcholine receptor.

Figure-35 (a)
Brdu-NeuN co-labelling studies in the spinal cord of control and experimental rats



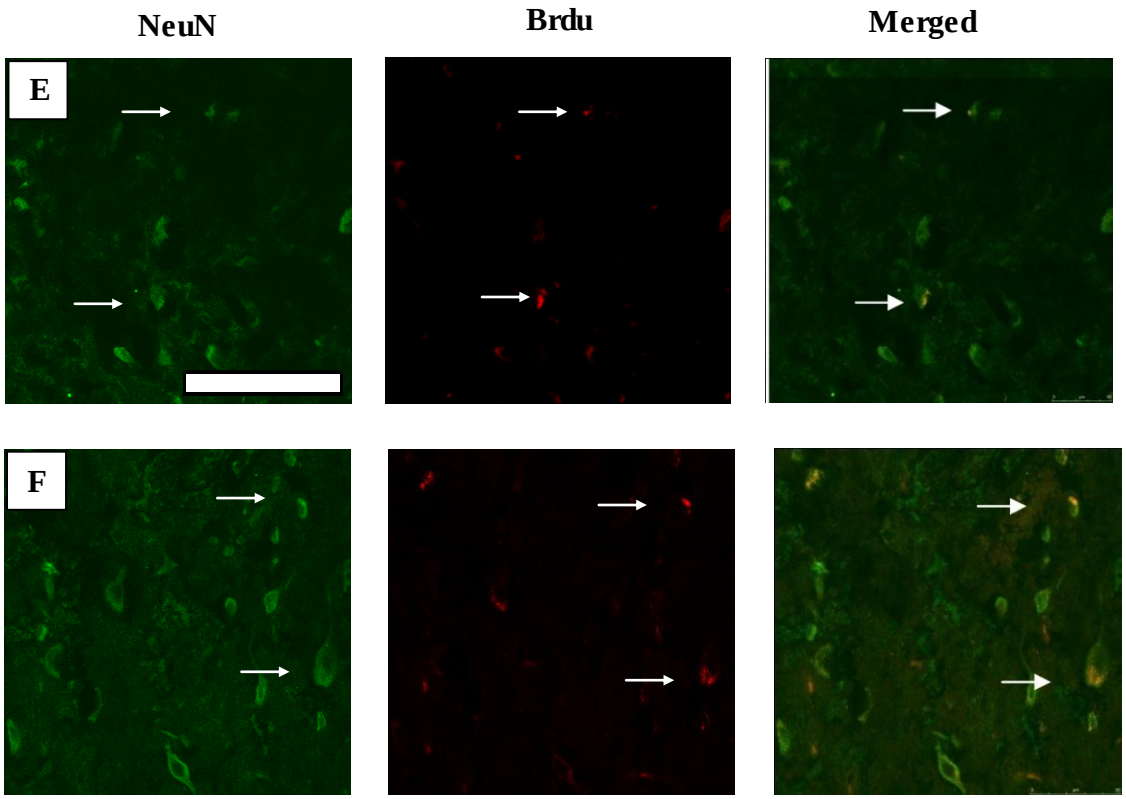
A - Control, B – Spinal cord injured rats. The scale bar represents 50 µm.

Figure-35 (b)
Brdu-NeuN co-labelling studies in the spinal cord of control and experimental rats



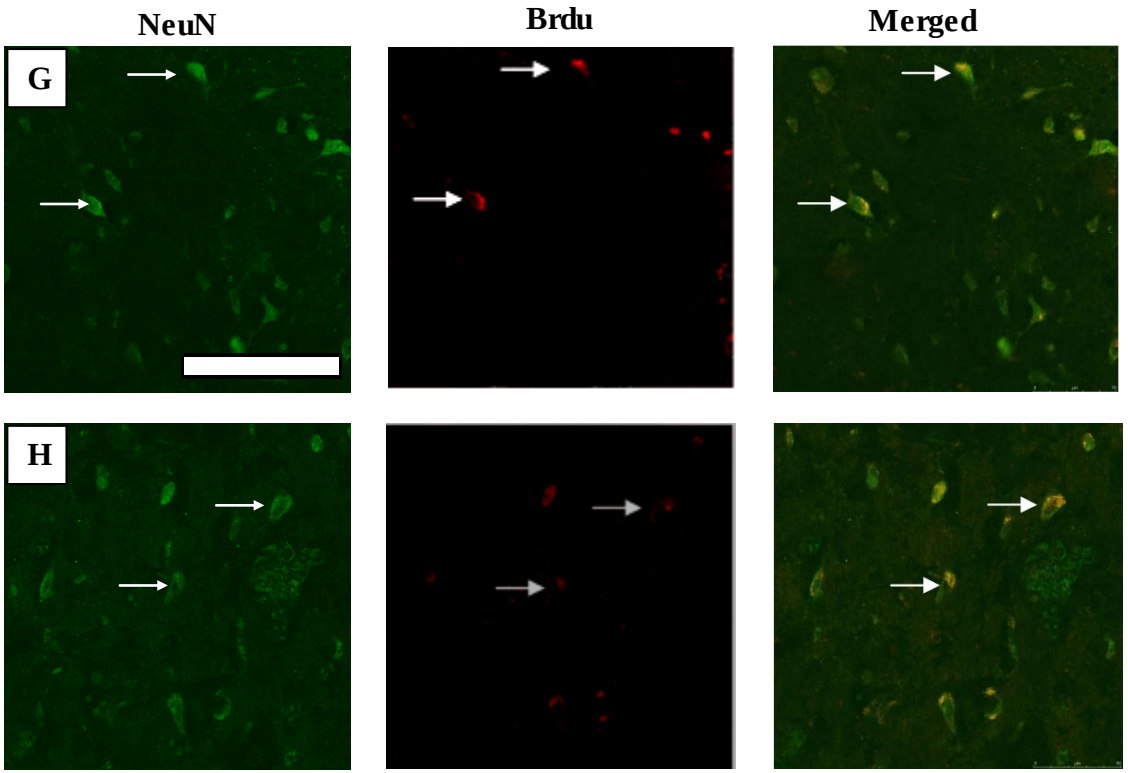
C – SCI treated with Serotonin, D – SCI treated with GABA. Scale bars represent 50 µm.

Figure-35 (c)
Brdu-NeuN co-labelling studies in the spinal cord of control and experimental rats



E– SCI treated with BMC, F- SCI treated with Serotonin and BMC. Scale bars represent 50 μm.

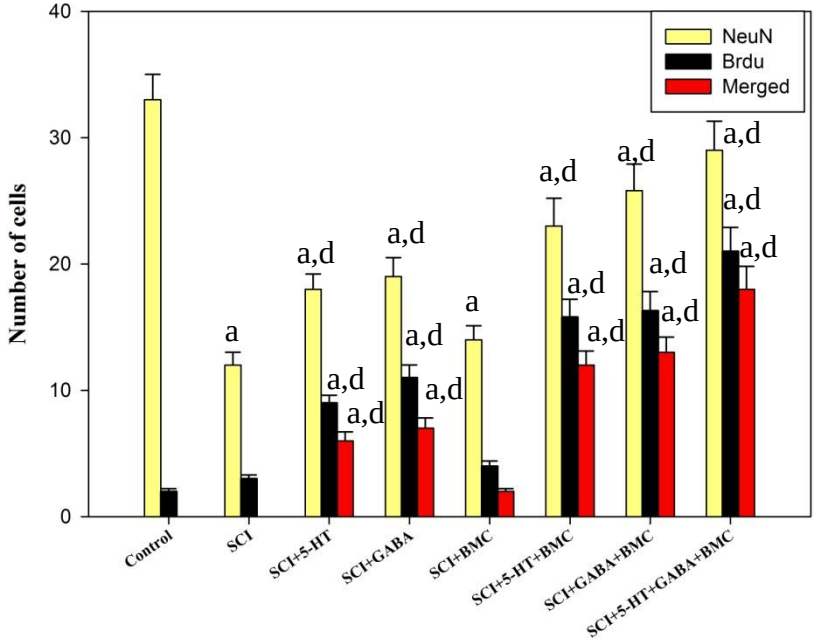
Figure-35 (d)
Brdu-NeuN co-labelling studies in the spinal cord of control and experimental rats



G- SCI treated with GABA and BMC, H -SCI treated with Serotonin, GABA and BMC.
Scale bars represent 50 µm.

Table-33

Brdu-NeuN co-labelling studies in the spinal cord of control and experimental rats



Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]p<0.001 when compared to Control. [d]p<0.001 when compared to SCI group.
C – Control, SCI – Spinal cord injured rats, SCI+5-HT – SCI treated with Serotonin, SCI+GABA – SCI treated with GABA, SCI+BMC– SCI treated with BMC, SCI+5-HT+BMC- SCI treated with Serotonin and BMC, SCI+GABA+BMC- SCI treated with GABA and BMC, SCI+5-HT+GABA+ BMC- SCI treated with Serotonin, GABA and BMC.

Figure-36

Scatchard analysis of [^{3}H] QNB binding against atropine to total muscarinic receptor in the cerebral cortex of control and experimental rats

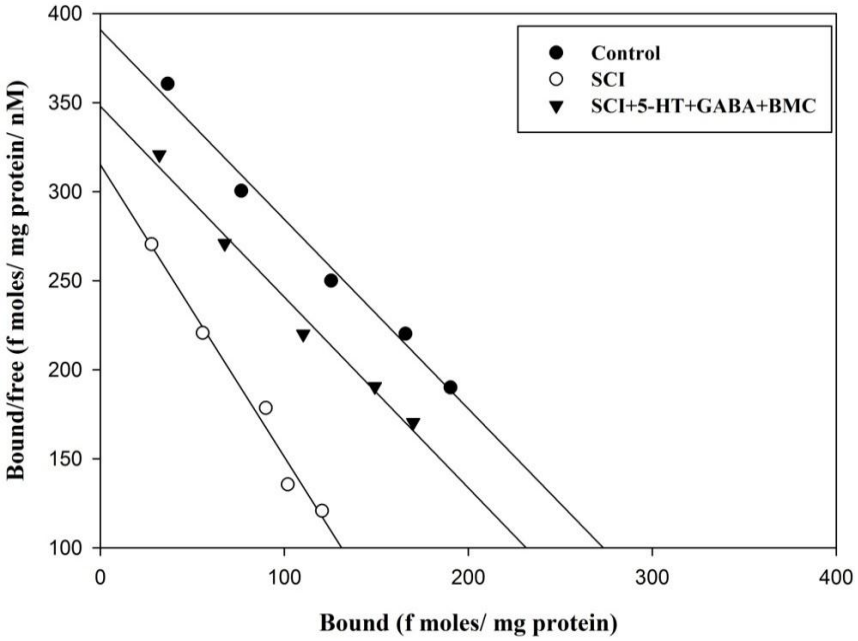


Table-34

Scatchard analysis of [^{3}H] QNB binding against atropine to total muscarinic receptor in the cerebral cortex of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
Control	273.44 ± 26.34	0.72 ± 0.04
SCI	131.57 ± 12.3[a]	0.45 ± 0.03[a]
SCI + 5-HT + GABA + BMC	231.09 ± 21.4[c,d]	0.66 ± 0.05[d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
[a] $p<0.001$, [c] $p<0.05$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-37

Scatchard analysis of [^{3}H] QNB binding against pirenzepine to muscarinic M1 receptor in the cerebral cortex of control and experimental rats

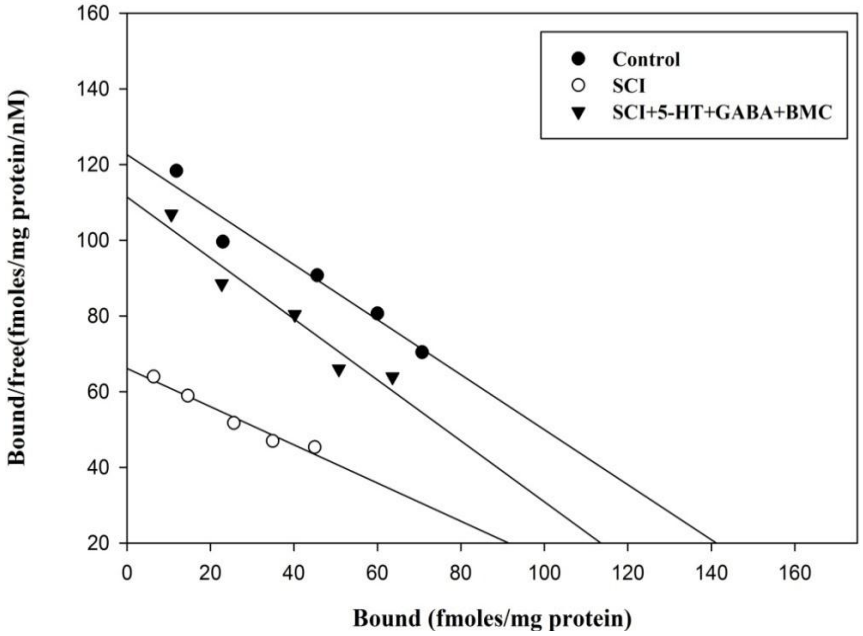


Table-35

Scatchard analysis of [^{3}H] QNB binding against pirenzepine to muscarinic M1 receptor in the cerebral cortex of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
Control	141.09 ± 11.02	1.15 ± 0.07
SCI	91.14 ± 6.50 [a]	1.37 ± 0.08 [a]
SCI + 5-HT + GABA + BMC	113.75 ± 10.98 [b,f]	1.02 ± 0.08 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
[a] $p<0.001$, [b] $p<0.01$ when compared to Control. [d] $p<0.001$, [f] $p<0.05$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-38

Scatchard analysis of [^{3}H] DAMP binding against 4-DAMP mustard to muscarinic M3 receptor in the cerebral cortex of control and experimental rats

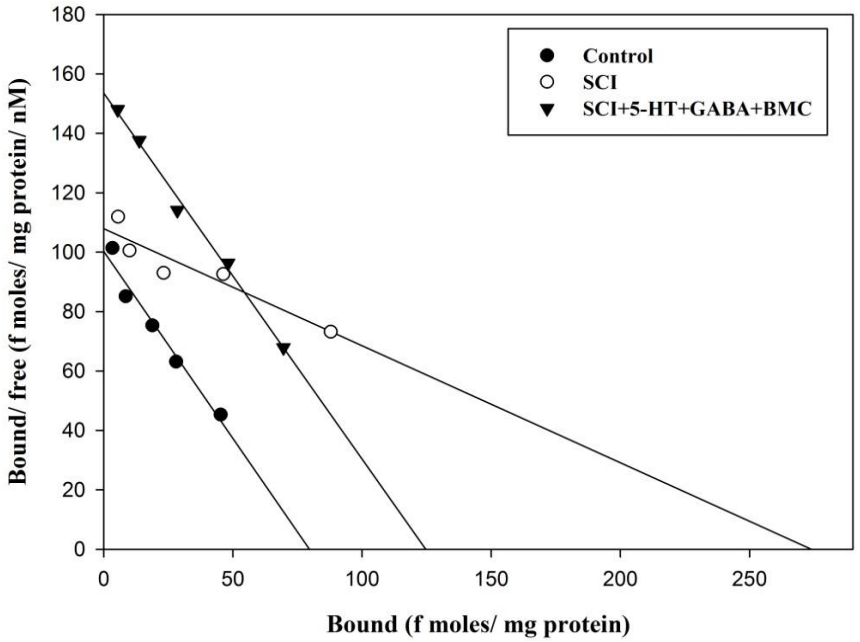


Table-36

Scatchard analysis of [^{3}H] DAMP binding against 4-DAMP mustard to muscarinic M3 receptor in the cerebral cortex of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
Control	80.38 ± 6.6	0.81 ± 0.06
SCI	272.49 ± 22.8 [a]	2.5 ± 0.02[a]
SCI + 5-HT + GABA + BMC	125.17 ± 10.27[b,d]	0.81 ± 0.07[d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-39

Real Time PCR amplification of acetylcholine esterase mRNA in the cerebral cortex of control and experimental rats

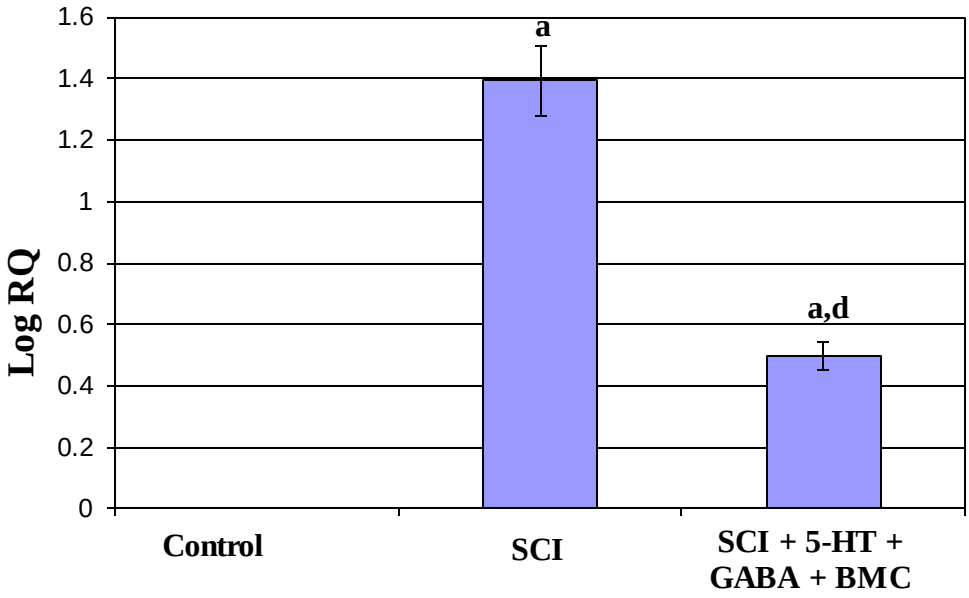


Table-37

Real Time PCR amplification of acetylcholine esterase mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	1.39 ± 0.09 [a]
SCI + 5-HT + GABA + BMC	0.49 ± 0.04 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control . [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-40

Real Time PCR amplification of choline acetyl transferase mRNA in the cerebral cortex of control and experimental rats

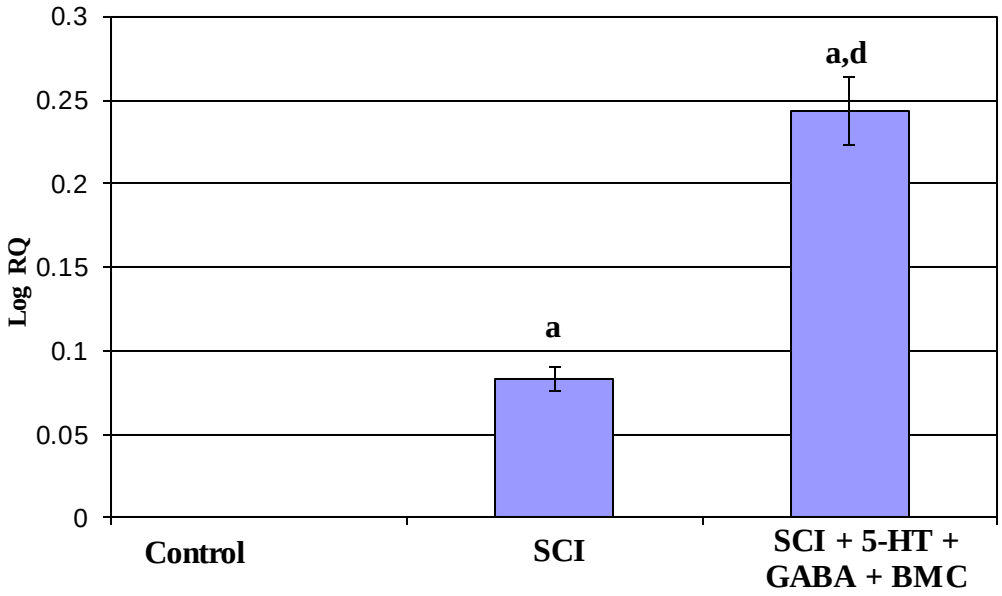


Table-38

Real Time PCR amplification of choline acetyl transferase mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	0.08 ± 0.007^{a}
SCI + 5-HT + GABA + BMC	$0.24 \pm 0.021^{a,d}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
$^{a}p<0.001$ when compared to Control . $^{d}p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-41

Real Time PCR amplification of muscarinic M1 receptor mRNA in the cerebral cortex of control and experimental rats

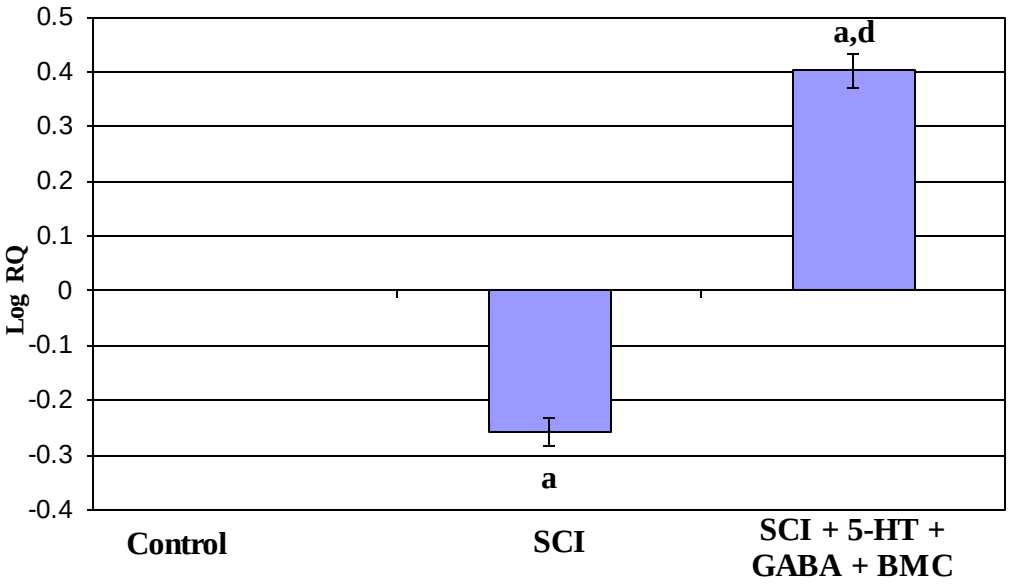


Table-39

Real Time PCR amplification of muscarinic M1 receptor mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-0.26 ± 0.02 [a]
SCI + 5-HT + GABA + BMC	0.41 ± 0.03 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-42

Real Time PCR amplification of muscarinic M2 receptor mRNA in the cerebral cortex of control and experimental rats

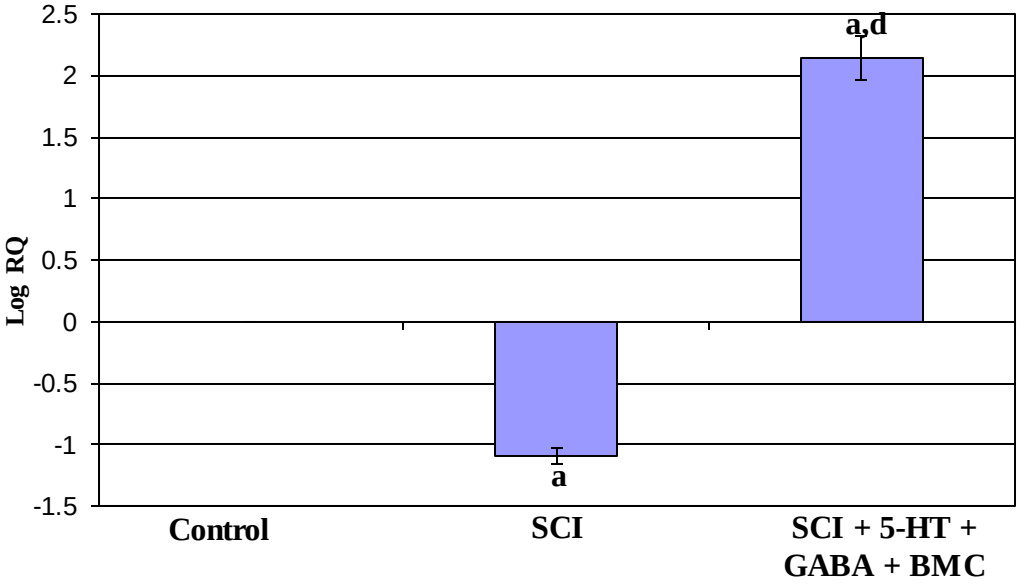


Table-40

Real Time PCR amplification of muscarinic M2 receptor mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-1.09 ± -0.10[a]
SCI + 5-HT + GABA + BMC	2.13 ± 0.19 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]p<0.001 when compared to Control. [d]p<0.001 when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-43

Real Time PCR amplification of muscarinic M3 receptor mRNA in the cerebral cortex of control and experimental rats

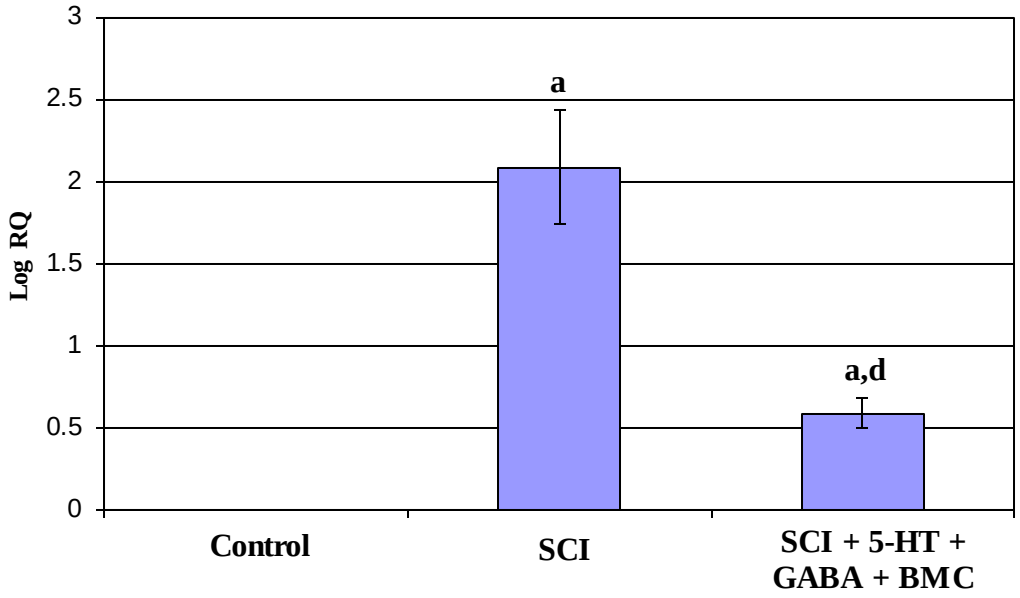


Table-41

Real Time PCR amplification of muscarinic M3 receptor mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	2.08 ± 0.182 [a]
SCI + 5-HT + GABA + BMC	0.58 ± 0.032 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-44

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the cerebral cortex of control and experimental rats

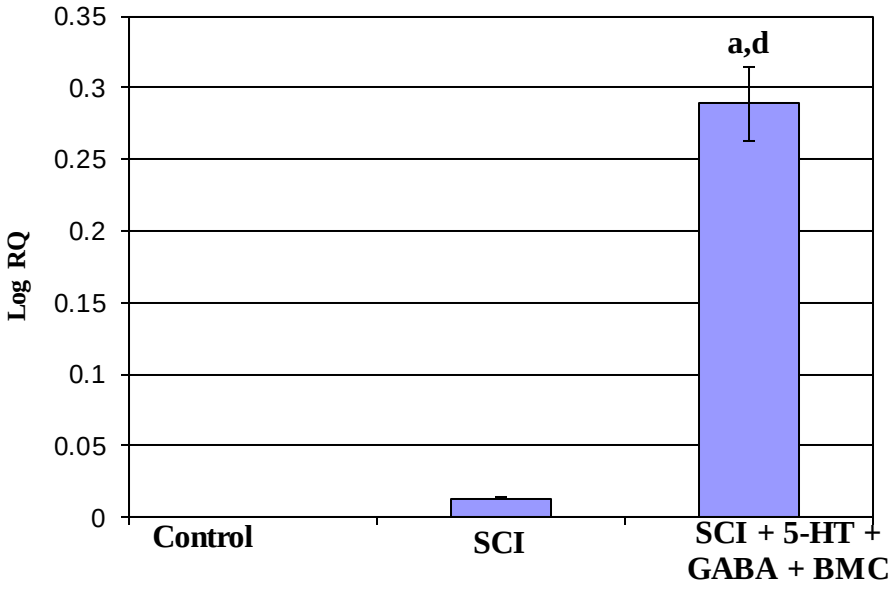


Table-42

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the cerebral cortex of control and experimental rat

Experimental groups	Log RQ
Control	0
SCI	0.01 ± 0.001
SCI + 5-HT + GABA + BMC	0.28 ± 0.027 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-45

Real Time PCR amplification of phospholipase C mRNA in the cerebral cortex of control and experimental rats

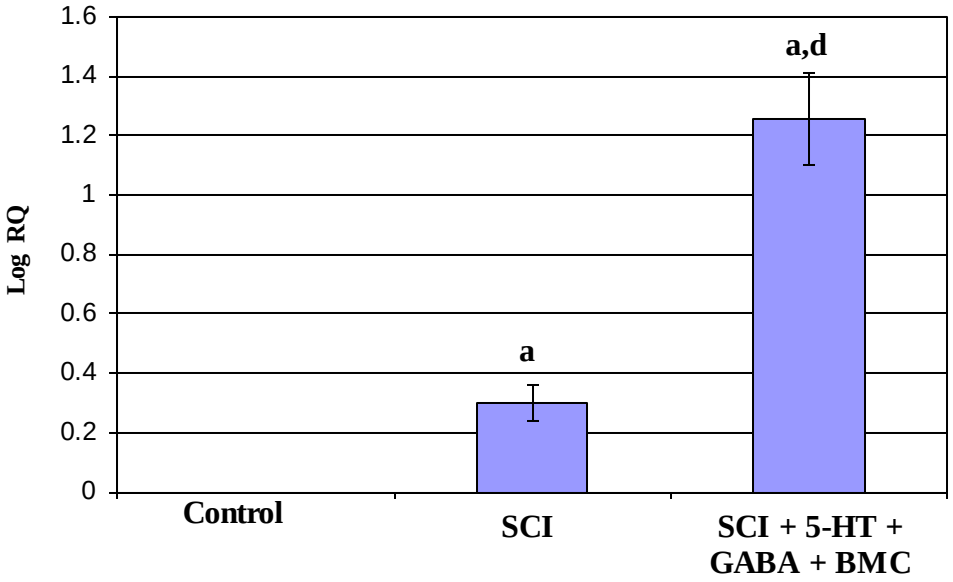


Table-43

Real Time PCR amplification of phospholipase C mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	0.30 ± 0.02 [a]
SCI + 5-HT + GABA + BMC	1.25 ± 0.10 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-46

Real Time PCR amplification of CREB mRNA in the cerebral cortex of control and experimental rats

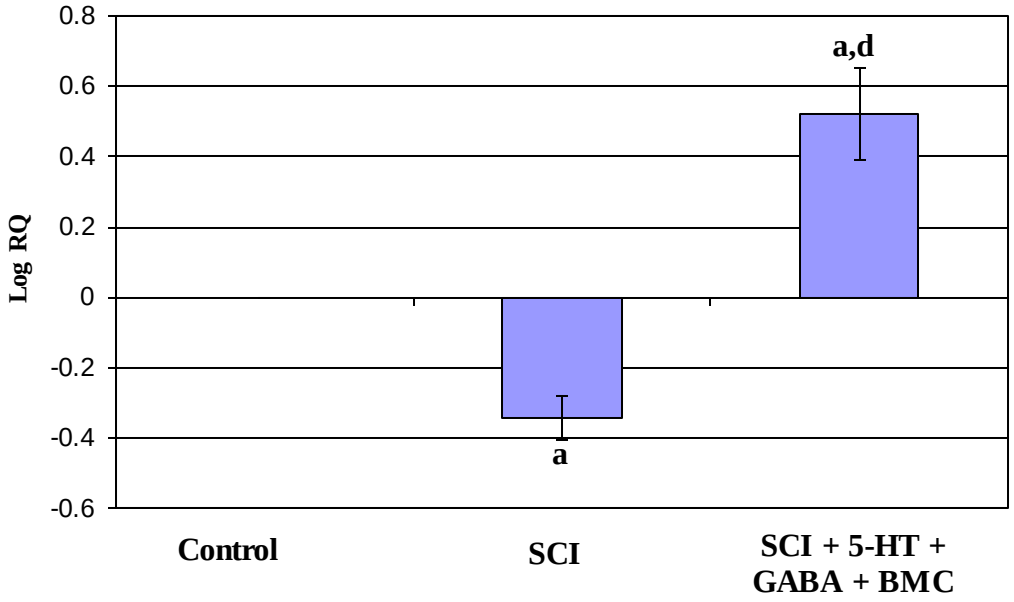


Table-44

Real Time PCR amplification of CREB mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-0.34 ± 0.02 [a]
SCI + 5-HT + GABA + BMC	0.52 ± 0.05 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-47

Real Time PCR amplification of Bax mRNA in the cerebral cortex of control and experimental rats

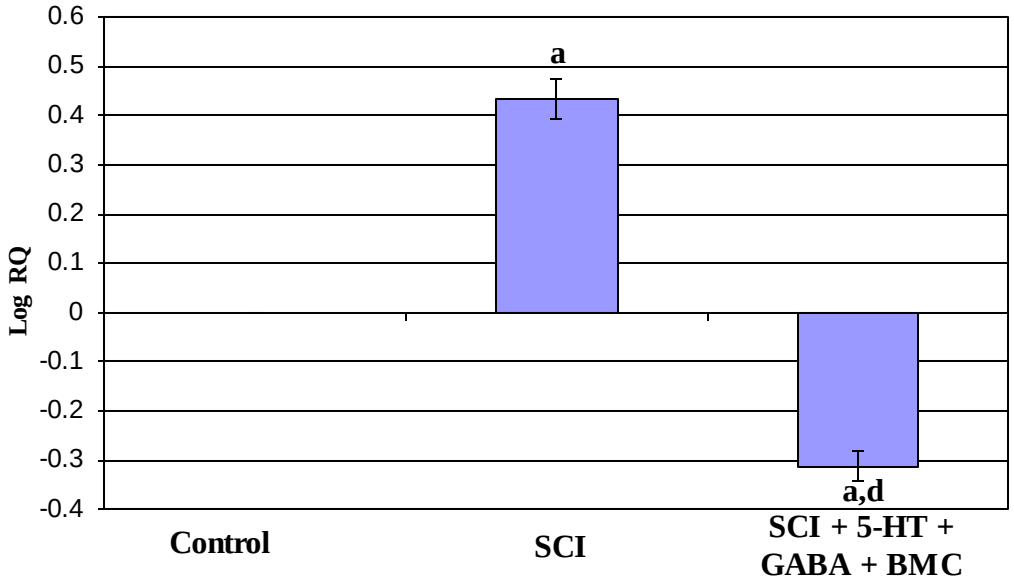


Table-45

Real Time PCR amplification of Bax mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	0.43 ± 0.03 [a]
SCI + 5-HT + GABA + BMC	-0.31 ± 0.02 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-48

Real Time PCR amplification of caspase-8 mRNA in the cerebral cortex of control and experimental rats

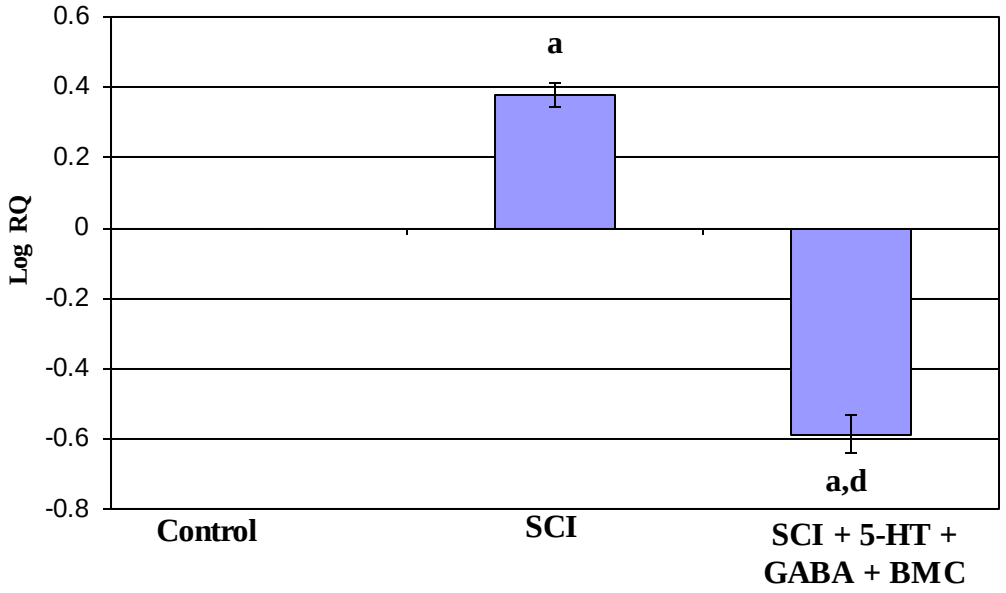


Table-46

Real Time PCR amplification of caspase-8 mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	0.38 ± 0.03 [a]
SCI + 5-HT + GABA + BMC	-0.59 ± 0.05 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-49

Real Time PCR amplification of superoxide dismutase mRNA in the cerebral cortex of control and experimental rats

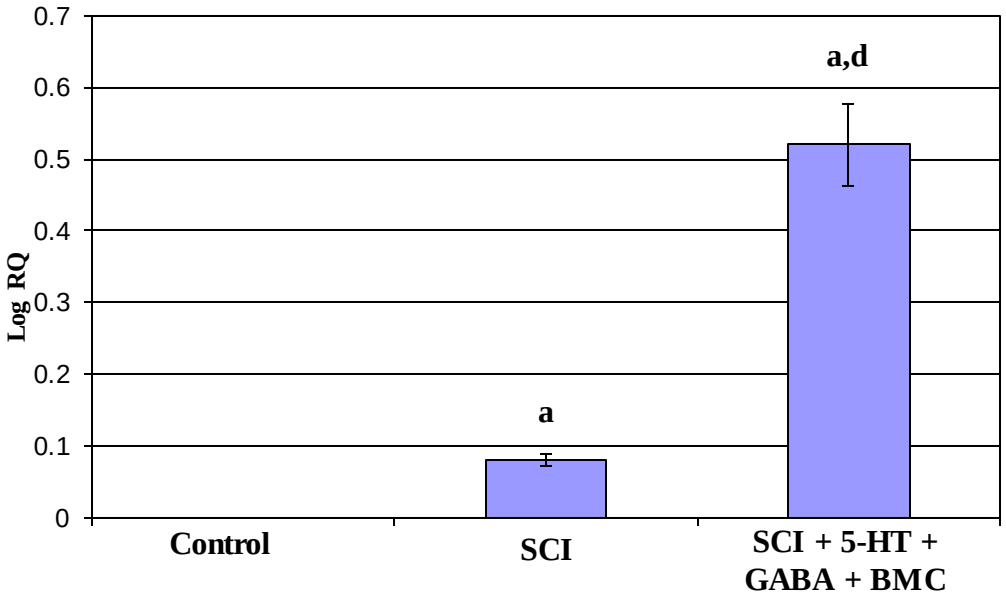


Table-47

Real Time PCR amplification of superoxide dismutase mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	0.08 ± 0.007[a]
SCI + 5-HT + GABA + BMC	0.52 ± 0.040 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-50

Real Time PCR amplification of glutathione peroxidase mRNA in the cerebral cortex of control and experimental rats

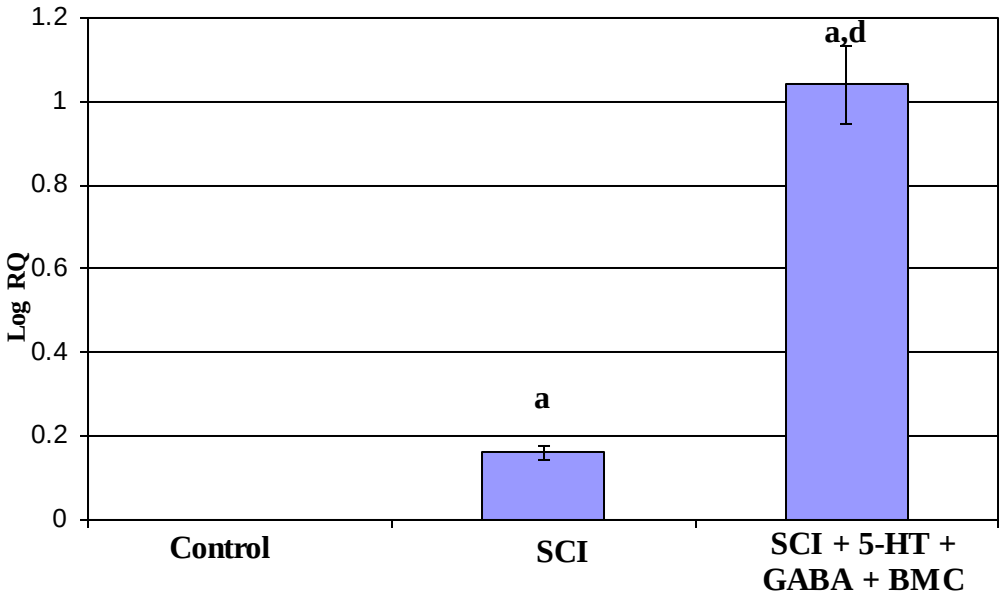


Table-48

Real Time PCR amplification of glutathione peroxidase mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	1.04 ± 0.09 [a]
SCI + 5-HT + GABA + BMC	0.17 ± 0.01 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-51
IP3 content in the cerebral cortex of control and experimental rats

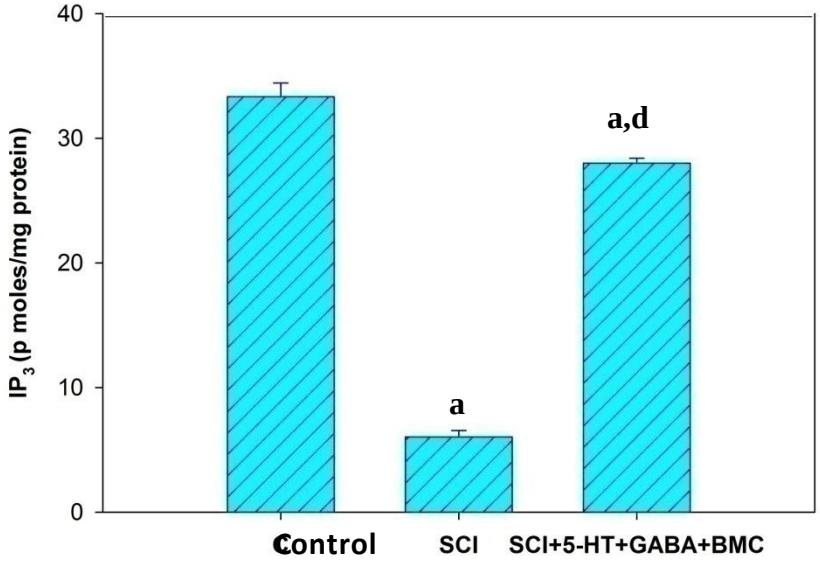


Table-49
IP3 content in the cerebral cortex of control and experimental rats

Experimental groups	IP3 Content (pmoles/mg protein)
Control	33.32± 1.10
SCI	6.04 ± 0.51 [a]
SCI + 5-HT + GABA + BMC	28.92± 0.39[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-52
cAMP content in the cerebral cortex of control and experimental rats

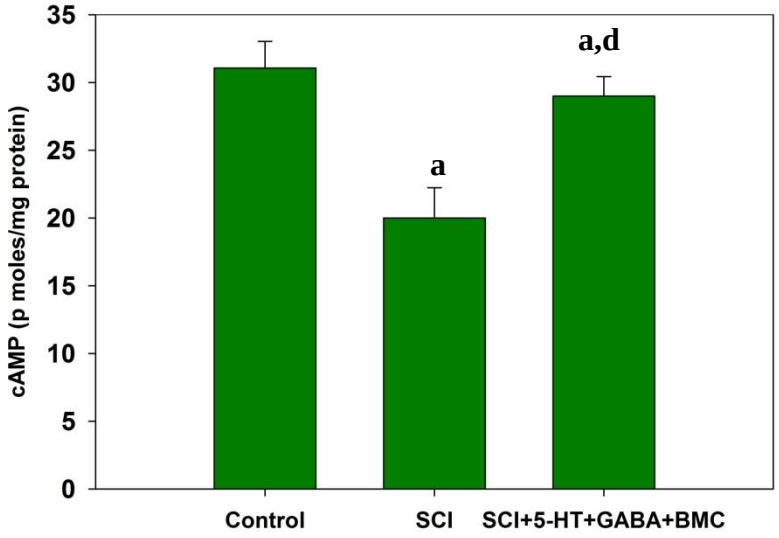


Table-50
cAMP content in the cerebral cortex of control and experimental rats

Experimental groups	cAMP Content (pmoles/mg protein)
Control	**31.07 ± 1.97**
SCI	**20.05± 2.24** [a]
SCI + 5-HT + GABA + BMC	**29.56± 1.43**[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-53
cGMP content in the cerebral cortex of control and experimental rats

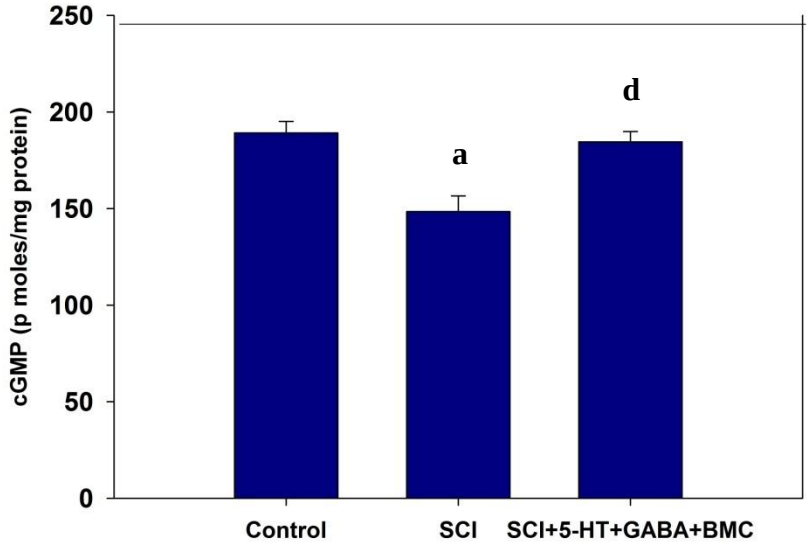
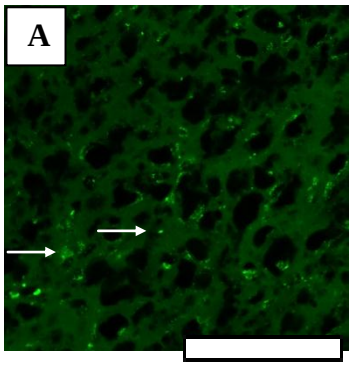


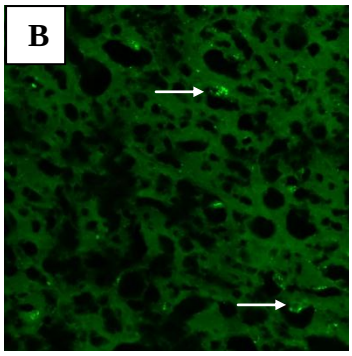
Table-51
cGMP content in the cerebral cortex of control and experimental rats

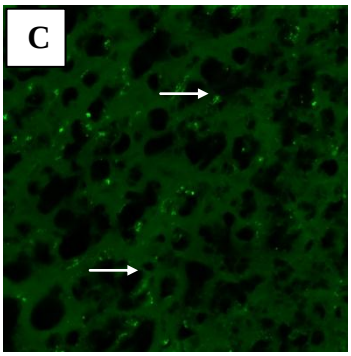
Experimental groups	cGMP Content (pmoles/mg protein)
Control	189.21 ± 5.94
SCI	148.41 ± 8.14 [a]
SCI + 5-HT + GABA + BMC	184.56 ± 5.32 [d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-54
Mucsarinic M1 receptor expression in the cerebral cortex of control and experimental rats



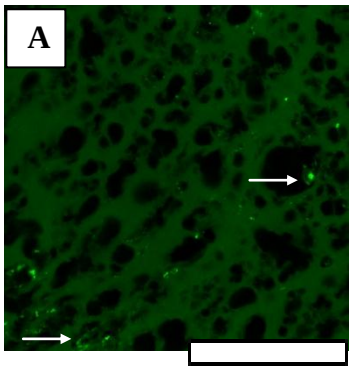


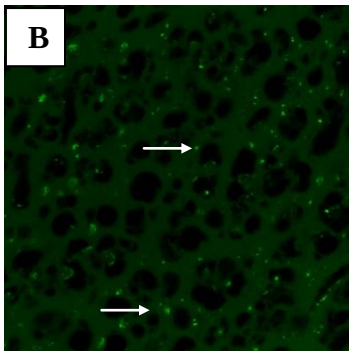


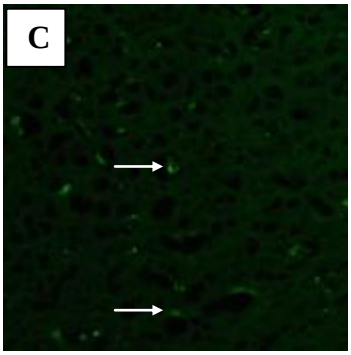
Experimental groups	Mean Pixel Intensity
CONTROL	46.31 ± 3.76
SCI	19.14 ± 1.27[a]
SCI + 5-HT + GABA + BMC	36.76±2.54[a,d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a]$p<0.001$ when compared to control, [d] $p<0.001$ when compared to SCI group.
A - Control, B – Spinal cord injured rats, C – Spinal cord injury treated with Serotonin.
Scale bars represent 150 µm. → in white shows muscarinic M1 receptor.

Figure-55
Mucsarinic M3 receptor expression in the cerebral cortex of control and experimental rats



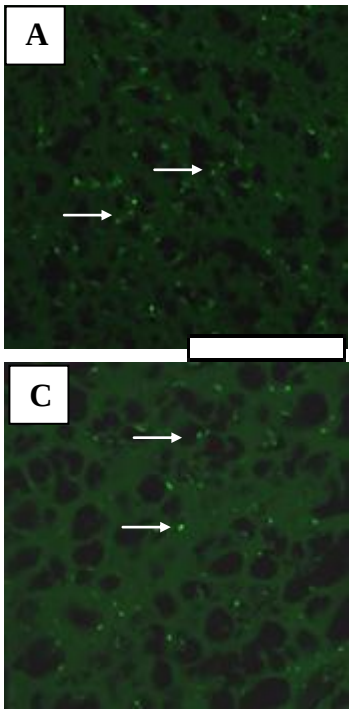




Experimental groups	Mean Pixel Intensity
CONTROL	21.29 ± 1.93
SCI	52.61 ± 4.21[a]
SCI + 5HT + GABA + BMC	32.48±2.82[a,d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a]$p<0.001$ when compared to control, [d] $p<0.001$ when compared to SCI group.
A - Control, B – Spinal cord injured rats, C – Spinal cord injury treated with Serotonin.
Scale bars represent 150 μm. → in white shows muscarinic M3 receptor.

Figure-56
α7 nicotinic acetylcholine receptor expression in the cerebral cortex of control and experimental rats



Experimental groups	Mean Pixel Intensity
CONTROL	49.54 ± 3.23
SCI	17.83 ± 1.32[a]
SCI + 5-HT + GABA + BMC	35.94 ± 2.82[a,d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a]p<0.001 when compared to control, [d] p<0.001 when compared to SCI group.
A - Control, B – Spinal cord injured rats, C – Spinal cord injury treated with Serotonin.
Scale bars represent 150 µm. → in white shows α7 nicotinic acetylcholine.

Figure-57

Scatchard analysis of [^{3}H] QNB binding against atropine to total muscarinic receptor in the cerebellum of control and experimental rats

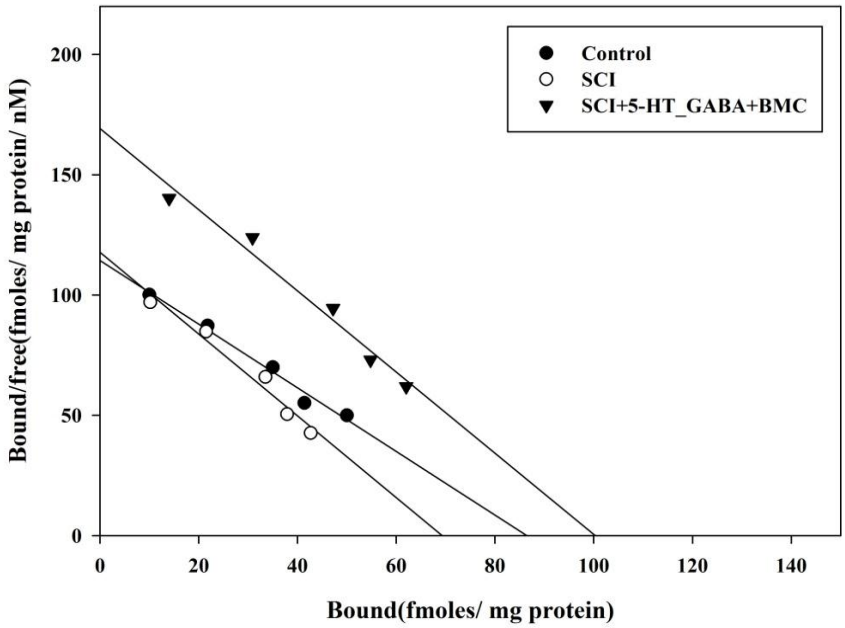


Table-55

Scatchard analysis of [^{3}H] QNB binding against atropine to total muscarinic receptor in the cerebellum of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
Control	86.01 ± 6.5	0.76 ± 0.06
SCI	69.80± 5.3 [b]	0.60 ± 0.05 [b]
SCI + 5-HT + GABA + BMC	100.01 ± 7.5 [c,d]	0.59 ± 0.04 [b]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
[b] $p<0.01$, [c] $p<0.05$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-58

Scatchard analysis of [^{3}H] QNB binding against pirenzepine to muscarinic M1 receptor in the cerebellum of control and experimental rats

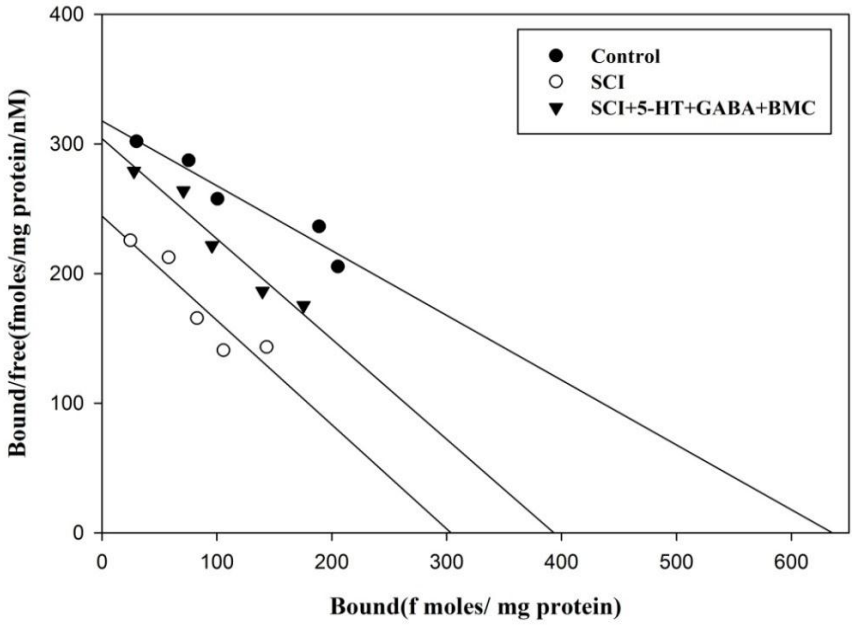


Table-56

Scatchard analysis of [^{3}H] QNB binding against pirenzepine to muscarinic M1 receptor in the cerebellum of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
Control	**633.75 ± 45.14**	**1.98 ± 0.18**
SCI	**304.72 ± 24.49[a]**	**1.24 ± 0.11[a]**
SCI + 5-HT + GABA + BMC	**392.73 ± 25.82[a,e]**	**1.28 ± 0.10[a]**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
[a] $p<0.001$ when compared to Control. [e] $p<0.01$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-59

Scatchard analysis of [^{3}H] DAMP binding against4-DAMP mustard to muscarinic M3 receptor in the cerebellum of control and experimental rats

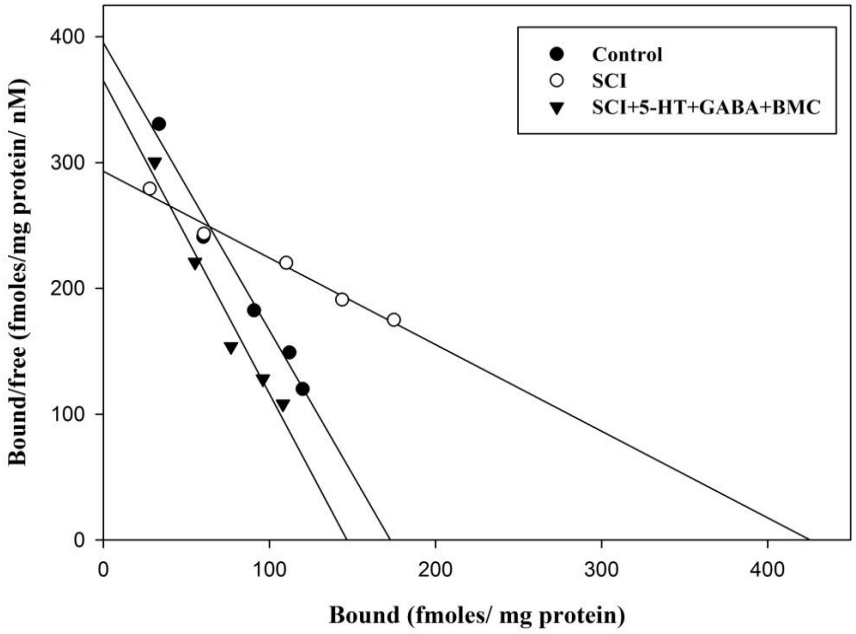


Table-57

Scatchard analysis of [^{3}H] DAMP binding against4-DAMP mustard to muscarinic M3 receptor i in the cerebellum of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
Control	173.19 ± 11.03	0.44± 0.03
SCI	425.67 ± 34.4[a]	1.46 ± 0.11[a]
SCI + 5-HT + GABA + BMC	147.5 ± 14.3[a,d]	0.40 ± 0.02[d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-60

Real Time PCR amplification of acetylcholine esterase mRNA in the cerebellum of control and experimental rats

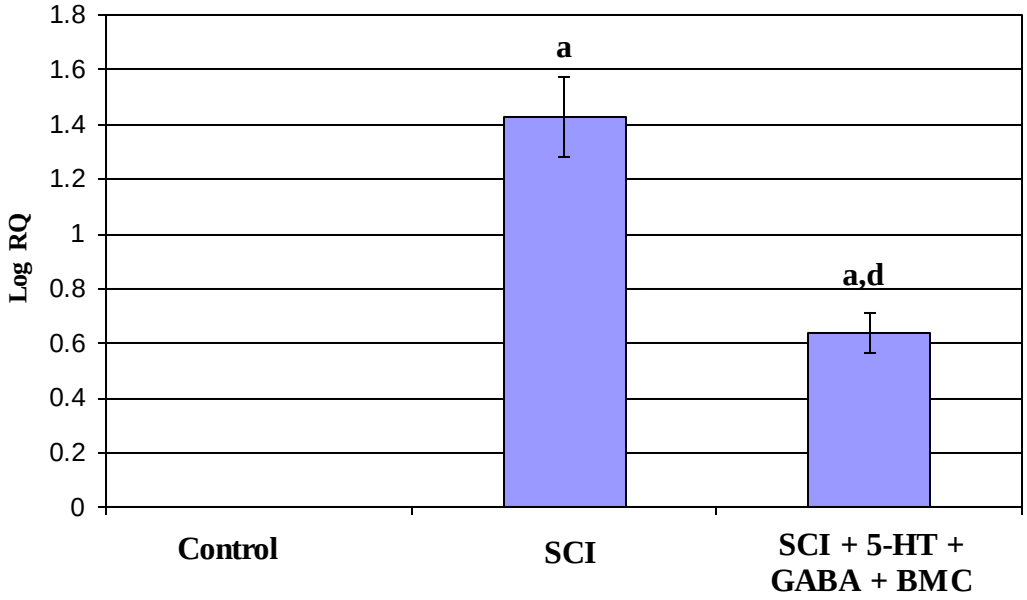


Table-58

Real Time PCR amplification of acetylcholine esterase mRNA in the cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	1.42 ± 0.13 [a]
SCI + 5-HT + GABA + BMC	0.64 ± 0.05 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-61
Real Time PCR amplification of choline acetyl transferase mRNA in the cerebellum of control and experimental rats

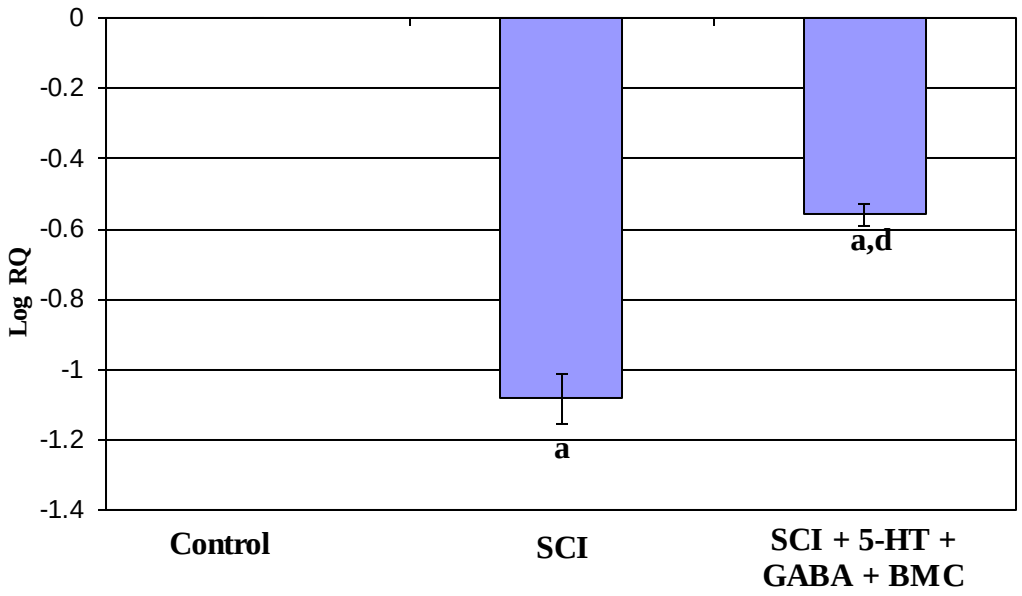


Table-59
Real Time PCR amplification of choline acetyl transferase mRNA in the cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-1.08 ± 0.09[a]
SCI + 5-HT + GABA + BMC	-0.58 ± 0.05 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-62

Real Time PCR amplification of muscarinic M1 receptor mRNA in the cerebellum of control and experimental rats

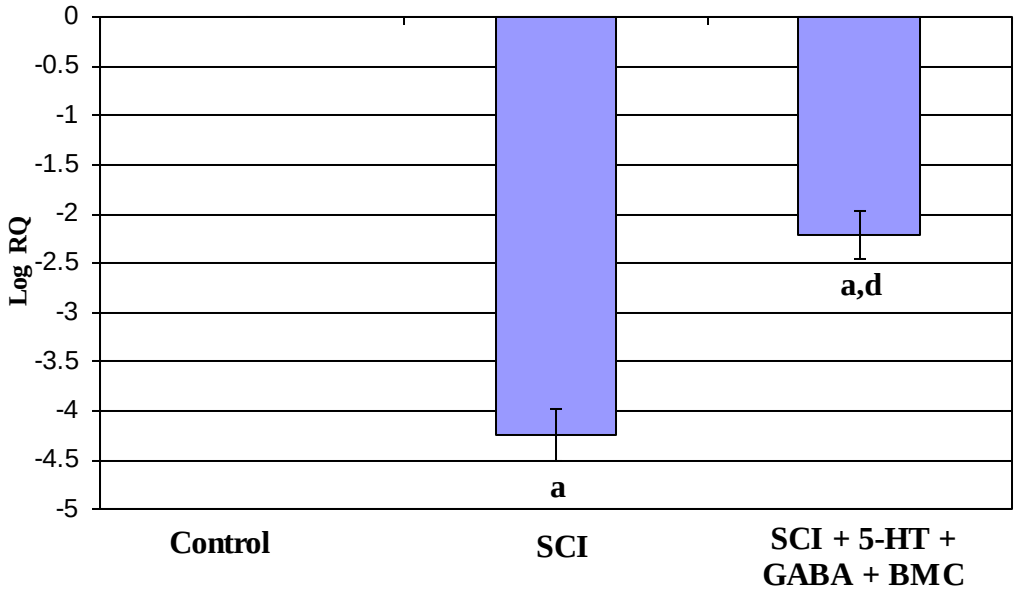


Table-60

Real Time PCR amplification of muscarinic M1 receptor mRNA in the cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-4.24 ± 0.26 [a]
SCI + 5-HT + GABA + BMC	-2.21 ± 0.19 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-63

Real Time PCR amplification of muscarinic M2 receptor mRNA in the cerebellum of control and experimental rats

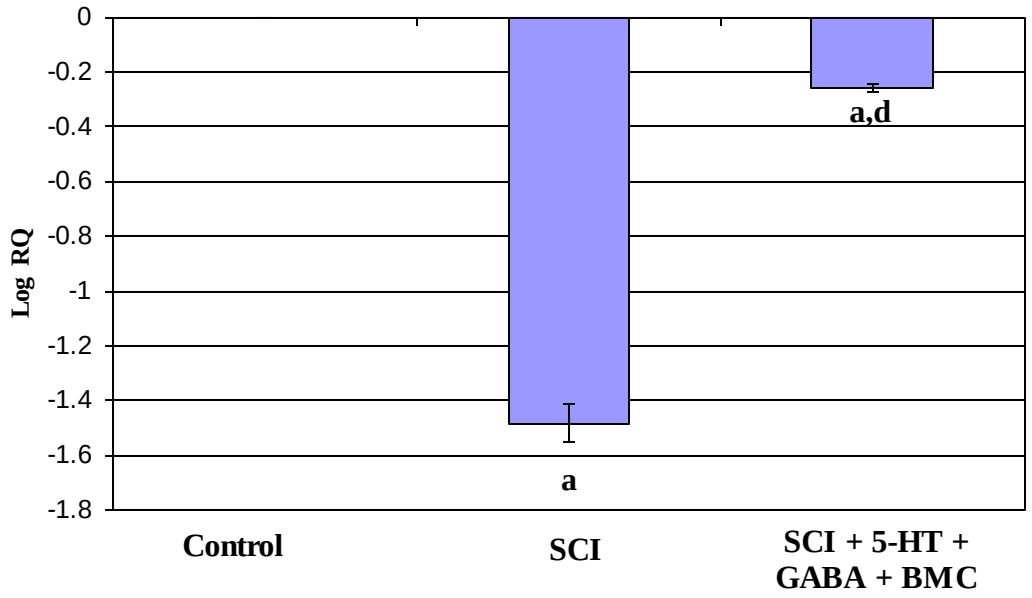


Table-61

Real Time PCR amplification of muscarinic M2 receptor mRNA in the cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-1.48 ± 0.13 [a]
SCI + 5-HT + GABA + BMC	-0.26 ± 0.01 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC

Figure-64

Real Time PCR amplification of muscarinic M3 receptor mRNA in the cerebellum of control and experimental rats

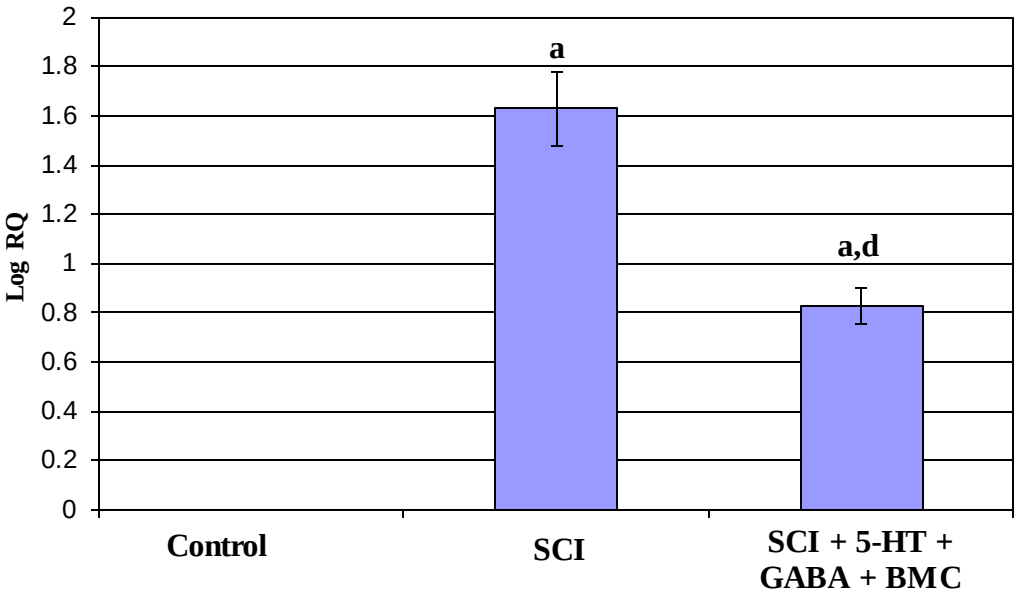


Table-62

Real Time PCR amplification of muscarinic M3 receptor mRNA in the cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	1.62 ± 0.14 [a]
SCI + 5-HT + GABA + BMC	0.82± 0.07 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-65

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the cerebellum of control and experimental rats

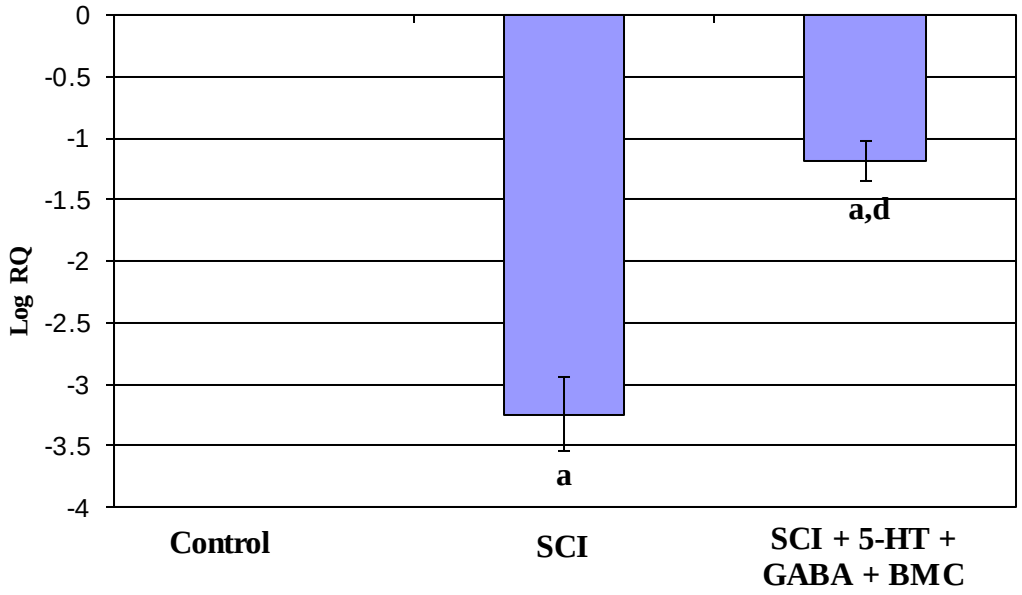


Table-63

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-3.24 ± 0.14 [a]
SCI + 5-HT + GABA + BMC	-1.18± 0.07 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-66

Real Time PCR amplification of phospholipase C mRNA in the cerebellum of control and experimental rats

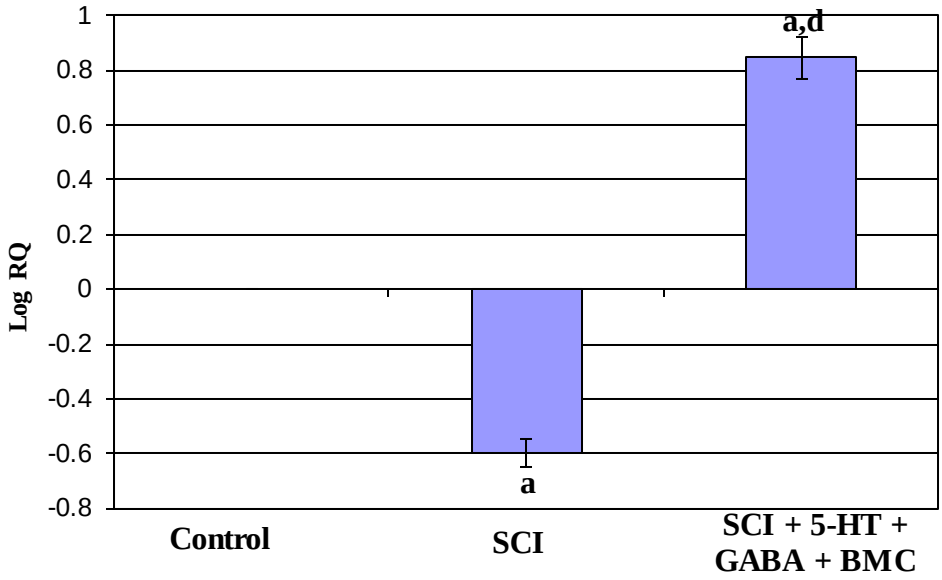


Table-64

Real Time PCR amplification of phospholipase C mRNA in the cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-0.60 ± 0.04 [a]
SCI + 5-HT + GABA + BMC	0.84 ± 0.07 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-67

Real Time PCR amplification of CREB mRNA in the cerebellum of control and experimental rats

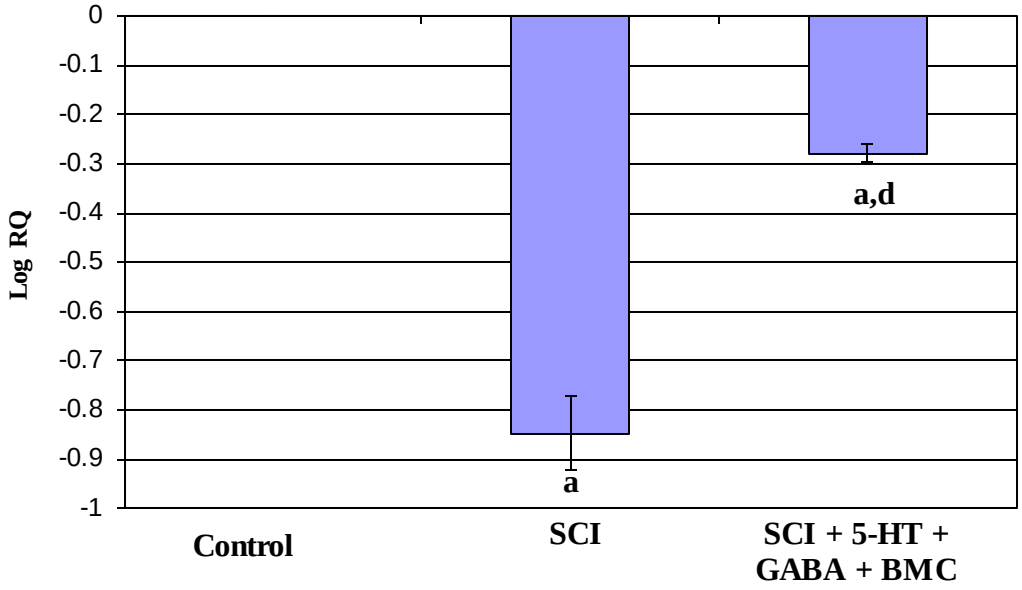


Table-65

Real Time PCR amplification of CREB mRNA in the cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-0.84 ± 0.07 [a]
SCI + 5-HT + GABA + BMC	-0.28 ± 0.02 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-68

Real Time PCR amplification of Bax mRNA in the cerebellum of control and experimental rats

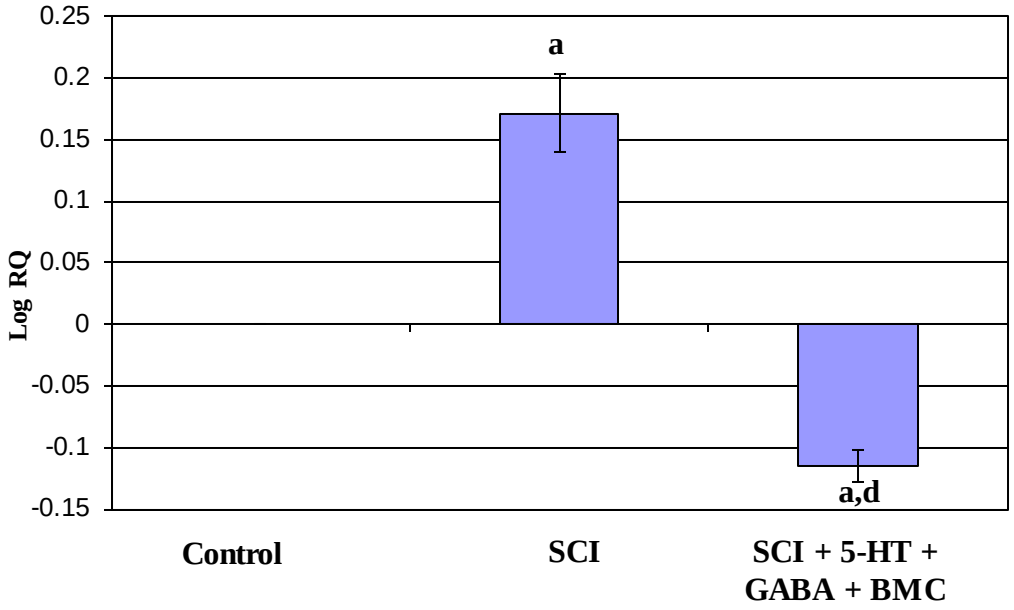


Table-66

Real Time PCR amplification of Bax mRNA in the cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	0.17 ± 0.01 [a]
SCI + 5-HT + GABA + BMC	-0.11 ± 0.01 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-69

Real Time PCR amplification of caspase-8 mRNA in the cerebellum of control and experimental rats

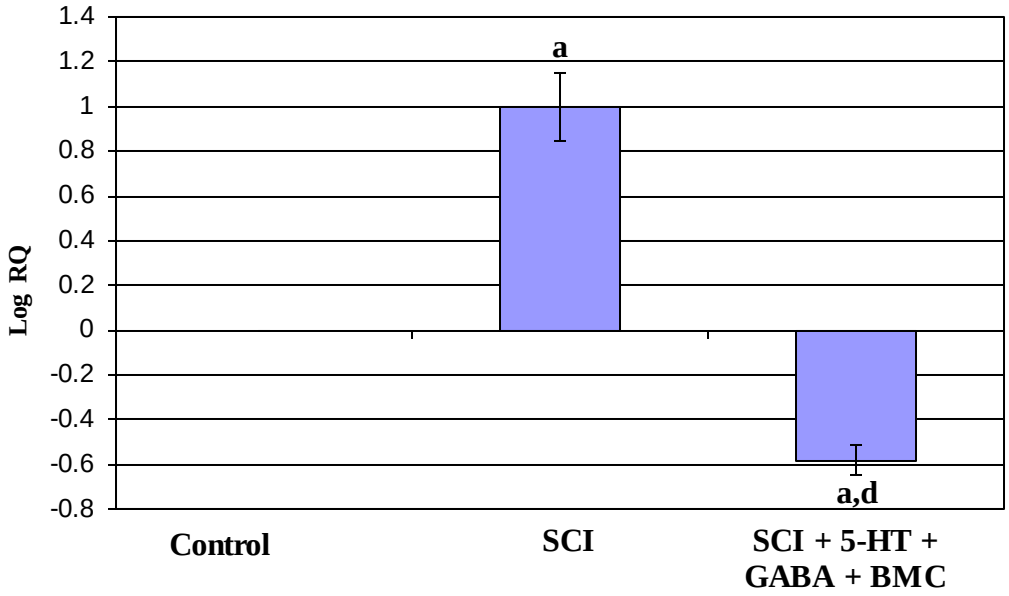


Table-67

Real Time PCR amplification of caspase-8 mRNA in the cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	1.00 ± 0.08 [a]
SCI + 5-HT + GABA + BMC	-0.59 ± 0.04 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-70

Real Time PCR amplification of superoxide dismutase mRNA in the cerebellum of control and experimental rats

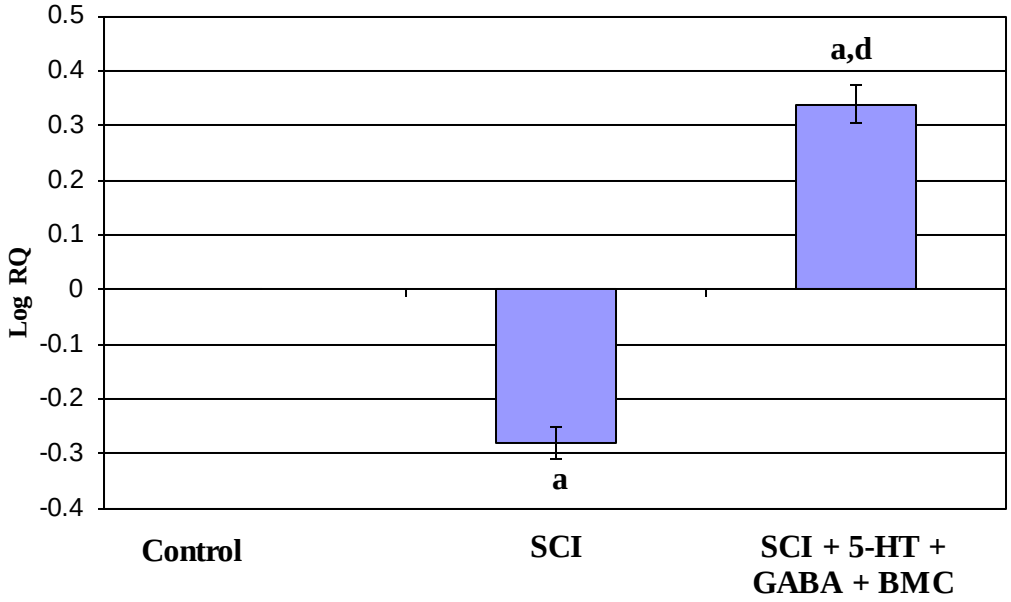


Table-68

Real Time PCR amplification of superoxide dismutase mRNA in the cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-0.27 ± 0.02 [a]
SCI + 5-HT + GABA + BMC	0.32 ± 0.09 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-71

Real Time PCR amplification of glutathione peroxidase mRNA in the cerebellum of control and experimental rats

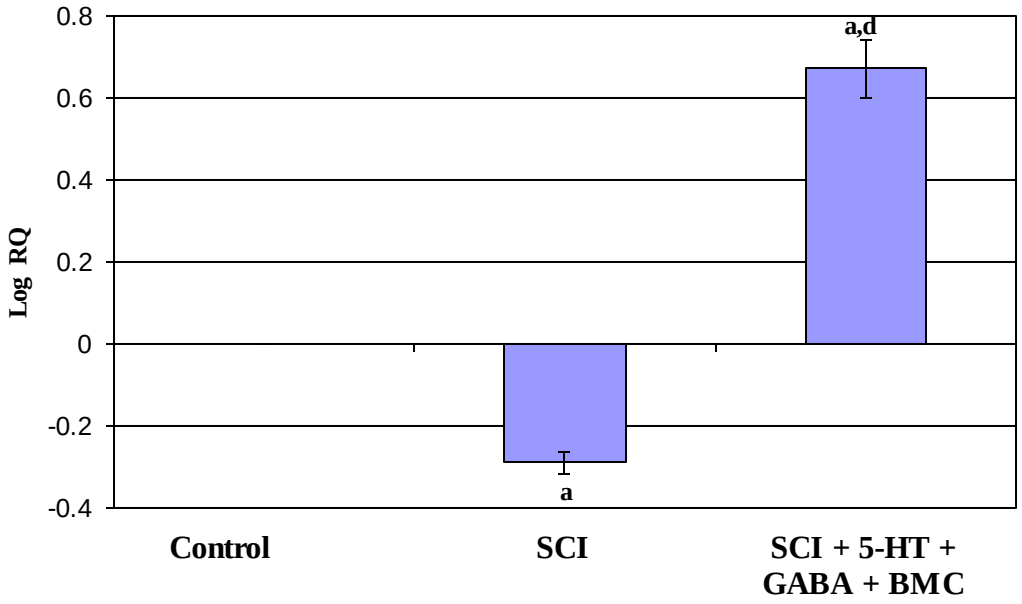


Table-69

Real Time PCR amplification of glutathione peroxidase mRNA in the cerebellum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-0.28 ± 0.02 [a]
SCI + 5-HT + GABA + BMC	0.67 ± 0.06 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-72
IP3 content in the cerebellum of control and experimental rats

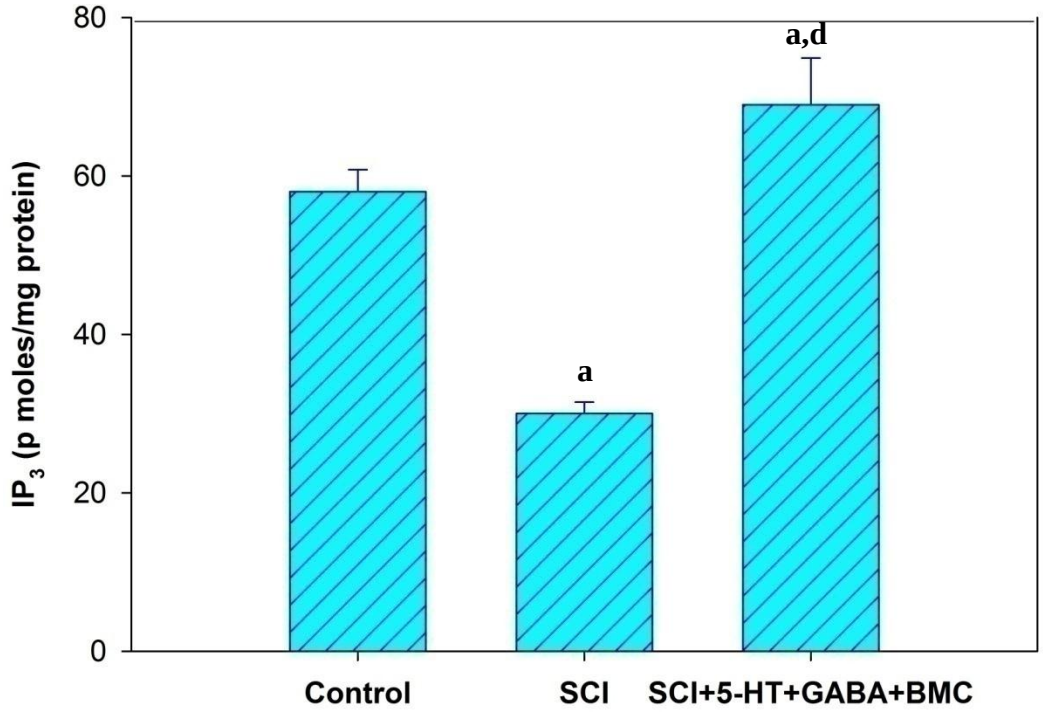


Table-70
IP3 content in the cerebellum of control and experimental rats

Experimental groups	IP3 Content (p moles/mg protein)
Control	58.12 ± 2.78
SCI	30.78 ± 1.43 [a]
SCI + 5-HT + GABA + BMC	69.44 ± 5.88 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-73
cAMP content in the cerebellum of control and experimental rats

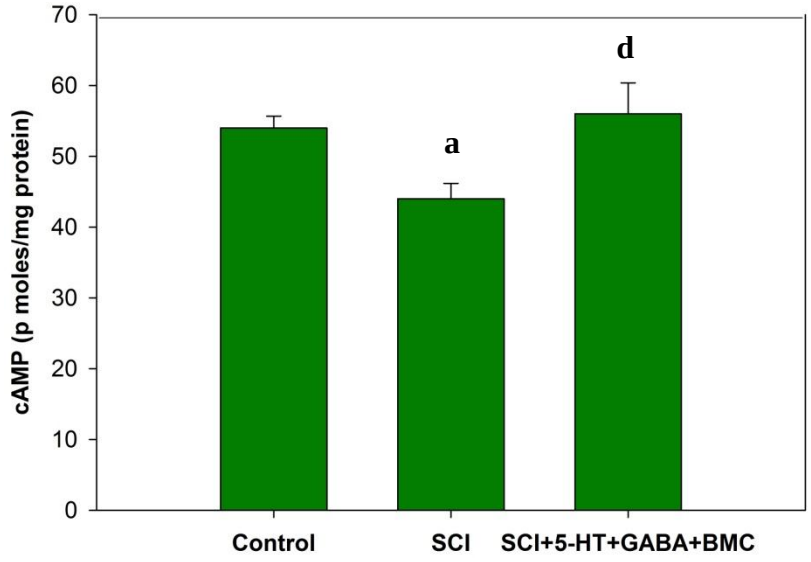


Table-71
cAMP content in the cerebellum of control and experimental rats

Experimental groups	cAMP Content (p moles/mg protein)
Control	**54.45 ± 2.12**
SCI	**44.59 ± 2.61[a]**
SCI + 5-HT + GABA + BMC	**56.23 ± 3.65 [d]**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-74
cGMP content in the cerebellum of control and experimental rats

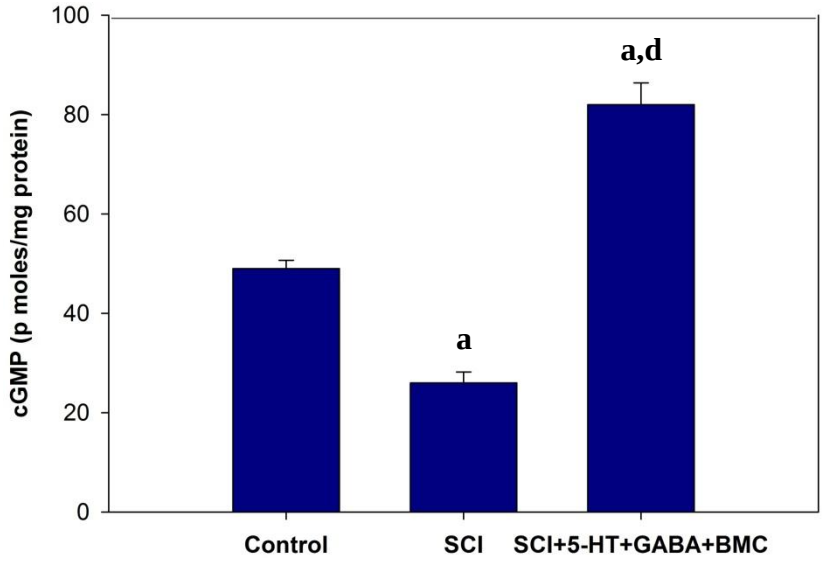


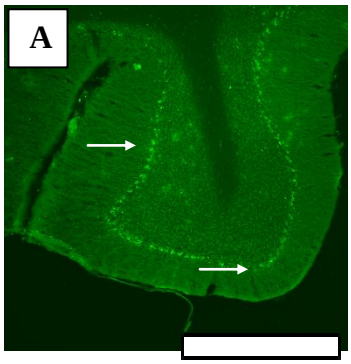
Table-72

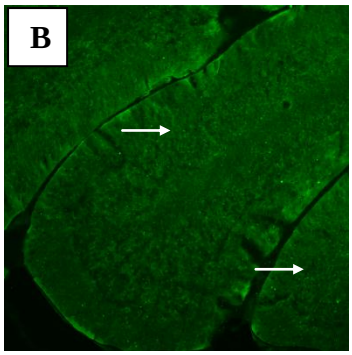
cGMP content in the cerebellum of control and experimental rats

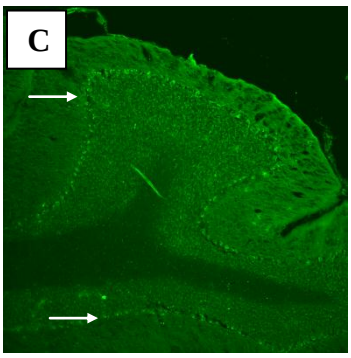
Experimental groups	cGMP Content (p moles/mg protein)
Control	**49.32 ± 2.34**
SCI	**26.89 ± 2.97** [a]
SCI + 5-HT + GABA + BMC	**82.02 ± 4.41** [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-75
Mucsarinic M1 receptor expression in the cerebellum of control and experimental rats



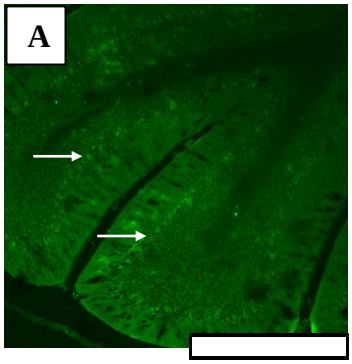


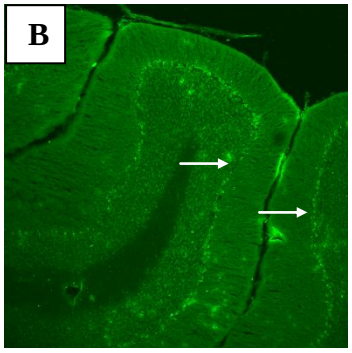


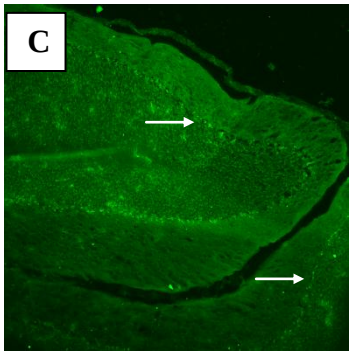
Experimental groups	Mean Pixel Intensity
CONTROL	72.59 ± 5.12
SCI	54.78 ± 4.39 [a]
SCI + 5-HT + GABA + BMC	70.82 ±6.72 [d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
A - Control, B – Spinal cord injured rats, C – Spinal cord injury treated with Serotonin.
Scale bars represent 250 µm. → in white shows muscarinic M1 receptor.

Figure-76
Mucsarinic M3 receptor expression in the cerebellum of control and experimental rats



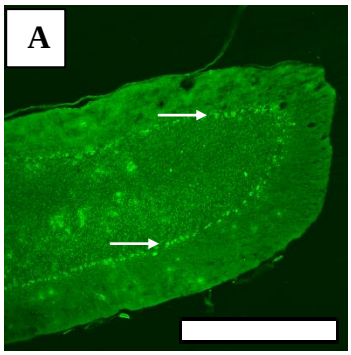


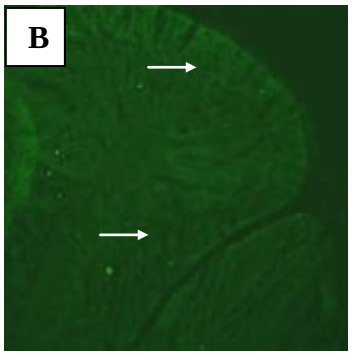


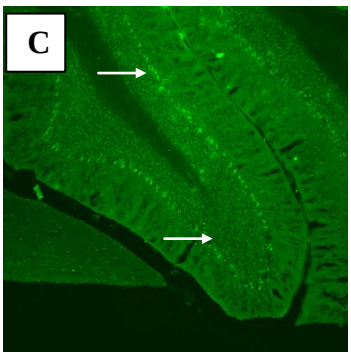
Experimental groups	Mean Pixel Intensity
CONTROL	54.30 ± 4.67
SCI	82.43 ± 7.23 [a]
SCI + 5-HT + GABA + BMC	67.71 ± 5.82 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
A - Control, B – Spinal cord injured rats, C – Spinal cord injury treated with Serotonin.
Scale bars represent 250 µm. → in white shows muscarinic M3 receptor.

Figure-77
α7 nicotinic acetylcholine receptor expression in the cerebellum of control and experimental rats







Experimental groups	Mean Pixel Intensity
CONTROL	81.77 ± 6.92
SCI	52.82 ± 4.56[a]
SCI + 5-HT + GABA + BMC	79.89 ± 6.21[d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]p<0.001 when compared to Control. [d]p<0.001 when compared to SCI group.
A - Control, B – Spinal cord injured rats, C – Spinal cord injury treated with Serotonin.
Scale bars represent 250 µm. → in white shows α7 nicotinic acetylcholine.

Figure-78

Scatchard analysis of [^{3}H] QNB binding against atropine to total muscarinic receptor in the brain stem of control and experimental rats

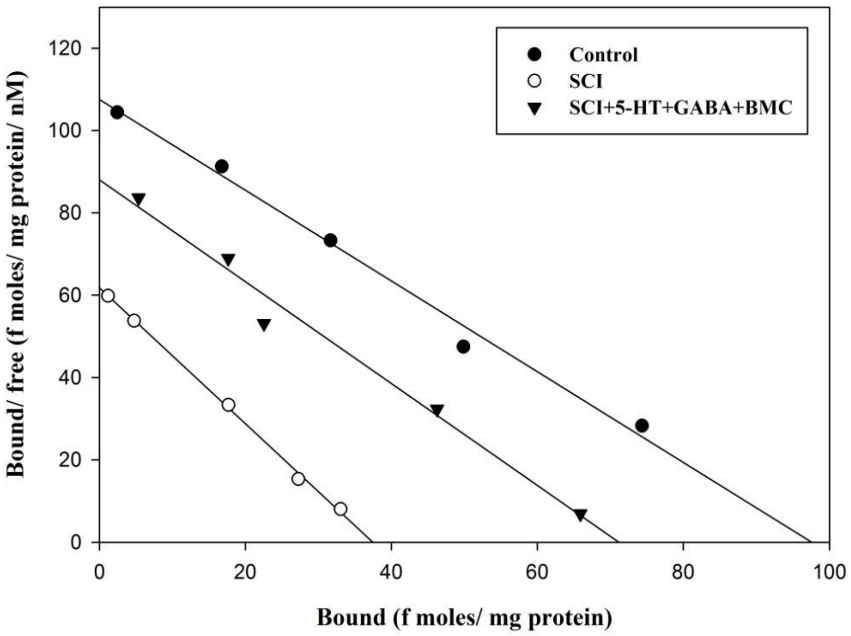


Table-76

Scatchard analysis of [^{3}H] QNB binding against atropine to total muscarinic receptor in the brain stem of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
Control	97.74 ± 8.19	0.91 ± 0.07
SCI	37.88 ± 3.22[a]	0.62 ± 0.05[a]
SCI + 5-HT + GABA + BMC	71.08 ± 4.78 [a,d]	0.80 ± 0.06[c,e]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
[a]p<0.001, [c]p<0.05 when compared to Control. [d]p<0.001, [e]p<0.01 when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-79

Scatchard analysis of [^{3}H] QNB binding against pirenzepine to muscarinic M1 receptor in the brain stem of control and experimental rats

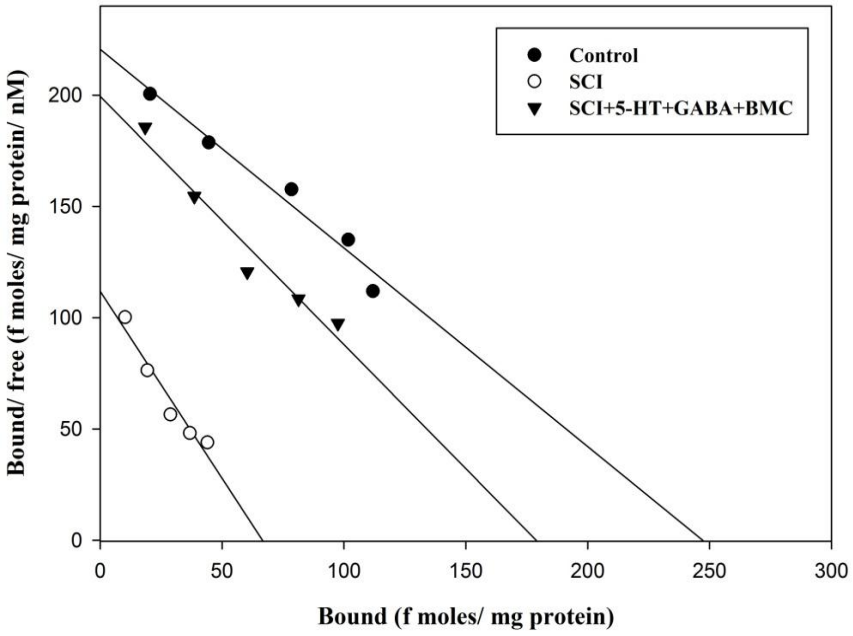


Table-77

Scatchard analysis of [^{3}H] QNB binding against pirenzepine to muscarinic M1 receptor in the brain stem of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
Control	247.57± 20.82	1.12 ± 0.09
SCI	66.57 ± 4.38[a]	0.63 ± 0.04[a]
SCI + 5-HT + GABA + BMC	178.76 ± 13.76[a,d]	0.89 ± 0.06[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-80

Scatchard analysis of [^{3}H] DAMP binding against 4-DAMP mustard to muscarinic M3 receptor in the brain stem of control and experimental rats

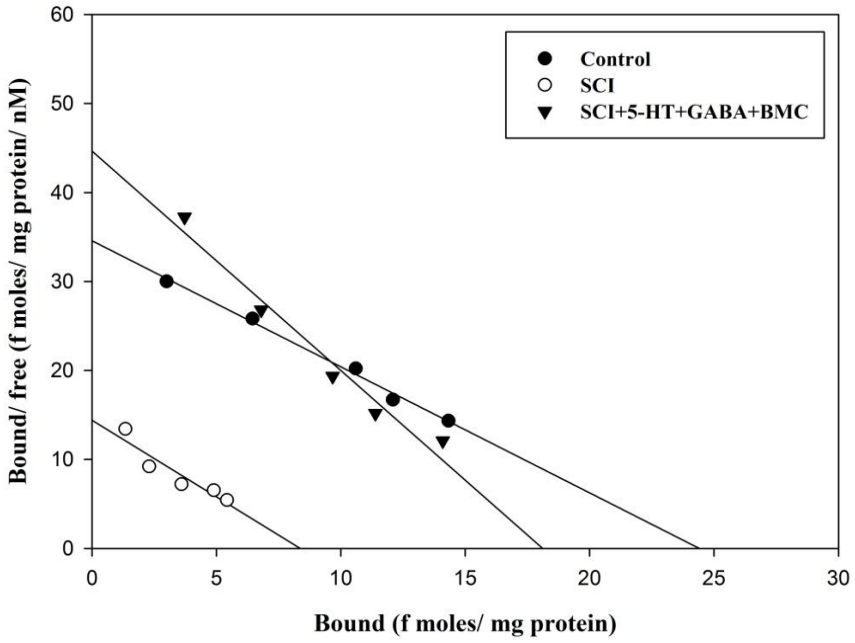


Table-78

Scatchard analysis of [^{3}H] DAMP binding against 4-DAMP mustard to muscarinic M3 receptor in the brain stem of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
Control	24.56 ± 2.00	0.71 ± 0.05
SCI	8.43 ± 0.61 [a]	0.60 ± 0.04 [a]
SCI + 5-HT + GABA + BMC	18.08 ± 1.47 [a,d]	0.41 ± 0.03 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-81

Real Time PCR amplification of acetylcholine esterase mRNA in the brain stem of control and experimental rats

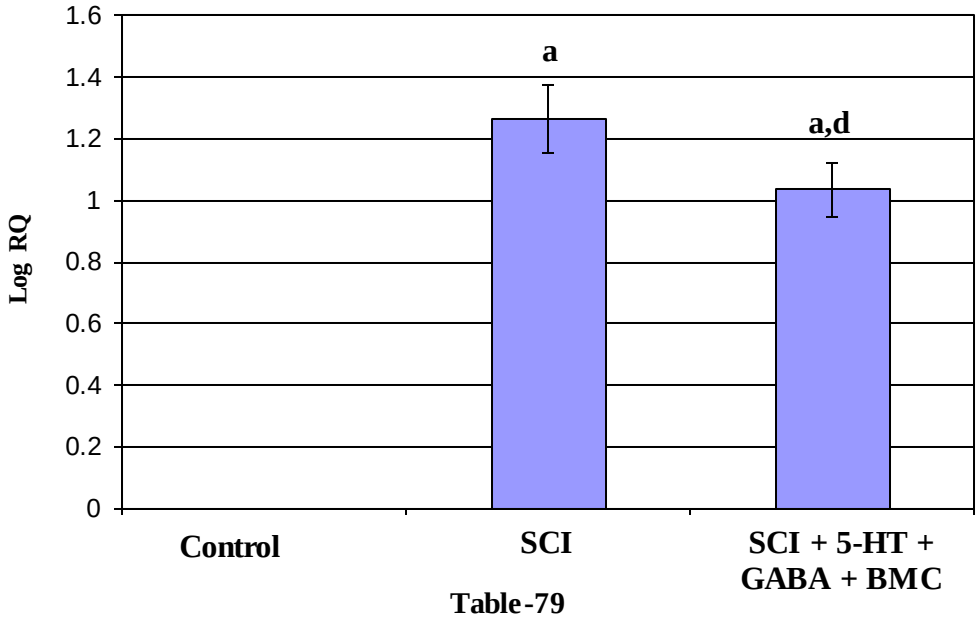


Table-79

Real Time PCR amplification of acetylcholine esterase mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	1.26 ± 0.11^{a}
SCI + 5-HT + GABA + BMC	$1.03 \pm 0.08^{a,d}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
$^{a}p<0.001$ when compared to Control. $^{d}p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-82

Real Time PCR amplification of choline acetyl transferase mRNA in the brain stem of control and experimental rats

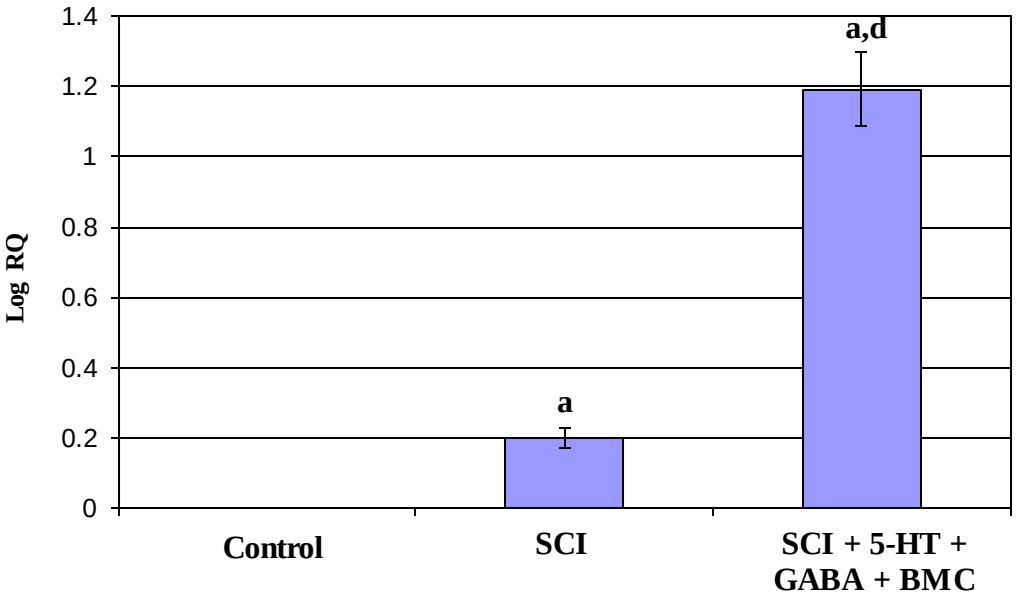


Table-80

Real Time PCR amplification of choline acetyl transferase mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	0.20 ± 0.02^{a}
SCI + 5-HT + GABA + BMC	$1.19 \pm 0.10^{a,d}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
$^{a}p<0.001$ when compared to Control. $^{d}p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-83

Real Time PCR amplification of muscarinic M1 receptor mRNA in the brain stem of control and experimental rats

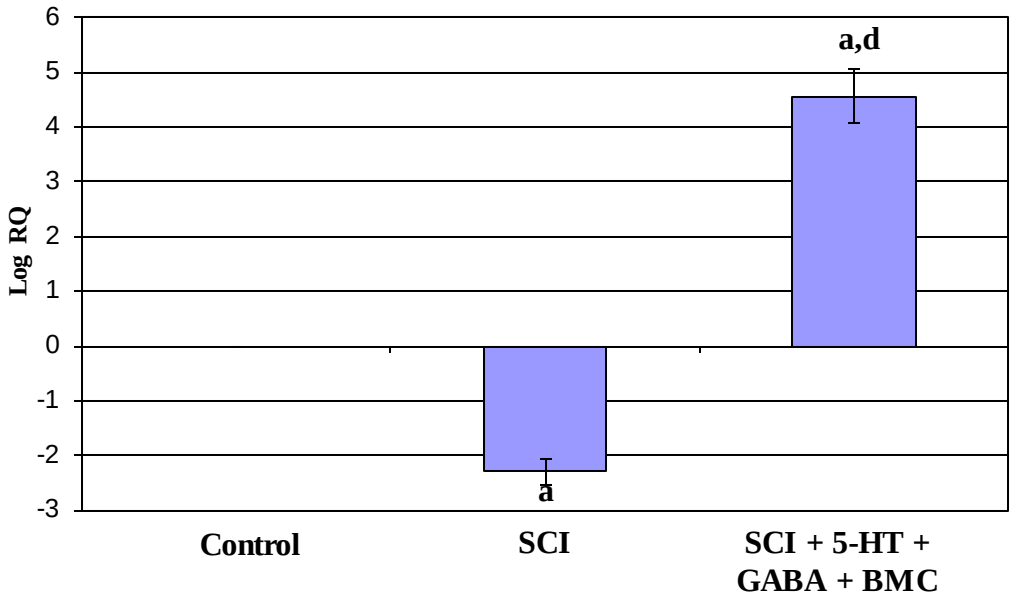


Table-81

Real Time PCR amplification of muscarinic M1 receptor mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-2.32 ± 0.21[a]
SCI + 5-HT + GABA + BMC	4.56 ± 0.41[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-84

Real Time PCR amplification of muscarinic M2 receptor mRNA in the brain stem of control and experimental rats

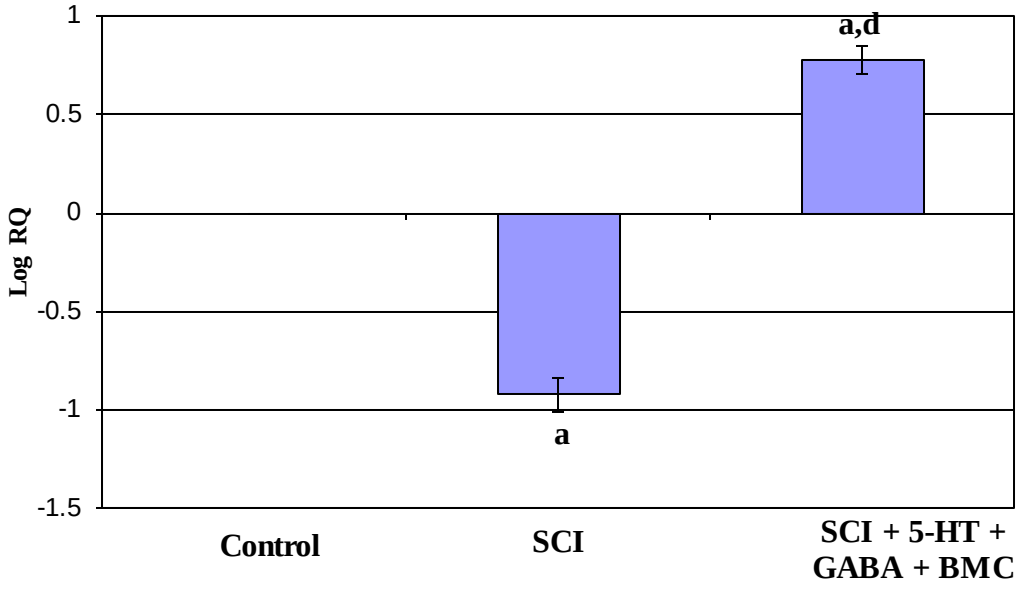


Table-82

Real Time PCR amplification of muscarinic M2 receptor mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-0.92 ± 0.08[a]
SCI + 5-HT + GABA + BMC	0.77± 0.06[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-85

Real Time PCR amplification of muscarinic M3 receptor mRNA in the brain stem of control and experimental rats

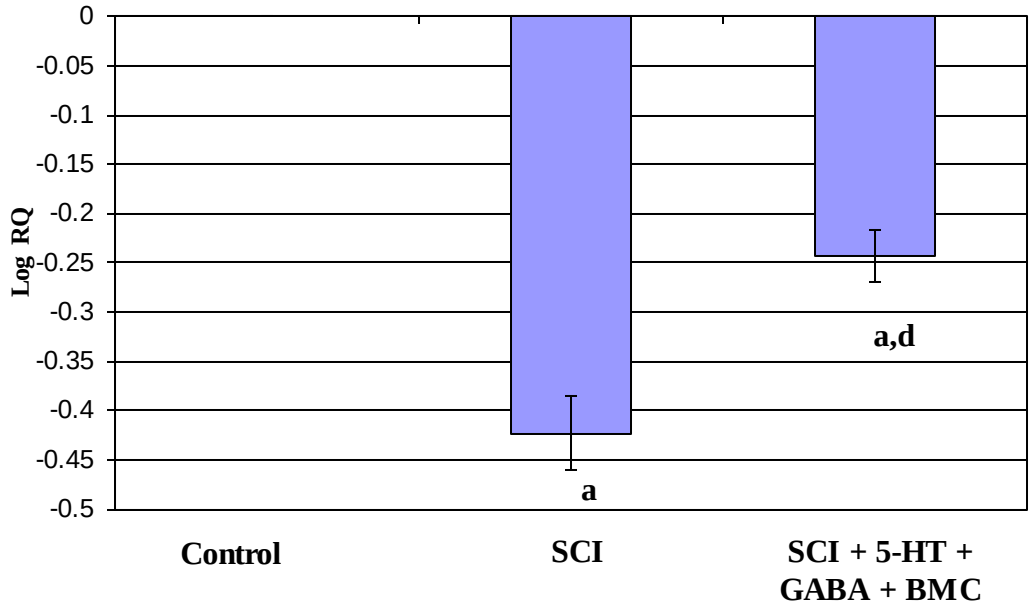


Table-83

Real Time PCR amplification of muscarinic M3 receptor mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-0.42 ± 0.03[a]
SCI + 5-HT + GABA + BMC	-0.24 ± 0.02[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]p<0.001 when compared to Control. [d]p<0.001 when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-86

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the brain stem of control and experimental rats

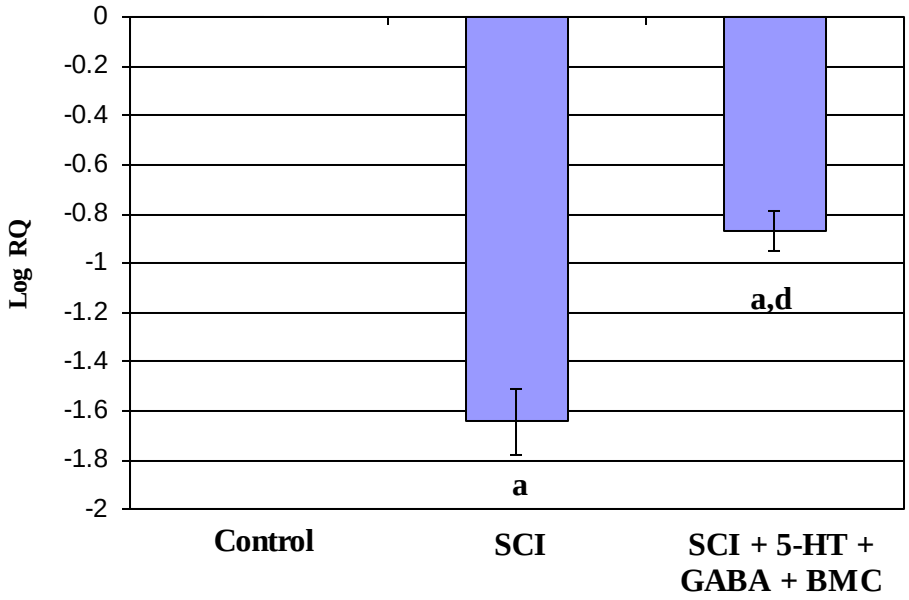


Table-84

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-1.64 ± 0.13[a]
SCI + 5-HT + GABA + BMC	-0.87 ± 0.08[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-87

Real Time PCR amplification of phospholipase C mRNA in the brain stem of control and experimental rats

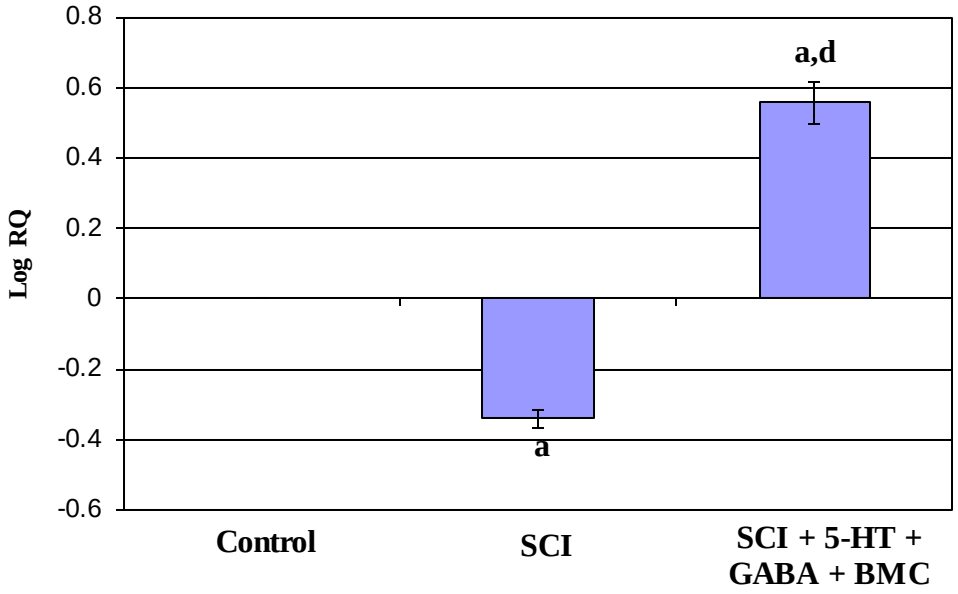


Table- 85

Real Time PCR amplification of phospholipase C mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-0.34 ± 0.02[a]
SCI + 5-HT + GABA + BMC	0.55± 0.04[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-88

Real Time PCR amplification of CREB mRNA in the brain stem of control and experimental rats

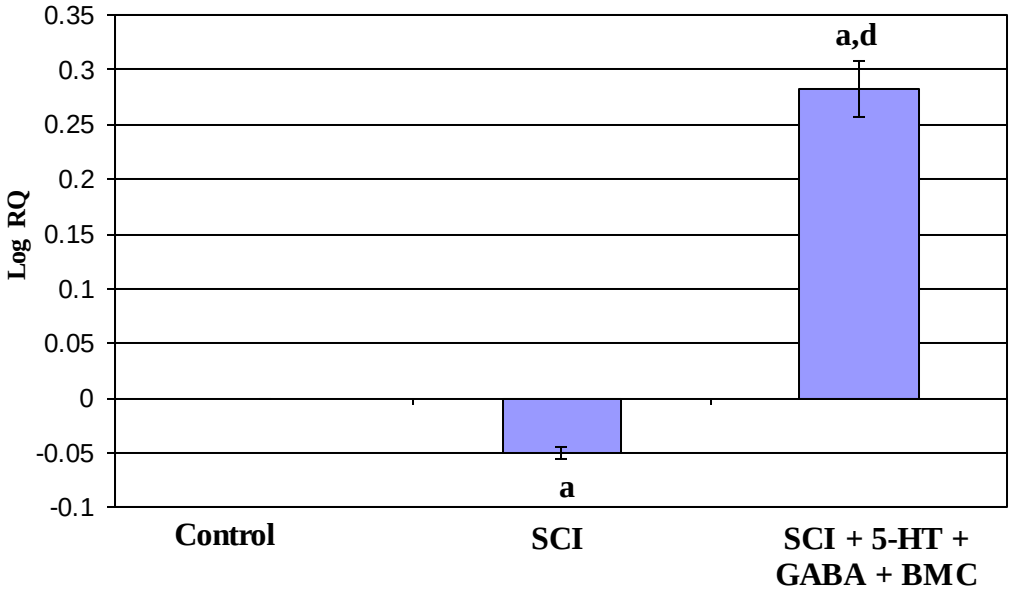


Table-86

Real Time PCR amplification of CREB mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-0.05 ± 0.005 [a]
SCI + 5-HT + GABA + BMC	0.28 ± 0.021 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-89

Real Time PCR amplification of Bax mRNA in the brain stem of control and experimental rats

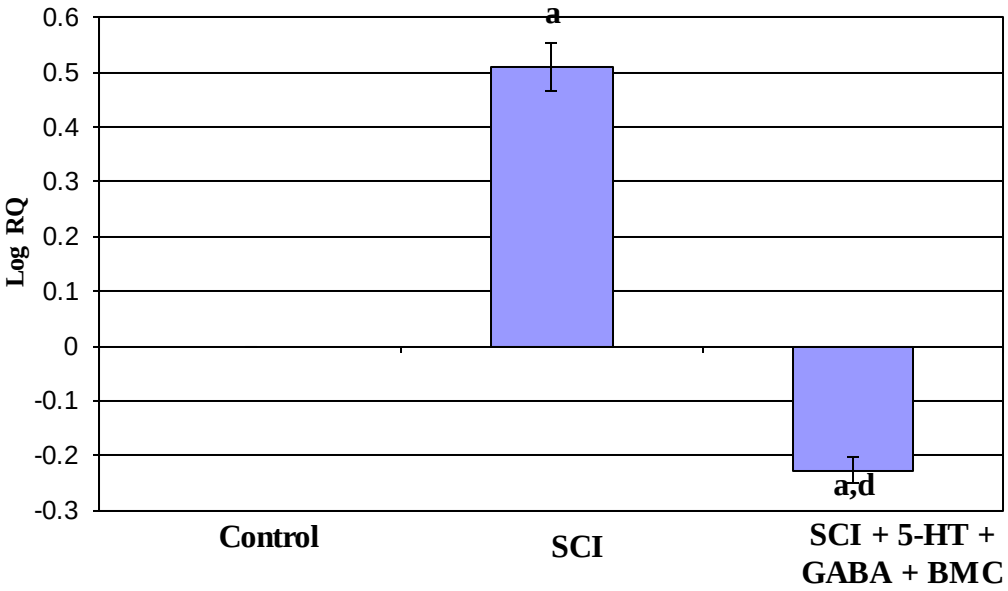


Table-87

Real Time PCR amplification of Bax mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	0.51 ± 0.04^{a}
SCI + 5-HT + GABA + BMC	$-0.22 \pm 0.02^{a,d}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCIgroup.C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC – Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-90

Real Time PCR amplification of Caspase-8 mRNA in the brain stem of control and experimental rats

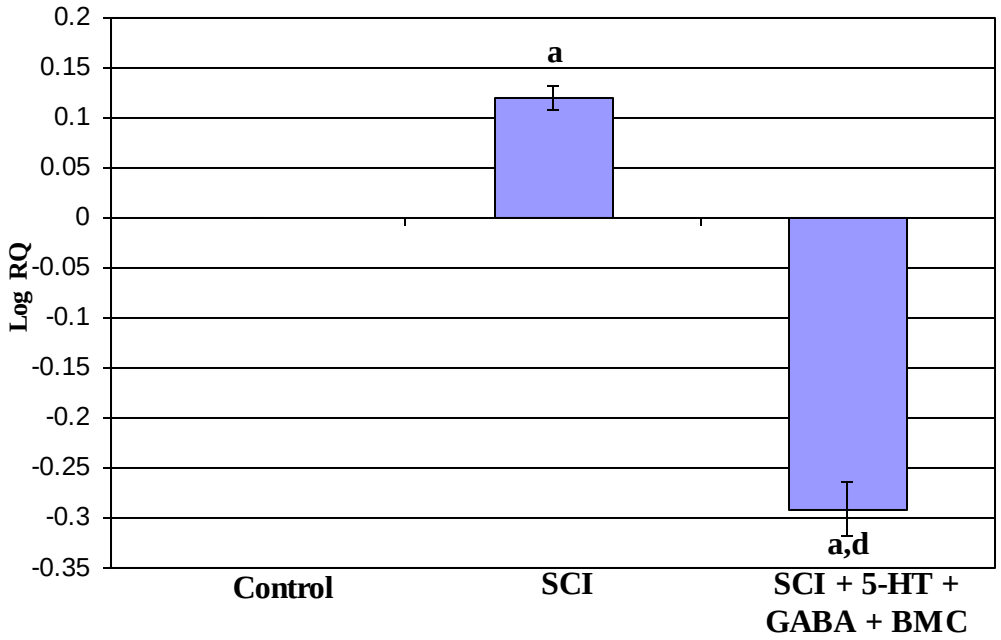


Table-88

Real Time PCR amplification of Caspase-8 mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	0.12 ± 0.01^{a}
SCI + 5-HT + GABA + BMC	$-0.29 \pm 0.02^{a,d}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-91

Real Time PCR amplification of superoxide dismutase mRNA in the brain stem of control and experimental rats

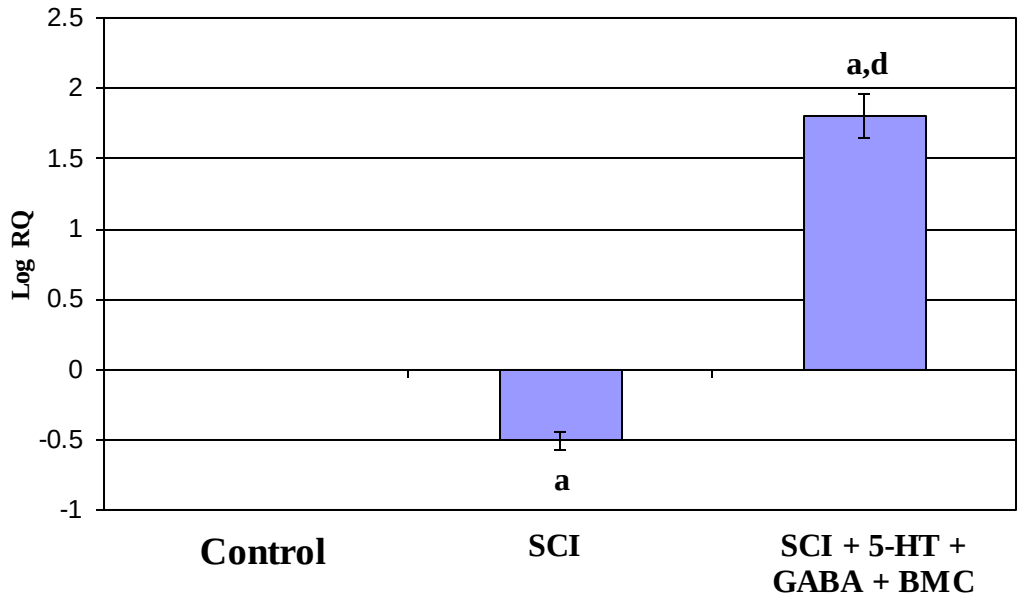


Table-89

Real Time PCR amplification of superoxide dismutase mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-0.50 ± 0.05 [a]
SCI + 5-HT + GABA + BMC	1.80 ± 0.16 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-92

Real Time PCR amplification of glutathione peroxidase mRNA in the brain stem of control and experimental rats

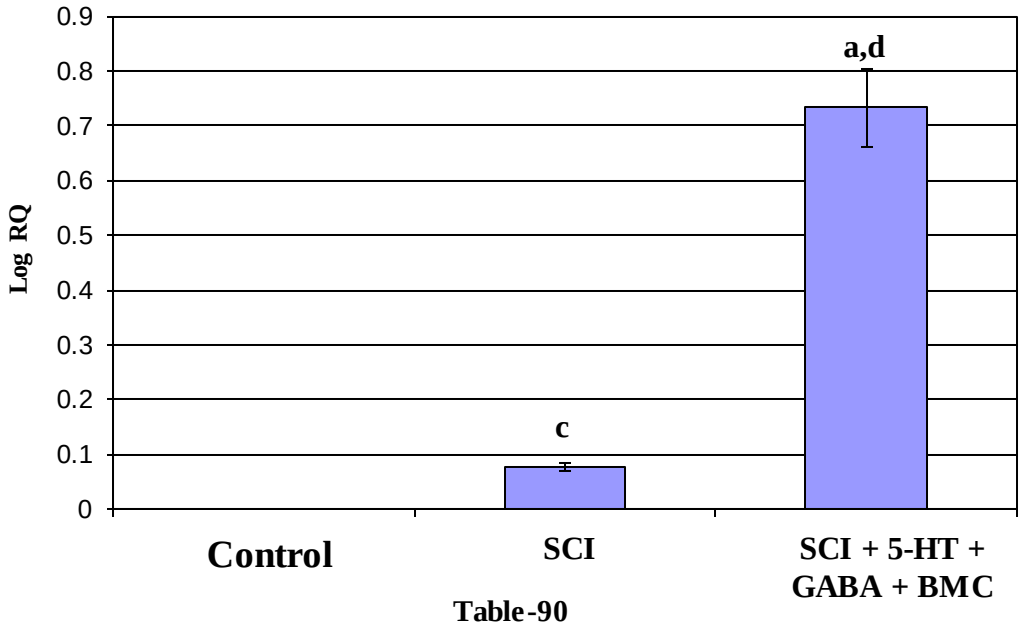


Table-90

Real Time PCR amplification of glutathione peroxidase mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	0.07 ± 0.006 [c]
SCI + 5-HT + GABA + BMC	0.73 ± 0.072 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$, [c] $p<0.05$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-93
IP3 content in the brain stem of control and experimental rats

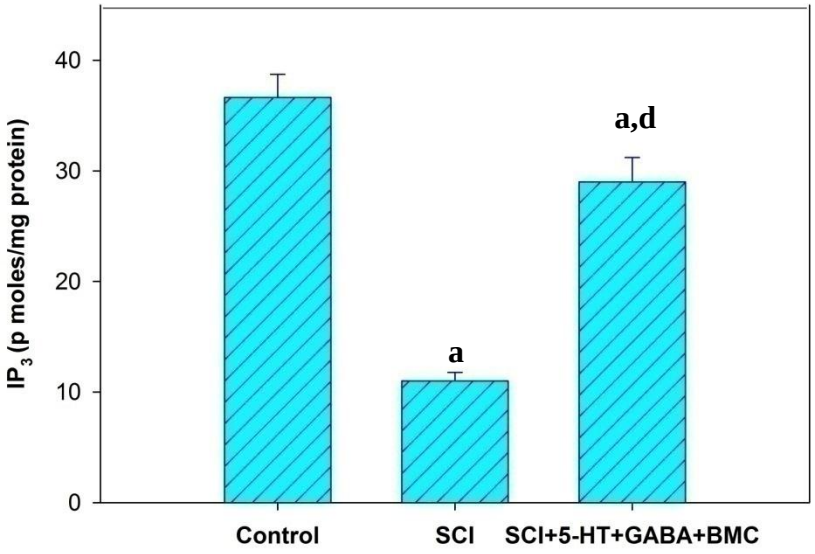


Table-91
IP3 content in the brain stem of control and experimental rats

Experimental groups	IP3 Content (p moles/mg protein)
Control	36.64 ± 2.09
SCI	11.52± 0.78 [a]
SCI + 5-HT + GABA + BMC	29.09 ± 2.21 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-94
cAMP content in the brain stem of control and experimental rats

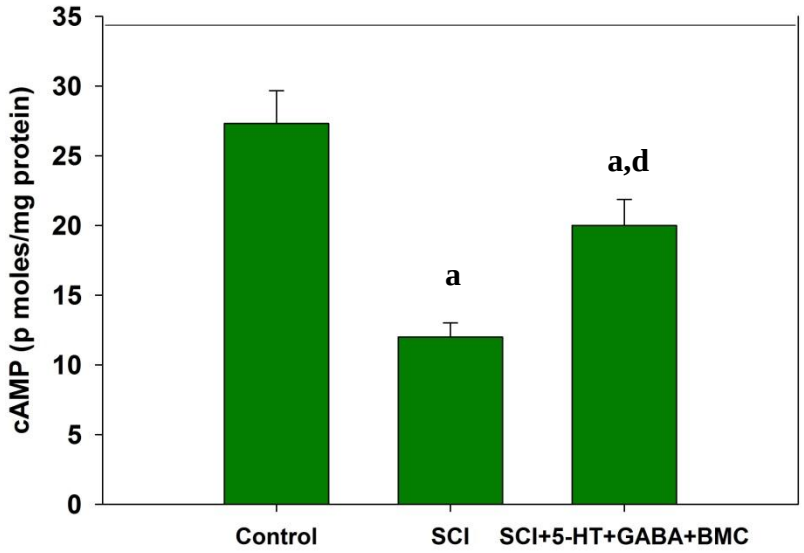


Table-92
cAMP content in the brain stem of control and experimental rats

Experimental groups	cAMP Content (p moles/mg protein)
Control	27.31± 2.36
SCI	12.45 ± 1.01 [a]
SCI + 5-HT + GABA + BMC	20.35 ± 1.87 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-95
cGMP content in the brain stem of control and experimental rats

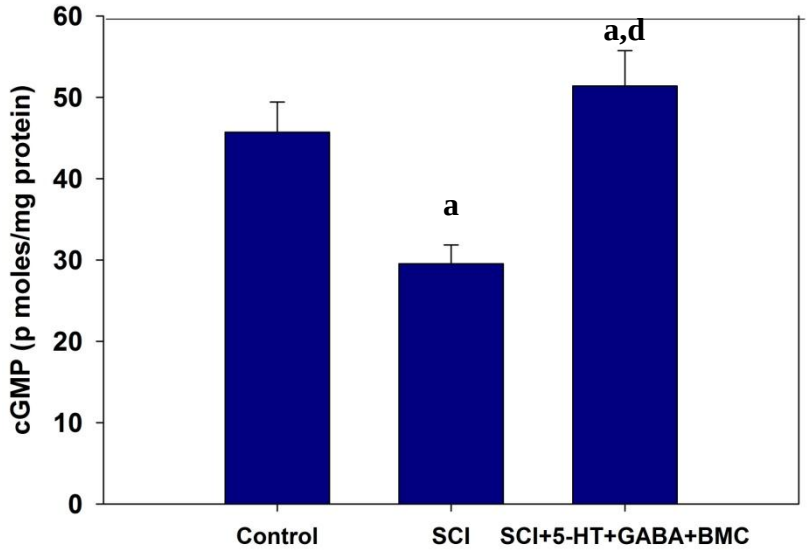
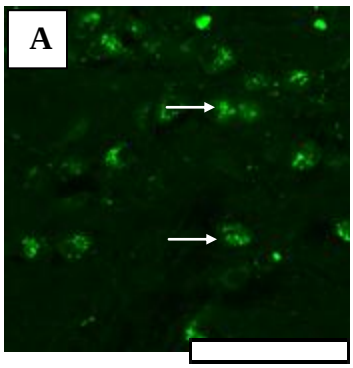


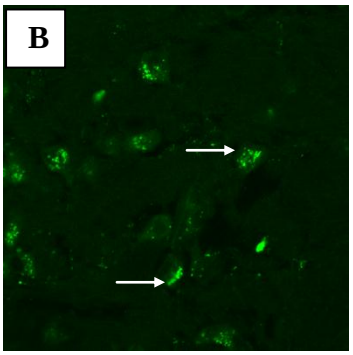
Table-93
cGMP content in the brain stem of control and experimental rats

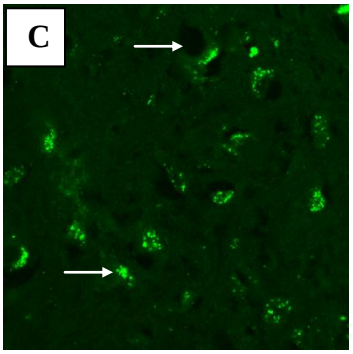
Experimental groups	cGMP Content (p moles/mg protein)
Control	45.72 ± 3.72
SCI	29.54 ± 2.31 [a]
SCI + 5-HT + GABA + BMC	51.39 ± 4.34 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-96
Mucsarinic M1 receptor expression in the brain stem of control and experimental rats



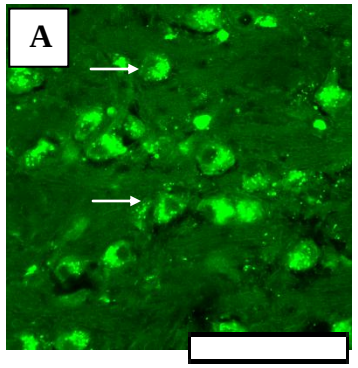


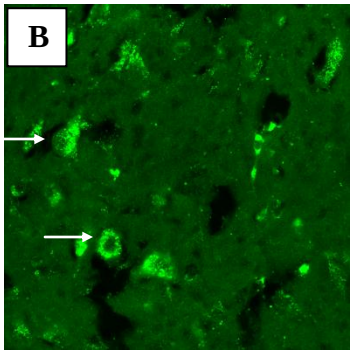


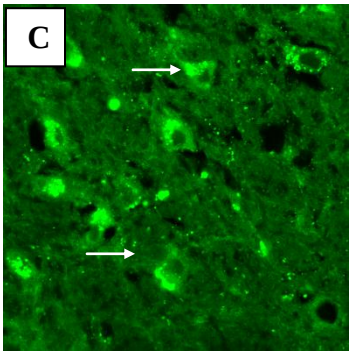
Experimental groups	Mean Pixel Intensity
CONTROL	49.34 ± 3.54
SCI	27.81 ± 1.58[a]
SCI + 5-HT + GABA + BMC	40.34 ± 3.61[c,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$, [c]$p<0.05$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
A - Control, B – Spinal cord injured rats, C – Spinal cord injury treated with Serotonin.
Scale bars represent 75 µm. → in white shows muscarinic M1 receptor.

Figure-97
Mucsarinic M3 receptor expression in the brain stem of control and experimental rats



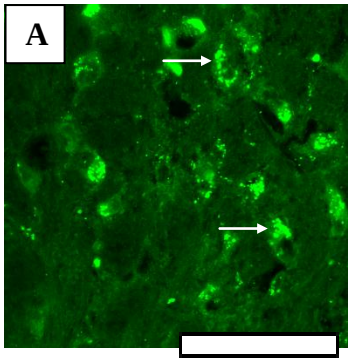


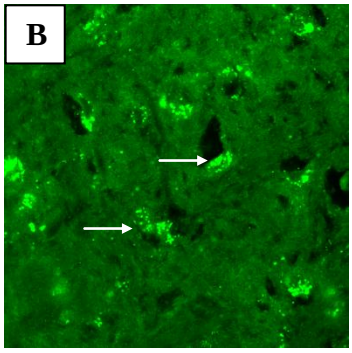


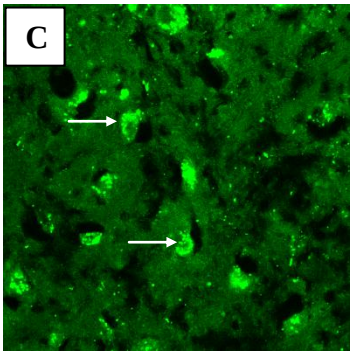
Experimental groups	Mean Pixel Intensity
CONTROL	87.06 ± 6.25
SCI	65.46 ± 5.43[a]
SCI + 5-HT + GABA + BMC	81.77 ± 7.12[d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
A - Control, B – Spinal cord injured rats, C – Spinal cord injury treated with Serotonin.
Scale bars represent 75 µm. → in white shows muscarinic M3 receptor.

Figure-98
α7 nicotinic acetylcholine receptor expression in the brain stem of control and experimental rats







Experimental groups	Mean Pixel Intensity
CONTROL	66.34 ± 5.39
SCI	42.71 ± 3.51[a]
SCI + 5-HT + GABA +BMC	58.83 ± 4.29[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]p<0.001 when compared to Control. [d]p<0.001 when compared to SCI group.
A - Control, B – Spinal cord injured rats, C – Spinal cord injury treated with Serotonin.
Scale bars represent 75 µm. → in white shows α7 nicotinic acetylcholine receptor.

Figure-99

Scatchard analysis of [^{3}H] QNB binding against total muscarinic receptor antagonist, atropine in the corpus striatum of control and experimental rats

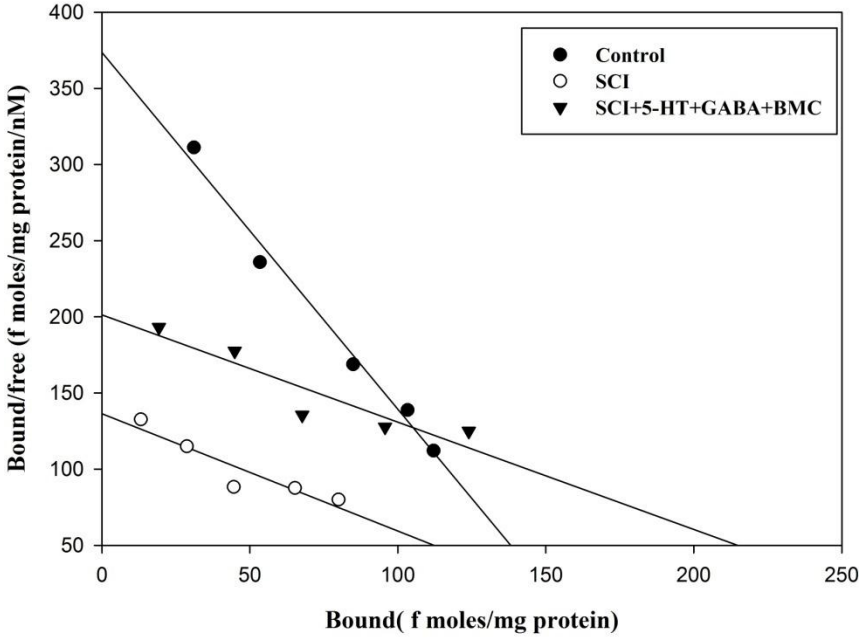


Table-97

Scatchard analysis of [^{3}H] QNB binding against total muscarinic receptor antagonist, atropine in the corpus striatum of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
Control	**137.52 ± 4.76**	**0.37 ± 0.02**
SCI	**111.05 ± 3.71^{b}**	**0.80 ± 0.05 ^{a}**
SCI + 5-HT + GABA + BMC	**213.72 ± 11.22^{a,d}**	**1.05 ± 0.08 ^{a,d}**

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
^{a}p<0.001, ^{b}p<0.01when compared to Control. ^{d}p<0.001 when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-100

Scatchard analysis of [^{3}H] QNB binding against pirenzepine to muscarinic M1 receptor in the corpus striatum of control and experimental rats

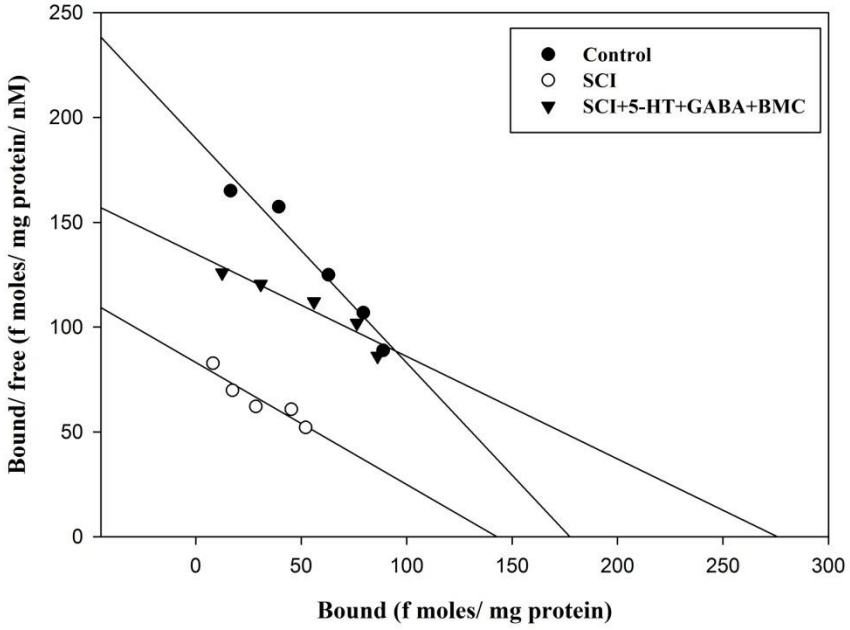


Table-98

Scatchard analysis of [^{3}H] QNB binding against pirenzepine to muscarinic M1 receptor in the corpus striatum of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
Control	178.36 ± 6.97	0.75 ± 0.05
SCI	143.30 ± 5.42 [a]	1.08 ± 0.09 [a]
SCI + 5-HT + GABA + BMC	275.45 ± 22.47 [a,d]	1.73 ± 0.13 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure- 101

Scatchard analysis of [^{3}H] DAMP binding against4-DAMP mustard to muscarinic M3 receptor in the corpus striatum of control and experimental rats

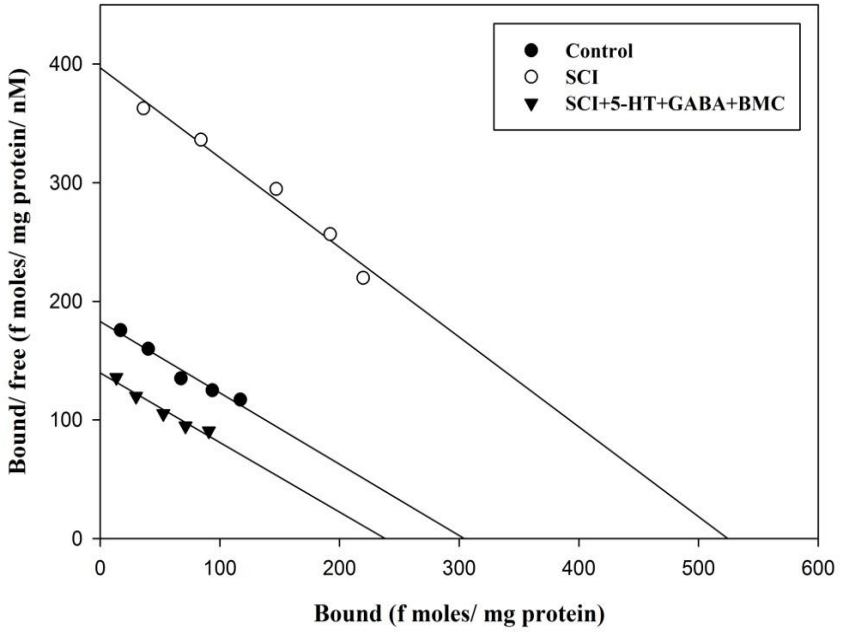


Table-99

Scatchard analysis of [^{3}H] DAMP binding against4-DAMP mustard to muscarinic M3 receptor in the corpus striatum of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
Control	305.91 ± 22.45	1.68 ± 0.12
SCI	524.40 ± 24.49 [a]	1.32 ± 0.08 [b]
SCI + 5-HT + GABA + BMC	239.82 ± 18.37 [a,d]	1.70 ± 0.12 [e]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
[a] $p<0.001$, [b] $p<0.01$ when compared to Control. [d] $p<0.001$, [e] $p<0.01$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-102

Real Time PCR amplification of acetylcholine esterase mRNA in the corpus striatum of control and experimental rats

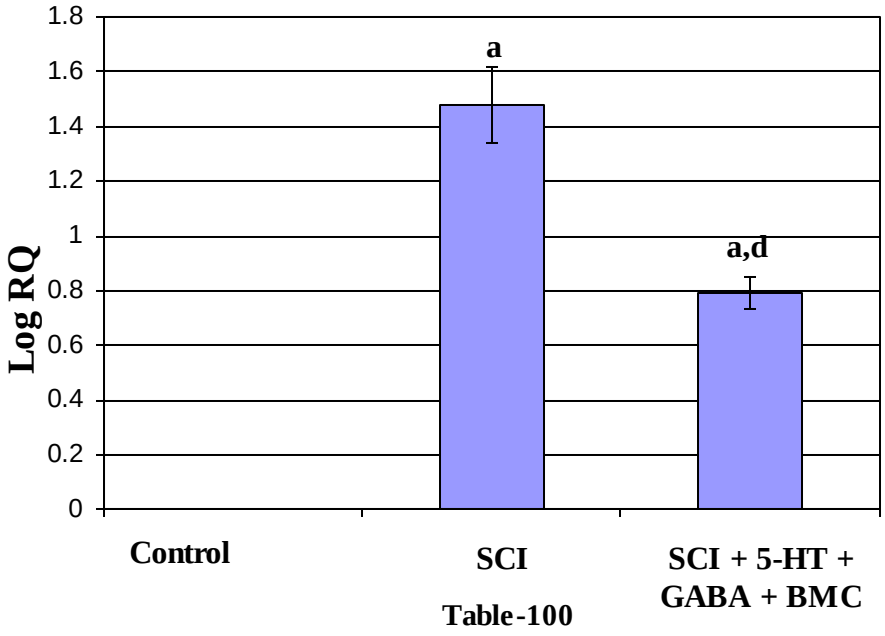


Table-100

Real Time PCR amplification of acetylcholine esterase mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	1.48 ± 0.14[a]
SCI + 5-HT + GABA + BMC	0.79 ± 0.07 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]p<0.001 when compared to Control. [d]p<0.001 when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-103

Real Time PCR amplification of choline acetyl transferase mRNA in the corpus striatum of control and experimental rats

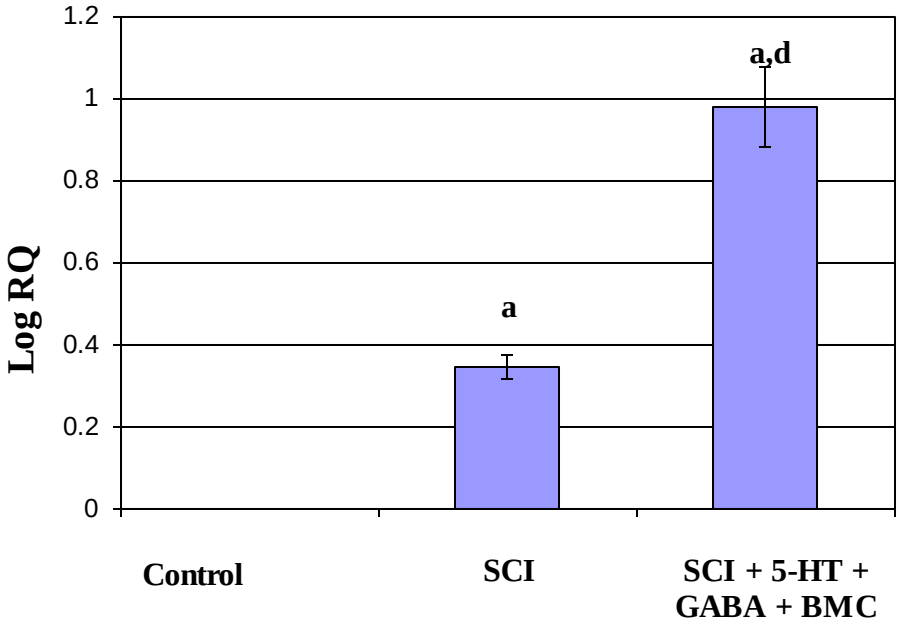


Table-101

Real Time PCR amplification of choline acetyl transferase mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	0.34 ± 0.03 [a]
SCI + 5-HT + GABA + BMC	0.98 ± 0.09 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-104

Real Time PCR amplification of muscarinic M1 receptor mRNA in the corpus striatum of control and experimental rats

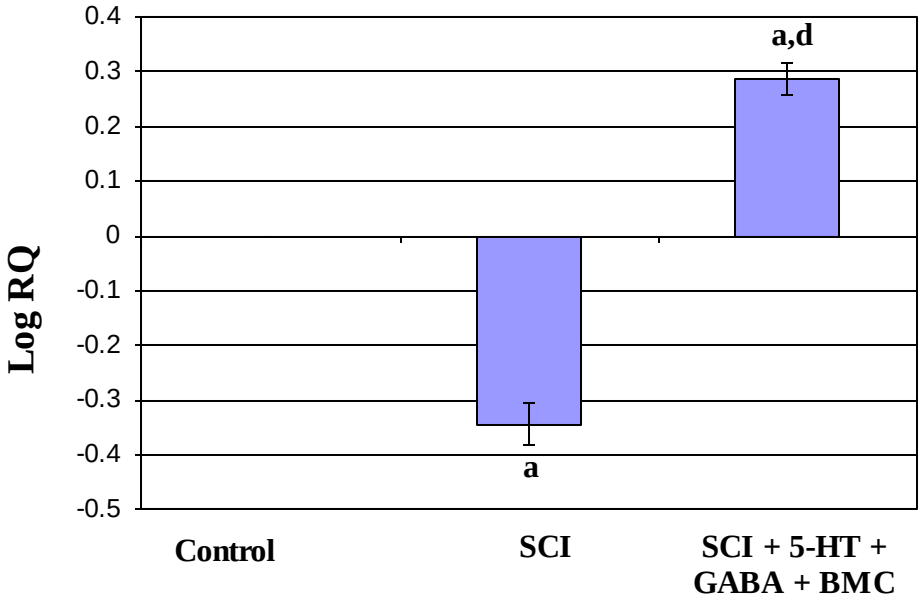


Table-102

Real Time PCR amplification of muscarinic M1 receptor mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-0.34 ± 0.03[a]
SCI + 5-HT + GABA + BMC	0.28 ± 0.02[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-105

Real Time PCR amplification of muscarinic M2 receptor mRNA in the corpus striatum of control and experimental rats

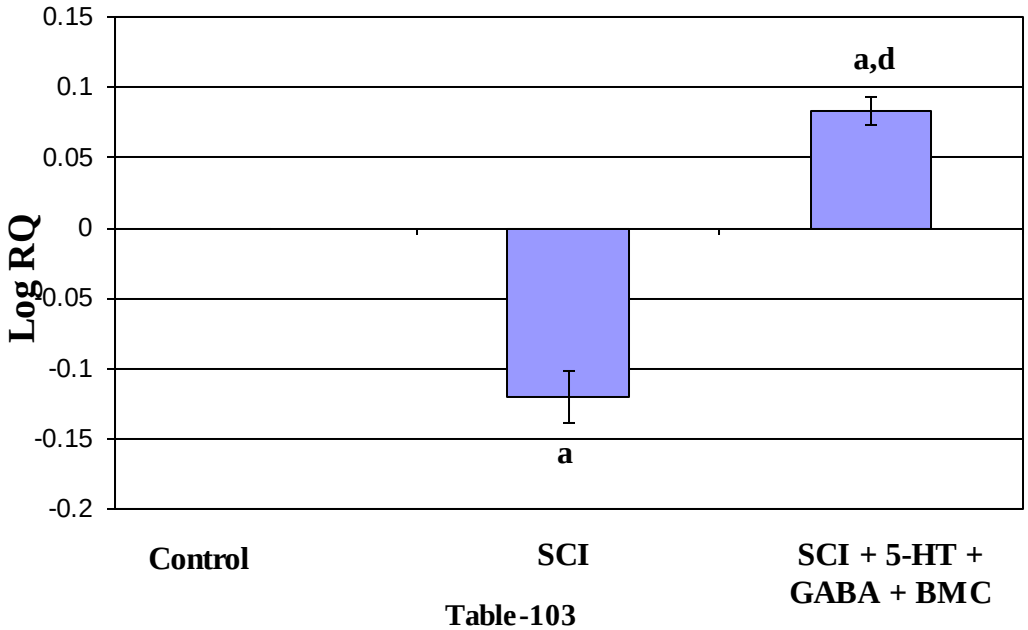


Table-103

Real Time PCR amplification of muscarinic M2 receptor mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-0.12 ± 0.011[a]
SCI + 5-HT + GABA + BMC	0.08 ± 0.007[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-106

Real Time PCR amplification of muscarinic M3 receptor mRNA in the corpus striatum of control and experimental rats

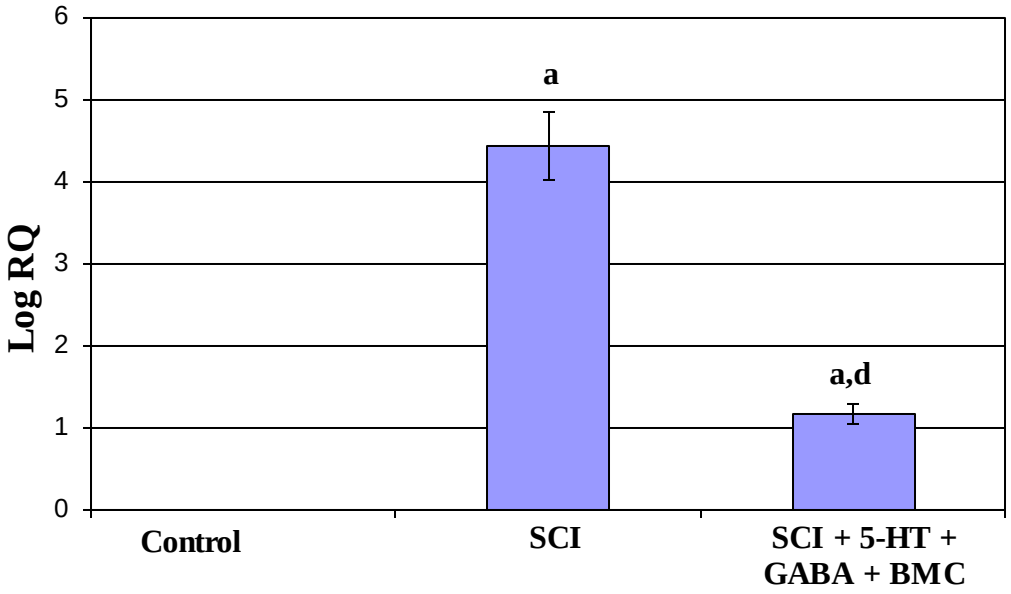


Table-104

Real Time PCR amplification of muscarinic M3 receptor mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	4.43 ± 0.41^{a}
SCI + 5-HT + GABA + BMC	$1.16 \pm 0.12^{a,d}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]p<0.001 when compared to Control. [d]p<0.001 when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-107

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the corpus striatum of control and experimental rats

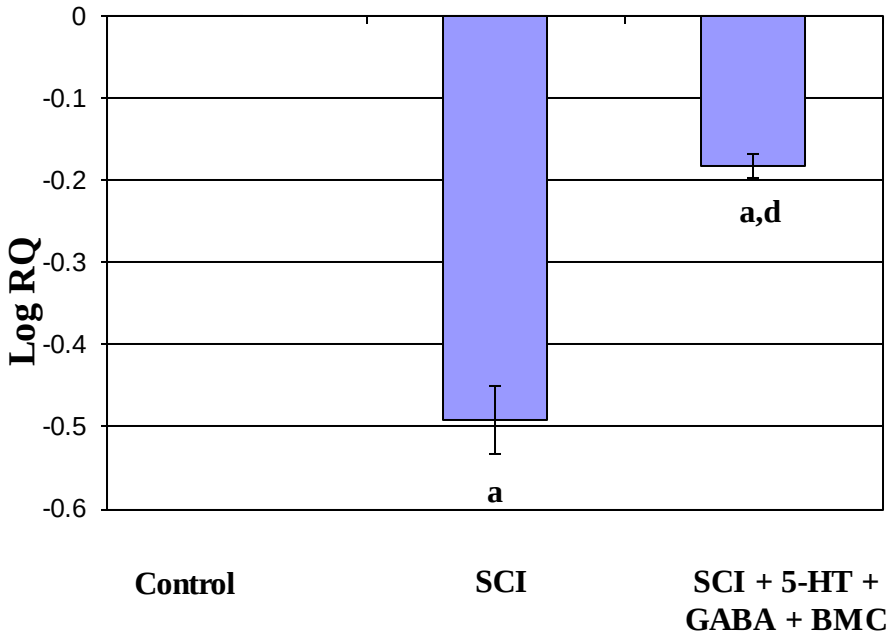


Table-105

Real Time PCR amplification of α7 nicotinic acetylcholine receptor mRNA in the corpus striatum of control and experimental rat

Experimental groups	Log RQ
Control	0
SCI	-0.49 ± 0.04[a]
SCI + 5-HT + GABA + BMC	-0.18 ± 0.01[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-108

Real Time PCR amplification of phospholipase C mRNA in the corpus striatum of control and experimental rats

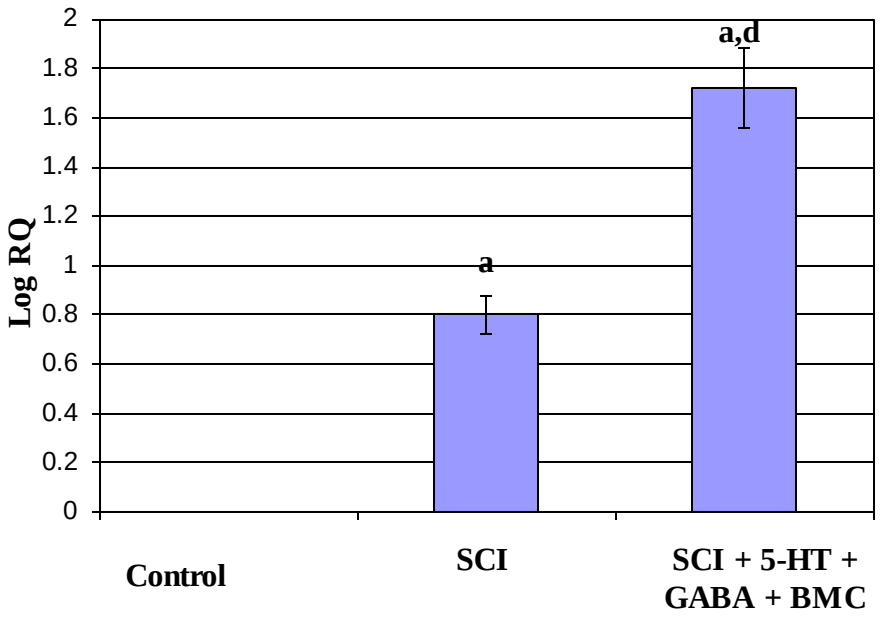


Table-106

Real Time PCR amplification of phospholipase C mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	0.80 ± 0.07^{a}
SCI + 5-HT + GABA + BMC	$1.71 \pm 0.16^{a,d}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-109

Real Time PCR amplification of CREB mRNA in the corpus striatum of control and experimental rats

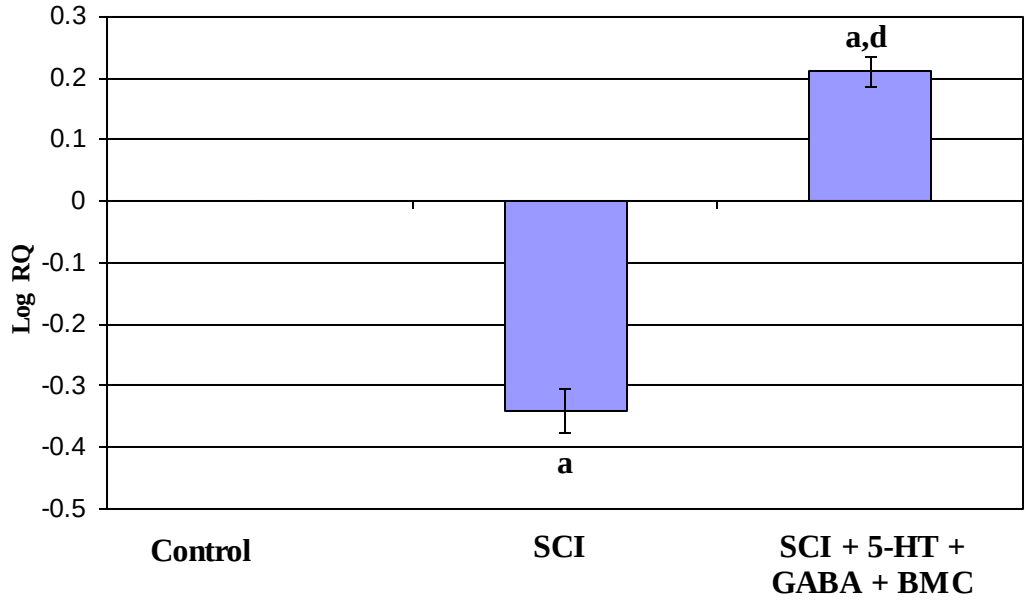


Table-107

Real Time PCR amplification of CREB mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	-0.34 ± 0.03^{a}
SCI + 5-HT + GABA + BMC	$0.21 \pm 0.02^{a,d}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
$^{a}p<0.001$ when compared to Control. $^{d}p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-110

Real Time PCR amplification of Bax mRNA in the corpus striatum of control and experimental rats

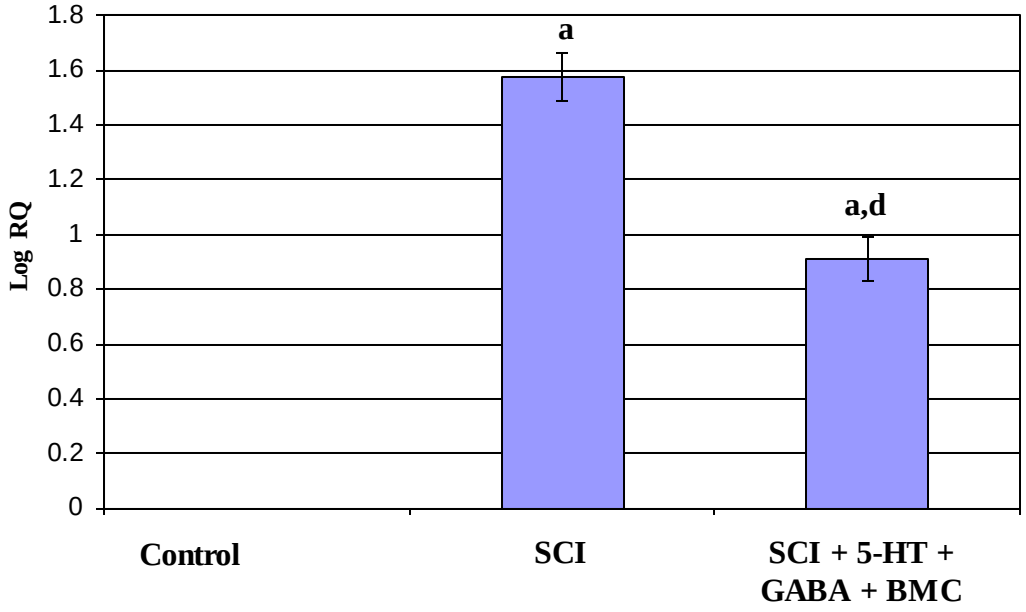


Table-108

Real Time PCR amplification of Bax mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	1.57 ± 0.09 [a]
SCI + 5-HT + GABA + BMC	0.91 ± 0.08 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-111

Real Time PCR amplification of Caspase-8 mRNA in the corpus striatum of control and experimental rats

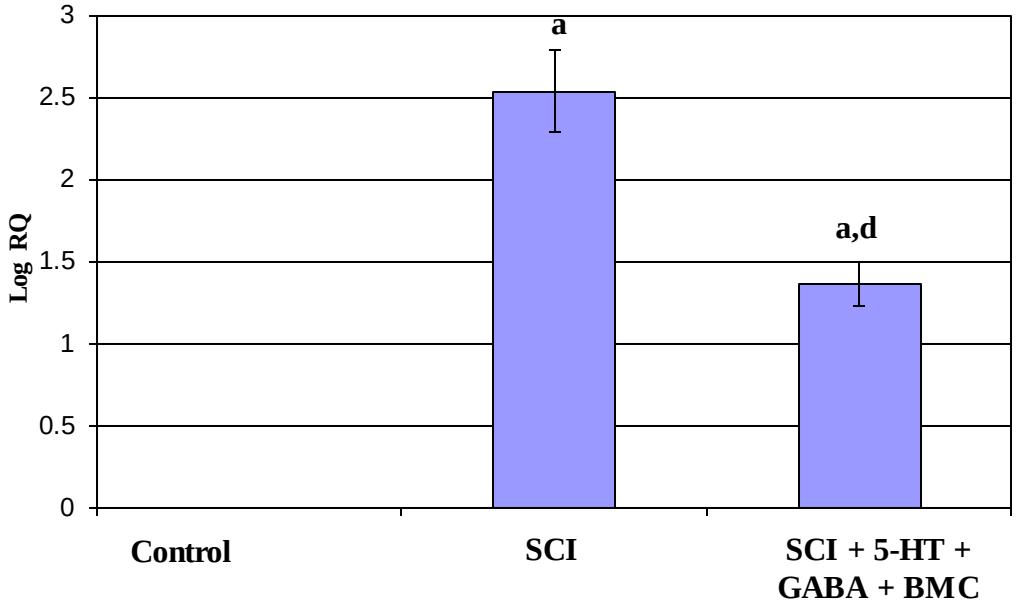


Table-109

Real Time PCR amplification of Caspase-8 mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	2.54 ± 0.23 [a]
SCI + 5-HT + GABA + BMC	1.36 ± 0.13 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-112

Real Time PCR amplification of Superoxide dismutase mRNA in the corpus striatum of control and experimental rats

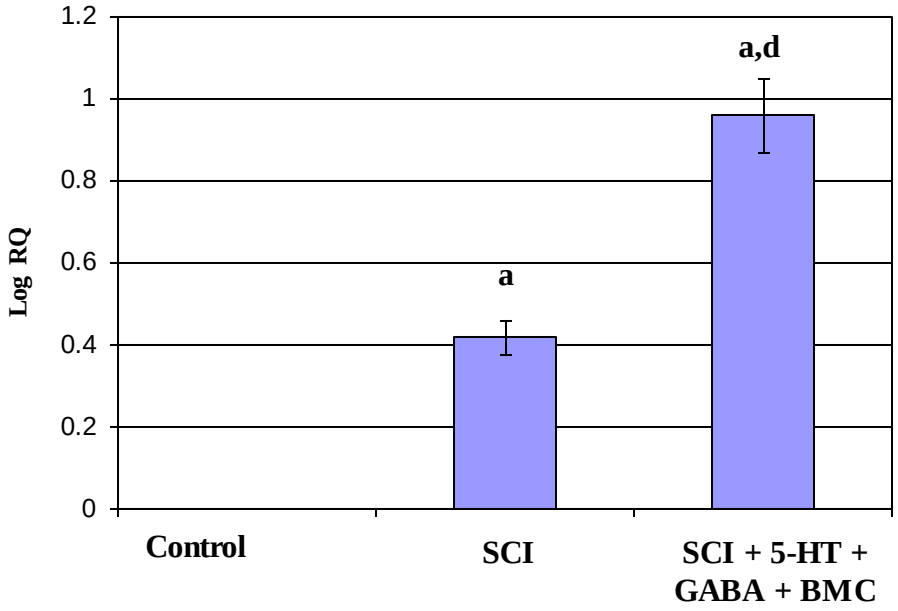


Table-110

Real Time PCR amplification of Superoxide dismutase mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	0.42 ± 0.04 [a]
SCI + 5-HT + GABA + BMC	0.96 ± 0.09 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-113

Real Time PCR amplification of Glutathione peroxidase mRNA in the corpus striatum of control and experimental rats

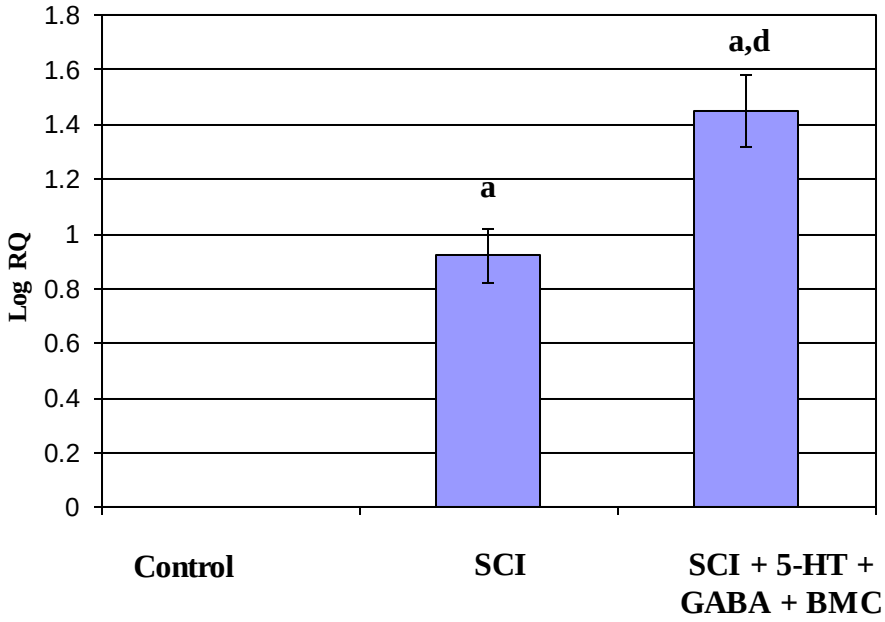


Table-111

Real Time PCR amplification of Glutathione peroxidase mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
Control	0
SCI	0.92 ± 0.09 [a]
SCI + 5-HT + GABA + BMC	1.45 ± 0.13 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-114
IP3 content in the corpus striatum of control and experimental rats

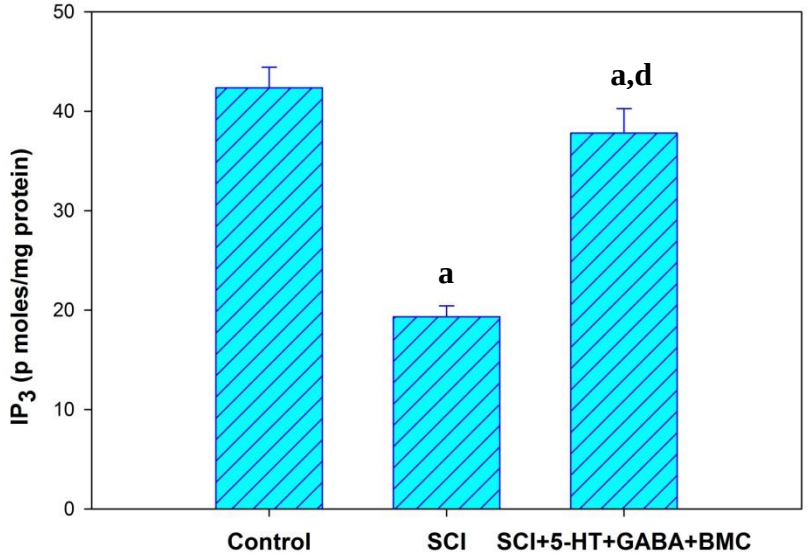


Table-112
IP3 content in the corpus striatum of control and experimental rats

Experimental groups	IP3 Content (p moles/mg protein)
Control	42.35 ± 2.06
SCI	19.34 ± 1.08 [a]
SCI + 5-HT + GABA + BMC	37.80 ± 2.45 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-115
cAMP content in the corpus striatum of control and experimental rats

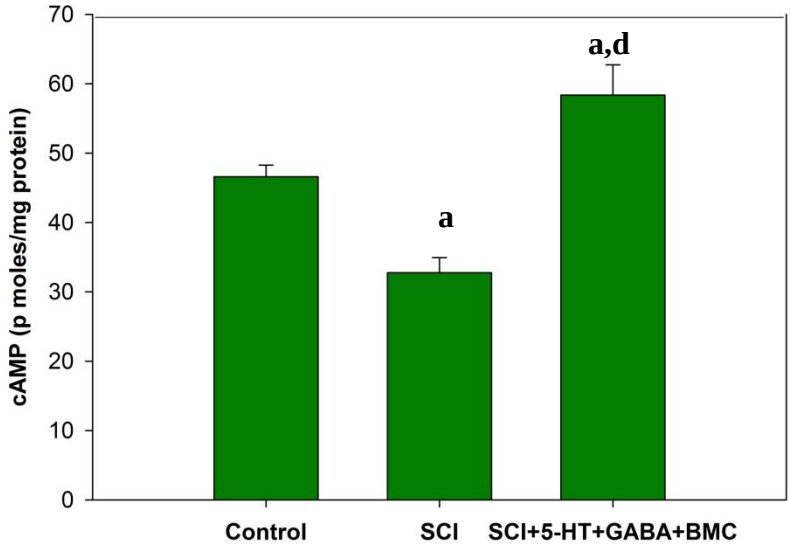


Table-113
cAMP content in the corpus striatum of control and experimental rats

Experimental groups	cAMP Content (p moles/mg protein)
Control	**46.60 ± 1.67**
SCI	**32.76 ± 2.18** [a]
SCI + 5-HT + GABA + BMC	**58.34 ± 4.37**[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-116
cGMP content in the corpus striatum of control and experimental rats

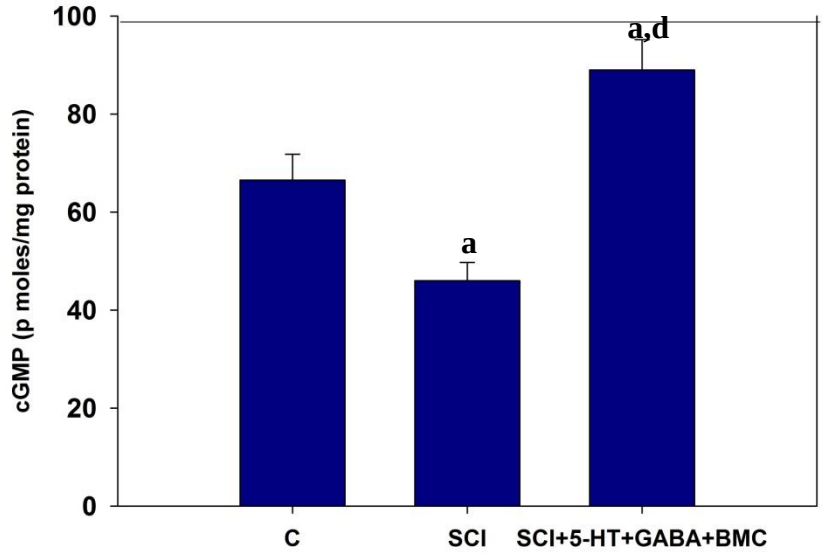
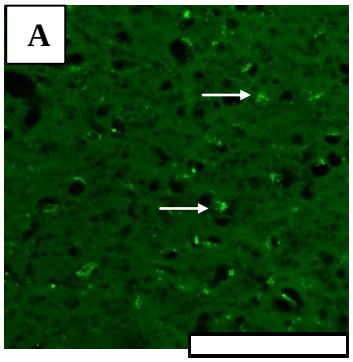


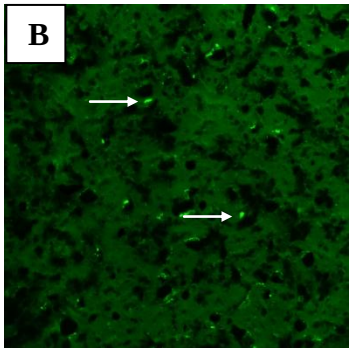
Table-114
cGMP content in the corpus striatum of control and experimental rats

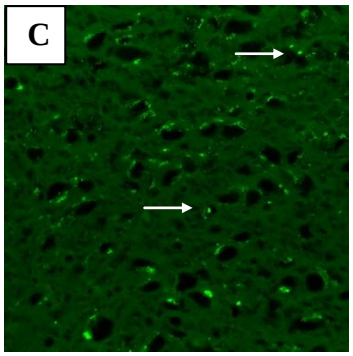
Experimental groups	cGMP Content (pmoles/mg protein)
Control	65.52 ±5.27
SCI	46.23 ± 3.69 [a]
SCI + 5-HT + GABA + BMC	89.12 ± 6.19 [a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a] $p<0.001$ when compared to Control. [d] $p<0.001$ when compared to SCI group.
C – Control, SCI – Spinal cord injury, SCI + 5-HT + GABA + BMC –Spinal cord injury treated with Serotonin, GABA and BMC.

Figure-117
Mucsarinic M1 receptor expression in the corpus striatum of control and experimental rats



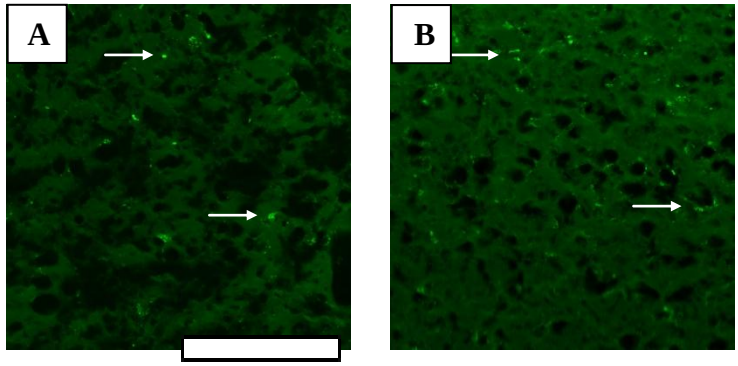


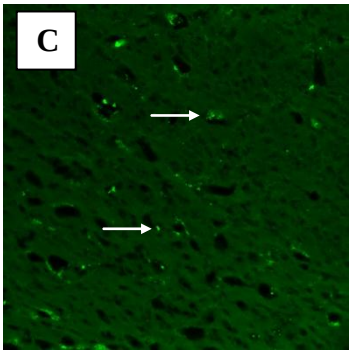


Experimental groups	Mean Pixel Intensity
CONTROL	39. 62 ± 2.56
SCI	28.44 ± 2.01[a]
SCI + 5-HT + GABA + BMC	51.45±4.31[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]p<0.001 when compared to Control. [d]p<0.001 when compared to SCI group.
A - Control, B – Spinal cord injured rats, C – Spinal cord injury treated with Serotonin.
Scale bars represent 150 μm. → in white shows muscarinic M1 receptor.

Figure-118
Mucsarinic M3 receptor expression in the corpus striatum of control and experimental rats

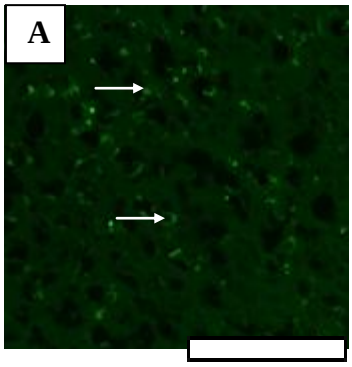


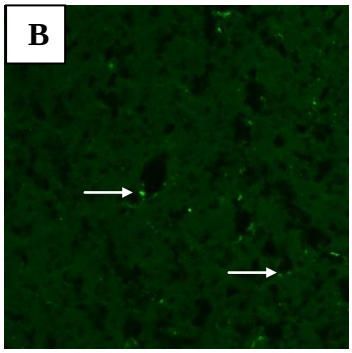


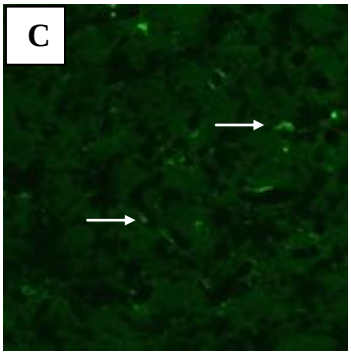
Experimental groups	Mean Pixel Intensity
CONTROL	21.12 ± 1.67
SCI	46.87 ± 4.21[a]
SCI + 5-HT + GABA + BMC	34.98 ± 2.68[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]p<0.001 when compared to Control. [d]p<0.001 when compared to SCI group.
A - Control, B – Spinal cord injured rats, C – Spinal cord injury treated with Serotonin.
Scale bars represent 150 μm. → in white shows muscarinic M3 receptor.

Figure-119
α7 nicotinic acetylcholine receptor expression in the corpus striatum of control and experimental rats







Experimental groups	Mean Pixel Intensity
CONTROL	44.90 ± 3.62
SCI	22.78 ± 1.58[a]
SCI + 5-HT + GABA + BMC	35.73 ± 2.92[a,d]

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 4-6 rats.
[a]$p<0.001$ when compared to Control. [d]$p<0.001$ when compared to SCI group.
A - Control, B – Spinal cord injured rats, C – Spinal cord injury treated with Serotonin.
Scale bars represent 150 µm. → in white shows α7 nicotinic acetylcholine receptor.

Discussion

Spinal cord injury is a distressing clinical problem that has irrevocable consequences, resulting in perpetual functional loss and life time disability (Sekhon & Fehlings, 2001). This devastating condition often affects young and healthy individuals at the prime of their life, creates enormous physical and emotional cost, and places a significant financial burden to society at large (Ackery *et al.,* 2004). Trauma to the spinal cord causes dysfunction of the cord, with loss of sensory and motor function distal to the point of injury. In case of SCI, the spinal cord is intact, but the cellular damage to it results in loss of functioning. In the mammalian CNS, the failure of spontaneous regeneration of injured axons leads to devastating consequences and poor functional recovery. SCI involves a primary mechanical injury followed by a secondary injury. Apoptosis and generation of free radicals occur as a consequence of secondary injury which worsens SCI. Severe injuries to CNS axons not only damage plasticity of synapses but also provoke complex degenerative cascades, leading to glial and neuronal apoptosis. Since spinal cord is the part of CNS, any damage to it affects the entire CNS including brain (Gomez *et al.,* 2012). ACh is one of the major neurotransmitters in the CNS. It binds to nAChRs and muscarinic ACh receptors. Both these receptors have roles in regulating locomotion (James *et al.,* 1981; Marc *et al.,* 1999). So the therapeutic interventions which modulate cholinergic receptors and reduce the secondary damage in CNS will open new windows for the treatment of SCI. Understanding of neuronal injury and neural regeneration also provide new promises for reversal of SCI that once was thought to be everlasting and irremediable (Carlson & Gorden, 2002).

BODY WEIGHT

In our experiment, SCI rats showed a significant decrease in body weight. After SCI, a loss in whole body weight accompanied by a large decrease of weight and volume in the forelimbs and the hindlimbs were observed (Landry *et al.,* 2004, Stefany *et al.,* 2007). After SCI, there is a rapid and dramatic loss of muscle mass below the level of the lesion (Wilmet *et al.,* 1995; Monroe *et al.,* 1998; Nuhlicek *et al.,* 1998). SCI animals show a diminished food intake and gastroparesis (Tong & Holmes, 2009; Qualls-Creekmore *et al.,* 2010). Cholinergic interneurons respond to motivationally relevant stimuli and are involved in appetitive learning. Muscarinic receptor antagonist reduced food intake (Pratt & Kelly, 2005; Pratt *et al.,* 2007). Mice deficient in the M3 muscarinic receptor display a significant decrease in food intake and reduced body weight (Yamada *et al.,* 2001). This indicates that a cholinergic pathway is involved in food intake. So the cholinergic alterations induced after SCI has affected food intake and has led to weight loss of SCI rats. SCI+5-HT, SCI+GABA, SCI+BMC rats did not show any significant change in the body weight of SCI rats. SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC treatment group showed improvement in body weight significantly which indicate recovery from SCI through the modulation of cholinergic receptors in this group of rats. More significant weight gain was seen in SCI+5-HT+GABA+BMC treatment group.

MOTOR DEFICITS IN SCI RATS

A number of assessments of motor, behavioural and electrophysiological function are available for adult rats (Kesslak & Keirstead, 2003; Basso, 2004; Nichols *et al.,* 2005; Webb & Muir, 2005). Importantly, most of these assessments evaluate the sensorimotor function rather than sensory or motor functions individually. Incomplete SCIs often lead to severe and persistent impairments of sensorimotor functions and are clinically the most frequent type of SCI.

Understanding the motor impairments and the possible functional recovery of upper and lower extremities is of great importance. Recovery from SCI is often monitored by manual evaluation of muscle function (Maynard *et al.,* 1997).

Motor skills and motor learning abilities are dependent on the integrity of the central neurotransmission (Thouvarecq *et al.,* 2001). Motor susceptibility is directly modulated by alterations in cholinergic system. We evaluated motor performance of control and experimental rats by rotarod, narrow beam walk and grid walk test Rotarod test has been used to examine the motor coordination (Cendelin *et al.,* 2008). The rotarod tests of motor performance provide quantitative, objective and reproducible measures of functional impairment of rats following SCI. The rotarod, beam walk and grid walk test experiments demonstrated the impairment of the motor function and coordination in SCI rats. SCI rats showed lower fall off time from the rotating rod when compared to control and increased number of foot slips grid walk test and decreased time spent in narrow beam test compared to control, suggesting impairment in their ability to integrate sensory input with appropriate motor commands to balance their posture. Moreover, SCI rats showed clear signs of deficiency in fine motor control as indicated by a reduced tendency to turn around and walk forward on the rotarod. At the same time, they were unable to adjust their limb movements on the metallic rod which is indicative of cerebellar dysfunction (Amee *et al.,* 2009; Anu *et al.,* 2010; Peeyush *et al.,* 2010; Sherin *et al.,* 2010). SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC showed an improved motor performance in rotarod, beam and grid walk test compared to SCI rats. Our findings indicate that 5-HT, GABA and BMC in combination reverses the motor abnormalities which assists in lowering their time for spatial recognition and thus helps to maintain their posture during movement on the rotating rod (rotarod).

CHOLINERGIC ENZYME ALTERATIONS IN SPINAL CORD AND BRAIN OF CONTROL AND EXPERIMENTAL RATS.

ACh is the primary neurotransmitter of the cholinergic system. It is a neurotransmitter that is involved in peripheral movements. ChAT, the enzyme responsible for the biosynthesis of ACh and AChE, the enzyme responsible for degradation of ACh are the most specific indicators for monitoring the functional state of cholinergic neurons in the central and peripheral nervous systems (Ellman *et al.,* 1961). The reduction of ChAT is correlated with the severity of dementia and pathologic changes (Rodrigo *et al.,* 2004). Changes in ChAT and AChE levels have been implicated in SCI.

Central cholinergic activity was studied in experimental rats after using ChAT and AChE as marker. Our results showed an increase in expression of AChE in spinal cord of SCI rats when compared to control. All treatment groups except SCI+BMC showed significant reversal when compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed prominent reversal when compared to all other treatment groups. The prominent reversal in this particular group is due to the co-mitogenic activity of 5-HT and GABA which can induce neuronal differentiation of BMC and restored neuronal connectivity and hence the cholinergic neurotransmission. Co-mitogenic activity of 5-HT and GABA has been reported earlier (Sudha & Paulose, 1998; Biju *et al.,* 2002).

ChAT expression was down regulated in SCI rats when compared to control. SCI+5-HT, SCI+GABA treated rats showed a significant reversal when compared to SCI rats. The combination treatment group of rats, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC treatment group significantly reversed and up regulated the gene expression of ChAT mRNA when compared to SCI rats. SCI+BMC did not show significant reversal when compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed prominent up regulation than

all the other group of rats. It has been demonstrated earlier that injury of the rabbit spinal cord causes alterations of the cholinergic enzyme activities. Decreased expression of ChAT has been observed due to the loss of neurons following SCI (Brambilla *et al.,* 1996). This is in concordance with our results. It has been reported earlier that from 2 to 12 weeks after spinal transection, a progressive loss of cholinergic motoneurons occurs (Kitzman,2006). Locomotor dysfunction occurs as a result of loss of cholinergic influence following spinal transection. This explains the locomotor deficit that we observed in SCI rats. Results suggest that cholinergic neurons, especially spinal motor neurons play an important role in the motor functional recovery following SCI. A strong correlation was found between the motor functional recovery and high levels of ChAT (Nakamura *et al.,* 1996). Thus administration of 5-HT and GABA along with BMC increased ChAT activity leading to improvement in motor co-ordination.

Our results also showed an increase in expression of AChE in cerebral cortex, cerebellum, brain stem and corpus striatum of SCI rats. Neurobehavioural deficit can be caused by increase in cerebellum and cortex AChE activity (Mohammed *et al.,* 2001, Peeyush *et al.,* 2011). This is evident in the behavioural tests of SCI rats. These altered expression of AChE was significantly reversed in SCI+5-HT+GABA+BMC treatment group. The decrease in AChE in SCI+5-HT+GABA+BMC treatment group increases ACh and hence locomotion. ChAT activity was up regulated in cerebral cortex, brain stem and corpus striatum and was significantly down regulated in cerebellum of SCI rats. ChAT activity was further up regulated in cerebral cortex, brain stem and corpus striatum of SCI+5-HT+GABA+BMC treatment group. It showed significant reversal in cerebellum of SCI+5-HT+GABA+BMC treatment group. The release of ACh from corpus striatum and cortex correlated with

the level of locomotor activity (Day *et al.,* 1991). Thus increase in ChAT, which synthesizes ACh attributes to the improvement in motor co-ordination in SCI+5-HT+GABA+BMC treatment group.

CENTRAL MUSCARINIC RECEPTOR ALTERATIONS

The role of cholinergic system in SCI has received much less attention. ACh has a role in the initiation and control of locomotion in vertebrates. In the *Xenopus* frog embryo, a portion of the drive to motoneurons during swimming is derived from a cholinergic input (Perrins & Roberts, 1995 a,b,c). Muscarinic receptors are a family of G protein-coupled receptors that have a primary role in central cholinergic neurotransmission. Specific agonists, which activate postsynaptic muscarinic receptors, stimulate cholinergic signaling (Valentin *et al.*, 2006). The muscarinic M1 receptor is one of five known muscarinic subtypes in the cholinergic nervous system (Bonner *et al.*, 1987; Hulme *et al.*, 1990; van Zwieten & Doods, 1995). The muscarinic M1, M2 and M4 subtypes of mAChRs are the predominant receptors in the CNS. These receptors activate a multitude of signaling pathways important for modulating neuronal excitability, synaptic plasticity and feedback regulation of ACh release (Volpicelli *et al.,* 2004).

SPINAL CORD

The spinal cord is a long, thin, tubular bundle of nervous tissue and support cells that extends from the brain (the medulla oblongata specifically). The brain and spinal cord together make up the CNS. The spinal cord functions primarily in the transmission of neural signals between the brain and the rest of the body but also contains neural circuits that can independently control numerous reflexes and CPG. Because the spinal cord acts as the main information pathway between the brain and the rest of the body, SCI can have significant physiological consequences (Gomez *et al.*, 2012). SCI involve

damage to the neurons of the spinal cord. Motor function is severely disrupted following SCI.

The spinal cord of vertebrates contains several types of cholinergic neurons, including motoneurons, preganglionic autonomic neurons, partition cells (lamina VII), central canal cluster cells (lamina X), and small dorsal horn cells scattered in lamina III–V (Houser *et al.* 1983; Barber *et al.* 1984; Phelps *et al.* 1984; Borges & Iversen 1986; Sherriff and Henderson 1994). There is consensus that these cholinergic cells form an extensive propriospinal system of interconnected neurons (Sherriff & Henderson 1994) and may therefore play a role in activities requiring coordination among several spinal segments. Spinal motoneurons receive prominent cholinergic terminals (Nagy *et al.,* 1993; Li *et al.,* 1995; Arvidsson *et al.,* 1997; Welton *et al.,* 1999) and so it seems likely that some of the intrinsic spinal cholinergic cells are involved in the control of movement. Furthermore, the large cholinergic terminals, termed C terminals (Nagy *et al.,* 1993; Li *et al.,* 1995) have been implicated in spinal cord plasticity (Pullen & Sears 1983; Feng-Chen & Wolpaw 1996).

Intrinsic cholinergic innervation has been demonstrated in the spinal cord (Ribeiro-da-Silva & Cuello, 1990; Wetts & Vaughn, 1994). The spinal cholinergic system is important for locomotion. Since ACh serve as excitatory neurotransmitter within the spinal cord, it could contribute to the functional neurologic impairment that follows injury (Faden *et al.,* 1986). ACh acting through muscarinic and nicotinic receptors is involved in the function of spinal locomotor networks in several vertebrate preparations (Smith *et al.,* 1988; Panchin *et al.,* 1991; Kiehn *et al.,* 1996; Fok & Stein, 2002; Quinlan *et al.,* 2004).

Binding studies in our experiment using [^{3}H] QNB and muscarinic general antagonist, atropine revealed that total muscarinic receptors were decreased in the spinal cord of SCI rats. Muscarinic M1 receptor changes during SCI were studied using subtype specific antagonist, pirenzepine and [^{3}H] QNB and muscarinic M3 receptor changes were studied using subtype specific antagonist, [^{3}H] 4- DAMP

mustard and [^{3}H] DAMP. Muscarinic M1 receptors were decreased in SCI rats, with a decrease in K_d indicating an increase in the affinity of receptors during SCI condition Real Time-PCR analysis also revealed a down regulation of the muscarinic M1 and M2 receptors mRNA level in SCI rats. Also, in SCI rats, the mRNA level and binding parameters of muscarinic M3 receptors showed a decrease in the spinal cord when compared to control. K_d did not show any significant change when compared to control. Immunohistochemistry study using confocal microscope confirmed a similar expression pattern in localization of muscarinic M1 amd M3 receptor in the spinal cord of control and experimental rats. This is concordant with our receptor binding and gene expression studies. Traumatic SCIs in rats are associated with decrease in muscarinic receptor binding. Reductions in muscarinic receptors were highly correlated with the degree of motor neuron loss found (Peter *et al.,* 1983). There has been evidence that muscarinic receptor binding did not recover in three weeks following trauma to spinal cord (Faden *et al.,* 1986) which is consistent with our result.

Significant reversal in total muscarinic receptor binding, muscarinic M1 and M3 binding parameters were seen in all treatment groups when compared to SCI rats. K_d of total muscarinic, muscarinic M1 receptor binding also significantly reversed in all treatment groups except in SCI+BMC rats when compared to SCI rats. K_d of muscarinic M3 receptor binding showed significant change in SCI+BMC group but not in the other treatment groups when compared to SCI rats. Real Time-PCR analysis of the muscarinic M1 receptor showed significant reversal in all treatment groups. Muscarinic M2 and M3 receptor mRNA level revealed a reversal in all treatment groups except SCI+BMC rats when compared to SCI rats. This is concordant with our receptor binding studies. Immunohistochemistry study using confocal microscope confirmed a similar expression pattern in localization of muscarinic M1 amd M3 receptor in the spinal cord of control and experimental rats. Prominent reversal in B_{max} as well as gene expression were seen in SCI+5-HT+GABA treated rats which show the

effectiveness of combination treatment in restoring muscarinic receptors in this group of rats.

5-HT and GABA are involved in a variety of cellular processes which includes neurogenesis and cell proliferation (Lauder & Krebs 1978; Kligman & Marshak, 1985; Ben & Golan, 2003). 5-HT can act as a potent hepatocyte comitogen and induce DNA synthesis in primary cultures of rat hepatocytes, which is suggested to be mediated through the 5-HT_2 receptors of hepatocytes (Sudha & Paulose ,1998) with which it can also trigger the cell division in the dopaminergic neurons in substantia nigra (Nandhu, 2011). Also, baclofen (a GABA agonist) is seen to act as a potent co-mitogen, triggering DNA synthesis in primary cultures of rat hepatocytes, mediated through the G(i) protein-coupled GABA(B) receptors (Biju *et al.,* 2002). Cell transplantation technology has demonstrated that BMCs differentiate into mature neurons or glial cells under specific experimental conditions and that the transplantation of BMCs can promote functional improvements after SCI (Jung *et al.,* 2011). Previous studies from our laboratory on Parkinson's disease and bone fracture healing established that administration of bone marrow cells alone without 5-HT and GABA for re-establishing neuronal connections were not satisfactory due to the slow differentiation of BMC. This was overcome by supplementing BMC along with neurotransmitters: 5-HT and GABA (Nandhu *et al.,* 2011), both having co mitogenic ability. So 5-HT and GABA administered along with BMC in SCI group of rats promote neurogenesis resulting in ameliorating the effect of SCI in the combination treatment group of rats. The excitability of spinal motoneurons is regulated by ACh *via* the activation of muscarinic receptors. Muscarinic-mediated effects of ACh, are reported at all neural levels involved in locomotion: in supraspinal structures (Garcia-Rill & Skinner, 1987; Le Ray *et al.,* 2004; Smetana *et al.,* 2007, Smetana *et al.,* 2010) and at the spinal cord level (Smith *et al.,* 1988; Kurihara *et al.,* 1993; Cowley & Schmidt, 1994; Alaburda *et al.,* 2002; Miles *et al.,* 2007; Anglister *et al.,* 2008). Since ACh is important in locomotion, the restoration of muscarinic receptors and

hence ACh through the administration of 5-HT and GABA in combination with BMC have led to the improvement in motor co-ordination as seen in the behavioural tests.

CEREBRAL CORTEX

In addition to intrinsic cholinergic neurons, the cerebral cortex and hippocampus receive extensive innervation from cholinergic neurons in the basal forebrain, beginning prenatally and continuing throughout the period of active growth and synaptogenesis. Cholinergic neurons project in a widespread ascending system from the medial septal nuclei to the hippocampus and from the nucleus basalis of Meynert to the cerebral cortex. The corticospinal tract serves as the motor pathway for upper motor neuronal signals coming from the cerebral cortex and from primitive brain stem motor nuclei. The RT-PCR and HPLC studies revealed that the M1 receptor was present in a relatively high density in the cerebral cortex (Jian *et al.,* 1993; Oki *et al.,* 2005). The muscarinic M1, M3 and M5 receptors are located predominantly on postsynaptic nerve terminals. Immunoprecipitation and immunofluorescence studies indicate that muscarinic M1 and M3 receptors are expressed in cortex (Levey, 1993).

Many areas of the cerebral cortex process sensory information or coordinate motor output necessary for control of movement. Flawless integration of limb movements and accompanying posture is a crucial factor for the execution of desired locomotor movements. Multiple cortical motor related areas are activated during monkey bipedal walking, similar to that observed in humans (Mori & Nakajima, 2010). Cortical inactivation studies revealed that each cortical region has an assigned functional role for the elaboration and refinements of its locomotor task (Mori & Nakajima, 2010). Thus, all kinds of sensory inputs which are determined by the external constraints of the locomotion can act *via* local as well as long neural loops including the sensory and motor cortex (Angel,

1977). Thus, a modulatory role of the cerebral cortex in normal locomotion has been suggested

Binding studies using [^{3}H] QNB and muscarinic general antagonist, atropine revealed that total muscarinic receptors are decreased in the cerebral cortex during SCI condition. Muscarinic M1 receptors were decreased in SCI rats, with a decrease in K_d indicating an increase in the affinity of receptors in SCI rats. Real Time-PCR analysis also revealed a down regulation of the muscarinic M1 and M2 receptors mRNA level in SCI rats. This is concordant with our receptor binding studies. Immunohistochemistry study using confocal microscope confirmed a similar expression pattern in localization of muscarinic M1 receptor in the cerebral cortex of control and experimental rats. Also, in SCI rats, the mRNA level and binding parameter, B_{max} and K_d of muscarinic M3 receptors showed an increase in the cerebral cortex when compared to control. Immunohistochemistry study using confocal microscope confirmed a similar expression pattern in localization of muscarinic M3 receptor in the cerebral cortex of control and experimental rats. Previous studies reported the role of muscarinic M1 receptors in regulating locomotion (Tsuyoshi *et al.,* 2001; Nathaniel *et al.,* 2008). Thus the disturbance in the cholinergic system have led to the locomotor dysfunction in SCI rats.

A significant reversal was seen in the binding studies, real time PCR analysis and immunohistochemical analysis of SCI+5-HT+GABA+BMC treatment group when compared to SCI rats. Functional brain imaging studies have shown that multiple cerebral sensorimotor cortices and the cerebellum are highly activated during human bipedal locomotion, suggesting that humans depend on the cerebrum and cerebellum for the elaboration of bipedal locomotion. Central muscarinic receptors are known to play key roles in memory and learning as well as in the regulation of many sensory, motor and autonomic processes (Levine & Birdsall, 1995; Brown & Taylor 1996; Levine & Birdsall, 1997). Thus, we speculated that 5-HT and GABA along with BMC have ability to

modulate muscarinic receptors in cerebral cortex, thereby ameliorating the impaired locomotor performance shown by SCI rats.

CEREBELLUM

Cerebellum is a region of the brain that plays an important role in coordination and motor control. It integrates information from vestibular system that indicates position and movement and uses this information to coordinate limb movements. In order to coordinate motor control, there are many neural pathways linking the cerebellum with the cerebral motor cortex and the spinocerebellar tract (Roberta & Peter, 2003).

As previously demonstrated by Barmack *et al.*, (1992 a,b), the predominant fiber system in the cerebellum that use ACh as a transmitter or a co-transmitter is formed by mossy fibers originating in the vestibular nuclei and innervating the nodulus and ventral uvula. In the cerebellar nuclei the beaded cholinergic fibers form a moderately dense network and could in principle have a significant effect on neuronal activity. For instance, the cholinergic neurons modulate the excitability of the cerebellonuclear neurons in relation to sleep and arousal (McCormick, 1989). The cerebellar cortex contains cholinergic fibers and both muscarinic receptors and nAChR (Andre *et al.*, 1995). The cholinergic system is essential for brain development, acting as a modulator of neuronal proliferation, migration and differentiation processes; its muscarinic receptors play pivotal roles in regulating important basic physiologic functions.

Sensory information ascends to the cerebellum along the spinal cord. The two main tracts that bring information from the periphery to the cerebellum are the ventro-spino-cerebellar tract and the dorso-spino-cerebellar tract**.** Cerebellum receives proprioceptive input from the spinal cord and controls the anti-gravity muscles of the body, thus regulating posture. When the cortex sends a message for motor movement to the lower motor neurons in the brain stem and spinal cord it also sends a copy of this message to the cerebellum. The cerebellum is attached to

the spinal cord by three peduncles that carry neural information. Cerebellum can influence spinal cord motor neurons through the corticospinal tract and also through the rubrospinal pathway and vice-versa.

The decrease in muscular activity represents a direct influence of SCI upon the neurons and neurotransmitters within the CNS as well as cerebellum. Gene expression studies showed that the mRNA level of muscarinic M1 and M2 in the cerebellum of SCI rats substantially decreased when compared to control. Binding parameters B_{max} and K_d of total muscarinic and muscarinic M1 receptors were decreased whereas B_{max} and K_d of muscarinic M3 were increased in SCI rats compared to control. These results suggest a cholinergic disturbance in cerebellum of SCI rats. Earlier studies reported that in resting conditions, transgenic animals expressing the muscarinic M3 receptor showed severe dysfunction of coordinated locomotion. We observed an increase in the gene expression of muscarinic M3 receptor in cerebellum of SCI rats which ascertains locomotor dysfunction as is evident from the behavioural test. This locomotor dysfunction is mediated through the disturbance in the cholinergic system in SCI rats. Cerebellar dysfuncton is associated with poor fine motor skills, hypotonia (Wassmer *et al.,* 2003). Earlier reports also suggest that lesion in cervical spinal cord causes corticospinal deficits. The cerebellum receives efferent fibres of CPG output to motoneurons *via* ventral spinocerebellar and spinoreticulocerebellar pathways, as well as information about the activity of the peripheral motor apparatus *via* the dorsal spinocerebellar tract (Orlovsky, 1991). So any damage to the spinocerebellar tract can cause motor dysfunction which is mediated through the disturbance in the cholinergic system which is evident in our results of SCI rats.

The current study also revealed the modulatory function of 5-HT, GABA along with BMC on total muscarinic, muscarinic M1 and M3 receptors by normalising the altered receptor gene expression and binding parameters to near control. Immunohistochemical analysis confirmed the result of mRNA expression and binding parameters. Thus 5-HT, GABA along with BMC administration in

SCI rats was able to restore cholinergic system. The spinocerebellar tract conveys the proprioception from extremities. It hence influences the coordination and the balance. The cerebellum, influences motoneurons indirectly *via* vestibulospinal, rubrospinal, reticulospinal and corticospinal pathways (Orlovsky, 1991). So the restoration of motor neurons which are cholinergic in nature lead to reinstatement of motor activity through spinocerebellar tract. This role is consistent with the sequelae to removal of the cerebellum—coarse, stereotyped movements with poor interlimb coordination and inaccurate foot placement as well as equilibrium deficits (Arshavsky *et al.,* 1983).

In the present study, we observed that the co-mitogenic effect of 5-HT, GABA along with BMC in injured spinal cord of rats can influence the re-establishment of neuronal connectivity between spinal cord and cerebellum or rather spinocerebellar tract which is otherwise damaged in SCI rats. The re-establishmant of spinocerebellar tract which modulates muscarinic receptors and muscarinic M1, M2 and M3 receptor subtypes gene expression in cerebellum is responsible for the coordination of voluntary motor movement, balance and equilibrium of posture in SCI+5-HT+GABA+BMC treated rats.

BRAIN STEM

The Brain Stem is a part of the brain located beneath the cerebrum and in front of the cerebellum. It connects the spinal cord to the rest of the brain. Brain stem along with hypothalamus serves as the key centre of the CNS regulating the body homeostasis. The neurogenesis for locomotor control involves the interaction of brain stem networks for generation of motor commands, spinal cord motor pattern-generating networks and sensorimotor circuits (Grillner, 1981; Feldman & Grillner, 1983; Grillner, 1985; Grillner & Walln, 1985). Locomotor functions of the brain stem are mainly mediated by ACh *via* cholinergic receptors. Muscarinic receptors are present in the brain stem of many vertebrate species and modulate neuronal and synaptic properties (McCormick 1992; Bal *et al.,* 1994; Klink &

Alonso 1997a,b; Segal & Auerbach 1997). Both metabotropic muscarinic cholinergic receptors and ionotropic nicotinic cholinergic receptors play crucial in motor control and coordination (James *et al.,* 1981; Marc *et al.,* 1999). Activity of the cholinergic muscarinic system is coupled with modulation of locomotor activity.

The binding parameters B_{max} and K_d of total muscarinic, muscarinic M1 and M3 receptors of the brain stem are found to be decreased in SCI condition. Gene expression of muscarinic M1, M2 and M3 receptors are decreased during SCI. This is in accordance with our receptor binding studies. Also, results of confocal studies using specific antibodies of muscarinic M1 and M3 in brain stem confirmed the results of real time PCR and scatchard analysis. The brain stem provides the main motor and sensory innervation *via* the cranial nerves. Muscarinic alterations in brain stem during SCI result in locomotor deficits. Muscarinic receptors are reported to activate locomotor mesencephalic neurons in brain stem which are involved in locomotor behaviour. The reduction in muscarinic receptor function in SCI can augment the locomotor disability and act as one of the molecular mechanism which contributes to the severity of monoplegic condition.

Cholinergic inputs are also believed to activate brain stem neurons in mammals (Garcia-Rill & Skinner, 1987; Homma *et al.,* 2002). Our results showed that 5-HT, GABA along with BMC in SCI rats restored the altered muscarinic functions associated with brain stem. Experimental evidence indicates that brain stem neurons which are connected with the spinal cord generate motor output that enables walking and other locomotion. The brainstem locomotor system is believed to be organized serially from the mesencephalic locomotor region to reticulospinal neurons, which in turn, project to locomotor neurons in the spinal cord. These newly discovered rhombencephalic muscarinoceptive neurons provide a powerful augmentation of locomotor drive. The boost requires a sustained excitation driven by muscarine (Roy *et al.,* 2010) which binds to muscarinic

receptors. So the restoration of muscarinic receptors is necessary for locomotor function. 5-HT, GABA along with BMC in SCI rats was capable of restoring the muscarinic receptor alteration which point towards the effectiveness of this treatment in restoring locomotor functions in SCI condition.

CORPUS STRIATUM

The corpus striatum is the largest component of the basal ganglia. It regulates movement with the assistance of dense bundles of motor neurons and associated neurotransmitters. Striatal dysfunction is associated with impairment of motor activities. Corpus striatum interacts with areas of cerebral cortex that give rise to descending motor pathways through a number of feedback loops. It connects to the extrapyramidal pathways and influences the output to the cord. Corpus striatum controls muscular movements by influencing the cerebral cortex. It doesn't have direct control through descending pathways to the brain stem and spinal cord. It helps to prepare for the movements that enable the trunk and limbs to be placed in appropriate positions before discrete movements of the hands and feet. Thus it is indirectly linked to spinal cord.

Striatal spiny neurones serve as a major anatomical locus for the relay of cortical information flow through the basal ganglia. These neurons also represent the main synaptic target of cholinergic interneurons. Cholinergic terminals within the striatum contain presynaptic muscarinic receptors that inhibit neurotransmitter release (Chesselet, 1984). Various anatomical, electrophysiological and pathological observations provide evidence that ACh plays a major role in the control of striatal function and in the regulation of motor control (Jabbari *et al.,* 1989). Striatal ACh is released from a population of large cholinergic interneurons that establish complex synaptic contacts with dopamine terminals, originating from the substantia nigra and with several striatal neuronal populations (Lehmann & Langer, 1982, 1983; Wainer *et al.,* 1984; Phelps *et al.,* 1985; Izzo & Bolam, 1988; Vuillet *et al.,* 1992). CNS muscarinic receptors s regulate a large number of

important central functions including cognitive, behavioural, sensory, motor and autonomic processes (Wess, 1996; Felder *et al.,* 2000; Eglen, 2005). Densities of muscarinic M1 receptor subtype were highest in the corpus striatum (Oki *et al.,* 2005). In the striatum, M2 receptor protein is associated mainly with cholinergic interneurons (Hersch *et al.,* 1994).

Scatchard analysis of total muscarinic and muscarinic M1 receptors revealed a decreased B_{max} in corpus striatum of SCI rats. Muscarinic M3 receptors showed an increased B_{max} in SCI rats. K_d increased significantly in total muscarinic and muscarinic M1 receptor binding studies and it decreased significantly in muscarinc M3 receptor binding studies. mRNA level revealed a down regulation of the muscarinic M1, M2 and up regulation of muscarinic M3 receptor during SCI condition. The results of confocal studies confirmed the alterations of muscarinic M1 and M3 receptor at protein level. The striatal cholinergic system has been implicated in the pathophysiology of movement disorders such as Parkinson's disease (Quik & Wonnacott, 2011; Kawamata *et al.,* 2012).

ACh mediated activation of striatal muscarinic ACh receptors is known to facilitate striatal dopamine release, as has been shown in both *in vitro* (Lehmann & Langer, 1982; Raiteri *et al.,* 1984; Schoffelmeer *et al.,* 1986; Kemel *et al.,* 1989) and *in vivo* (Xu *et al.,* 1989; De Klippel *et al.,* 1993; Smolders *et al.,* 1997) studies. A proper balance between striatal muscarinic cholinergic and dopaminergic neurotransmission is required for coordinated locomotor control (Calabresi *et al.,* 2000; Kaneko *et al.,* 2000; Quik & Wonnacott, 2011; Kawamata *et al.,* 2012). The altered expression of muscarinic receptor binding studies, gene expression was reversed in SCI+5-HT+GABA+BMC administered rats when compared to SCI rats. This would have resulted in improvement in motor control in this group of rats when comapred to SCI rats which was evident in the behavioural experiments that were done. Evidence suggests that ACh mediated mechanisms is of crucial importance in processing the cortical inputs to

the striatum (Paolo *et al.,* 2000). Thus the treatment with 5-HT, GABA and BMC increased the muscarinc receptor in the corpus striatum, aiding in cortical input, indirectly influencing the motor control. The present study suggests that activation of muscarinic receptors are of significant therapeutic benefit in SCI. Thus our results revealed the significance of central muscarinic receptor changes during SCI and the regulatory role of 5-HT, GABA and BMC on muscarinic receptors in corpus striatum.

*α*7 NICOTINIC RECEPTOR GENE EXPRESSION IN SPINAL CORD AND BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

Nicotinic receptors form a heterogeneous family of ion channels that are differently expressed in many regions of the CNS and peripheral nervous system. It binds to ACh and contribute to a wide range of brain activities and influence a number of physiological functions. Neuronal nAChRs are involved in neuronal survival and neuroprotection as well as in synaptic plasticity (Hong *et al.,* 2010; Quik & Wonnacott, 2011; Hernandez & Dineley, 2011; Kawamata *et al.,* 2012). Moreover, presynaptic nAChRs can modulate the release of many neurotransmitters, including dopamine, noradrenaline, 5-HT, ACh, GABA and glutamate. These neurotransmitter systems play an important role in cognitive and non-cognitive functions such as learning, memory, attention, locomotion, motivation, reward, reinforcement and anxiety. Thus, nAChRs are hopeful therapeutic targets for new treatments of these neurodegenerative disorders (Quik & Wonnacott, 2011; Hernandez & Dineley, 2011; Kawamata *et al.,* 2012).

One of the predominant nAChRs expressed in the mammalian CNS is homomeric α7 receptors. α7nAChRs have been identified on rat spinal motor neurons by immunohistochemical methods. It has an important role in motor control (Villégier *et al.,* 2010). α7 nAChRs are functionally different from alpha-beta receptors; for example, they have a greater calcium permeability and is gated either by ACh or by choline. However, the involvement of the α7 nAChR in SCI

has not been addressed. The major aim of the study was to further explore whether SCI is related to α7 nAChR modulation in spinal cord and brain regions and also to learn the role of 5-HT, GABA and BMC treatment in modulating α7 nAChR.

The gene expression of α7 nAChR was down regulated in SCI rats. SCI+5-HT and SCI+GABA treated rats showed significant reversal when compared to SCI rats. It was significantly reversed and up regulated in SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC treatment group. SCI+BMC rats showed no significant reversal in gene expression compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed prominent up regulation than all the other group of rats. The α7 nAChR is involved in the regulation of neuronal growth, differentiation and synapse formation (Hong *et al.*, 2010). So a decrease in these receptors affects the neuronal growth and survival in SCI rats. nAChRs are involved in the regulation of motoneuron survival (Ferreira *et al.*, 2001). This neuronal survival is mediated through calcium influx (Messi *et al.*, 1997). The marked up regulation of α7 nAChR in SCI+5-HT+GABA+BMC is indicative of neuronal survival in this group of rats.

In the brain, nicotinic receptors include several subtypes with differing properties and functions. Nicotinic receptors present in somatodendritic and terminal regions regulate cell excitability, dopamine release and neuronal integration influencing striatal outcome and eliciting different locomotor effects (Exley & Cragg, 2008; Livingstone & Wonnacott, 2009). Our results showed no significant change in the expression of α7 nAChR in cerebral cortex of SCI rats when compared to control. Our results showed a decreased expression of α7 nAChR in cerebellum, brain stem and corpus striatum of SCI rats compared to control. Confocal studies using specific antibody for α7 nAChR confirmed the mRNA expression in cerebral cortex, cerebellum, brain stem and corpus striatum. Gene expression of α7 nAChR was increased significantly in cerebral cortex of SCI+5-HT+GABA+BMC treatment group. In cerebellum, brain stem and corpus striatum it was significantly reversed in SCI+5-HT+GABA+BMC treatment

group. Confocal studies for α7 nAChR confirmed the mRNA expression in the brain regions. Neuronal nicotinic ACh receptors in the CNS are thought to play an important role during development of the fetal brain in differentiation, guidance of axons and synapse formation (Candy *et al.,* 1985; Lipton & Kater 1989; Kinney *et al.,* 1993; Coronas *et al.,* 1998; Utsugisawa *et al.,* 1999). An increase in locomotor activity occurs consistently after repeated nicotinic agonist administration followed by a subsequent challenge, a phenomenon known as behavioural sensitization (Menzaghi *et al.,* 1997; Pawlak *et al.,* 2005). Nicotinic ACh receptors influence striatal dopaminergic activity and its outcome on motor behavior (Wonnacott, 1997; Exley & Cragg, 2008; Livingstone & Wonnacott, 2009). nAChR also has a neuroprotective effect and protects the cell from neurotoxicity. It influences cell survival *via* activation of Akt (Shun *et al.,* 2009). In line with this, α7 nAChR functional difference in SCI is suggested to be one of the major factor causing behavioural deficit and neuronal degeneration. 5-HT and GABA along with BMC have modulated the α7 nAChR leading to improvement in motor function.

PHOSPHOLIPASE C EXPRESSION IN SPINAL CORD AND BRAIN

A large number of extracellular signals stimulate hydrolysis of PIP2 by PLC For the regulation of cellular processes, the best known consequence of this hydrolysis is the generation of two second messengers, IP3 and DAG involved in calcium release from intracellular stores and stimulation of protein kinase C isozymes (Berridge, 1993; Nakamura & Nishizuka, 1994). In addition to the second messenger production, regulation of PIP2 concentrations itself could be relevant for cell signalling since many proteins bind and/or require PIP2 to function. (Divecha & Irvine, 1995; Lee & Rhee, 1995). Activation of Gq coupled M1, M3 and M5 mAChR receptor subtypes stimulates mitogen activated protein kinase C by PLC dependent and PLC independent mechanisms (Wotta *et al.,* 1998).

In the present study, we observed SCI alterations in PLC expression in the spinal cord and brain regions- cerebral cortex, cerebellum, brain stem and corpus striatum. Further we extended the studies to PLC regulation with 5-HT and GABA along with BMC treatment for potential therapeutic drugs which modulate signal transduction pathway thereby contributing to the prevention of CNS dysfunction in SCI. Real Time-PCR analysis showed that the PLC gene expression was down regulated in spinal cord of SCI rats when compared to control. It was reversed and up regulated in all treatment groups when compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed prominent up regulation than all the other groups of rats. Thus it showed differential expression in spinal cord of SCI and treatment rats. Of the five subtypes of muscarinic receptors characterized by molecular cloning, the Ml, M3, and M5 receptors are coupled to membrane-associated phospholipases, such as phospholipases A2, C, and D, which generate the second messengers arachidonic acid, inositol phosphates, diacylglycerides and phosphatidic acid. Experiments with muscarinic M1 and M3 receptors suggested that the PLC serves as the primary effector for the muscarinic M1and M3 receptor (Caulfield, 1993 & Felder, 1995). We considered that the down regulation of the PLC in rat spinal cord during SCI contributes to the impaired signal transduction of G-protein coupled neurotransmitter receptors. Neurons of the spinal cord that control locomotion require neurotransmitters for their activity (Arshavsky, 2003). ACh is an important neuromodulator of CPG Activity. An agonist of ACh (Selverston & Moulins, 1985) can trigger the locomotor CPG in spinal cord (Grillner *et al.,* 1998; Hochman *et al.,* 1994; Kiehn *et al.,* 1996). Muscarinic receptors activate PLC in the brain (del Río *et al.,* 1994; Masgrau *et al.,* 2000; Se-Young Choi *et al.,* 2005). The prominent up regulation of PLC in SCI+5-HT+GABA+BMC treatment group is indicative of restoration of signal transduction *via* odd numbered muscarinic receptors and hence locomotion. This is attributed to the BMC differentiation into neurons by the co-mitogenic activity of 5-HT and GABA in spinal cord leading to increase in ACh levels. Co-

mitogenic activity of these neurotransmitters is supported by observations of Sudha & Paulose (1998) and Biju *et al.,* (2002).

Gene expression studies showed that it is up regulated in cerebral cortex and corpus striatum whereas it is down regulated in cerebellum and brain stem of SCI rats. In SCI+5-HT+GABA+BMC treatment group, it was further up regulated in cerebral cortex and corpus striatum. It was reversed and up regulated in cerebellum and brain stem region. Altered PLC expression in brain regions of SCI rats fails to modulate the activity of downstream proteins important for cellular signaling. Defective expression of PLC results in low levels of IP3 causing the impaired release of Ca^{2+} and bring down the level of intracellular calcium and thus failed to execute the normal neuronal function in brain regions of SCI rats. Administration of 5-HT and GABA along with BMC increased the PLC expression, which in turn influenced activation of muscarinic receptors and signal transduction pathway. Also, activation of PLC is a vital step in the neuronal protection in brain regions (Fahlman *et al.,* 2002; Rogel *et al.,* 2006). Thus 5-HT and GABA when administered with BMC increases the expression of PLC aiding in neuronal protection in SCI rats.

CREB EXPRESSION IN SPINAL CORD AND BRAIN

CREB is a transcription factor that is both a positive and a negative regulator of gene transcription that affects the expression of hundreds of genes depending upon cell phenotype and context. CREB forms homo- and heterodimers that interact with DNA in gene promoters. CREB activation is driven by the second messengers: cAMP and/or calcium (Giles *et al.,* 2007). Activation of adenylyl cyclases elevates the intracellular level of cAMP which activates cAMP-dependent protein kinase A, PKA. PKA phosphorylates and activates CREB. While CREB is expressed in numerous tissues, it plays a large regulatory role in the nervous system.

In the present study, Gene expression of CREB mRNA showed significant down regulation in the spinal cord of SCI rats compared to control. All other treatment groups except SCI+BMC showed significant reversal when compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed significant reversal and up regulation when compared to SCI rats. Deletion of CREB in neurons of the developing CNS results in apoptosis and postnatal ablation of these genes results in neuronal degeneration in adulthood. Thus the decrease in CREB expression in SCI rats lead to neuronal degeneration in spinal cord region. Injury to the spinal cord decreased the level of CREB (Gomez *et al.,* 2012). CREB is believed to play a key role in promoting neuronal survival, precursor proliferation, neurite outgrowth and neuronal differentiation in certain neuronal populations (Lee *et al.,* 1999; Lonze *et al.,* 2002; Redmond *et al.,* 2002; Zhang *et al.,* 2011). The upregulation of CREB in SCI+5-HT+GABA+BMC group is indicative of neuronal survival.

The cAMP/CREB signaling pathway has been strongly implicated in the regulation of a wide range of biological functions such as growth factor-dependent cell proliferation and survival Lee *et al.,* 1999; Lonze *et al.,* 2002). Our findings also showed a significant down regulation of CREB in cerebral cortex, cerebellum, brain stem and corpus striatum of SCI rats, when compared to control. The decreased expression of CREB in brain regions of SCI rats is indicative of decreased cell survival (Kempermann *et al.,* 1997; Hu-shan *et al.,* 2010). CREB expression was significantly reversed and up regulated in cerebral cortex, brain stem and corpus striatum of SCI+5-HT+GABA+BMC treatment group. It was significantly reversed in cerebellum of SCI+5-HT+GABA+BMC treatment group when compared to SCI rats. Neurons of the brain are particularly vulnerable to CREB deficiency (Kempermann *et al.,* 1997; Theo *et al.,* 2002; Hu-shan *et al.,* 2010). The richness of CREB signaling is greatly increased by its responsiveness to multiple intracellular signal transduction cascades and the potential for this family of transcription factors to induce and suppress gene expression renders

them ideally suited for regulating gene expression during the process of epidermal differentiation (Johannessen *et al.,* 2004). The up regulation of CREB in SCI+5-HT+GABA+BMC treatment group is due to the neuronal survival in brain regions. This study demonstrated that 5-HT and GABA along with BMC possess regulatory effect in the transcription factor CREB expression, which is crucial in maintaining the normal neuronal function. The study of the cholinergic receptors expression in relation with CREB phosphorylation in SCI is an important step toward elucidating the relationship between molecular adaptations and behavioural consequences.

Bax EXPRESSION IN SPINAL CORD AND BRAIN

SCI induces a series of endogenous biochemical changes that lead to secondary degeneration, including apoptosis (Bao *et al.,* 2006). Apoptosis, as a distinct type of cell death, is governed by a number of regulating genes mediated by apoptotic signals. Apoptosis in oligodendrocytes is induced by expression of Bax (Dong *et al.,* 2003). Bax is a proapoptotic gene which plays an important role in developmental cell death (Chittenden *et al.,* 1995) and in CNS injury (Bar-Peled *et al.,* 1999). Under stress conditions, it undergoes a conformation change that causes translocation to the mitochondrion membrane; leading to the release of cytochrome C that then triggers apoptosis. The present study analyses the relationship between expression of Bax gene and SCI in spinal cord and brain regions.

In our study, gene expression of Bax mRNA was up regulated in the spinal cord of SCI rats compared to control. All treatment groups except SCI+BMC showed significant reversal when compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed significant reversal and down regulation when compared to SCI rats. p53 and Bax have been reported to be up regulated in injured spinal neurons (Bai *et al.,* 2009; Kotipatruni *et al.,* 2011) which is consistent with our result in case of SCI rats. Decrease in expression of

bax is a survival mechanism (Li & Dou, 2000). This suggests that significance of 5-HT and GABA along with BMC in anti-apoptotic pathway. Thus 5-HT and GABA along with BMC promote cell survival.

SCI produced a significant apoptotic change and cell death not only in the spinal cord but also in the rat brain (Chang *et al.,* 2009). In the brain regions like cerebral cortex, cerebellum, brain stem and corpus striatum of SCI rats, Bax was up regulated. In SCI+5-HT+GABA+BMC treatment group, Bax expression was reversed and down regulated in cerebral cortex, cerebellum and brain stem. In Corpus striatum of SCI+5-HT+GABA+BMC treatment group, Bax showed a differential pattern where it was significantly reversed when compared to SCI rats. Bax is an activator of apoptosis and its up regulation in brain regions of SCI rats is indicative of apoptosis in these regions which occurs as a consequence of injury in the spinal cord. Neurons lacking Bax are protected against apoptosis (White *et al.,* 1998). Since SCI affects rat brain (Cheng *et al.,* 2009) the decreased expression of Bax in brain regions of SCI+5-HT+GABA+BMC treatment group is indicative of protection against apoptosis due to the re-establishment of neuronal network in injured spinal cord in this group of rats. The neuronal connectivity has been established due to the co-mitogenic activity of 5-HT (Sudha & Paulose, 1998) and GABA (Biju *et al.,* 2002) in inducing neuronal differentiation in BMC (Nandhu, 2011). Thus we report that rats with SCI show ascending brain injury that could be restricted by the administration of 5-HT and GABA along with BMC.

Caspase-8 EXPRESSION IN SPINAL CORD AND BRAIN

Cell loss induced by traumatic (Crowe *et al.,* 1997; Liu *et al.,* 1997) or ischemic (Kato *et al.,* 1997; Mackey *et al.,* 1997; Hayashi *et al.,* 1998; Sakurai *et al.,* 1998) SCI is attributed partly due to apoptotic mechanisms. Apoptosis plays an important role in determining neurological outcome following SCI (Crowe *et al.,* 1997; Emery *et al.,* 1998). It is well known that neural cell loss in SCI occurs

both at the time of injury and secondarily over a period of days to weeks after the traumatic event. Caspases are activated during the early phase of apoptosis and are markers of apoptosis (Darzynkiewicz *et al.,* 2011). Caspase-8 is seen in neurons undergoing cell death (Matsushita *et al.,* 2000). Caspase-8 bridges extrinsic and intrinsic apoptosis pathways (Vandenabeele *et al.,* 2006; Hou *et al.,*2010).

Cell death from moderate traumatic SCI is regulated by caspase family of cysteine proteases. Takagi *et al.,* (2003) identified apoptotic cells in mice after SCI, which showed caspase-8 in the injured spinal cord. Reports also suggest that caspase-8 mediated mechanisms of cell death are prominent in spinal cord ischemia (Matsushita *et al.,* 2000). In the current study, caspase-8 mRNA was up regulated in the spinal cord of SCI rats compared to control. This points to the apoptotic cell death in SCI model in our study as well. Caspase-8 (initiator of apoptosis) is expressed immediately after spinal injury and persists during apoptosis (Adjan *et al.,* 2007; Keane., *et al* 2001). All treatment groups except SCI+BMC showed significant reversal in gene expression of caspase-8 when compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed prominent reversal than all the other groups. So the decrease in caspase-8 expression in treatment groups except SCI+BMC rats is indicative of decrease in apoptosis. This pin points to the fact that 5-HT and GABA along with BMC protects the injured nerve tissues by inhibiting the expression of caspase-8, thereby inhibiting the neuronal apoptosis after SCI.

Apoptosis of brain cells likely also occurs after traumatic insults (Rink *et al.,* 1995). Widespread neuronal and glial apoptosis following injury to the CNS contribute to neurological dysfunction. We studied apoptosis in the brain regions after SCI with caspase-8, a marker of apoptosis. Caspase-8 is the initiator caspases in the death receptor and its activation is a tightly regulated process (Adjan *et al.,* 2007). Recent studies have shown that axotomy after a complete spinal cord transection induces death of spinal-projecting neurons of the lamprey brain stem (Shifman *et al.,* 2008; Busch & Morgan, 2012). Real Time-PCR analysis showed

that the caspase-8 gene expression up regulated in SCI condition in cerebral cortex, cerebellum, brain stem and corpus stratum. Thus there is apoptotic cell death in the brain regions as a result of damage of spinal cord during SCI. Caspase-8 gene expression was significantly reversed and down regulated in cerebral cortex, cerebellum and brain stem of SCI+5-HT+GABA+BMC treatment group. In corpus striatum of SCI+5-HT+GABA+BMC treatment group, it was significantly reversed when compared to SCI rats. Studies suggest that absence of caspase-8 renders protection from apoptosis in neural stem and progenitor cells (Ricci-vitiani *et al.,* 2004). Neuronal deletion of caspase-8 reduces brain damage and improves post-traumatic functional outcomes (Krajewska *et al.,* 2011). The decrease in caspase-8 expression in our study indicates an activation of anti-apoptotic pathway and protection from apoptosis in brain regions of SCI+5-HT+GABA+BMC treatment group. Thus we propose that 5-HT and GABA along with BMC has a role in regulating apoptosis in spinal cord and brain regions of SCI rats.

SUPEROXIDE DISMUTASE EXPRESSION IN SPINAL CORD AND BRAIN

SOD is an enzyme that repairs cells and reduces the damage that occurs to the cell by superoxide, the most common free radical in the body. SOD acts as both an antioxidant and anti-inflammatory agent. Tissue damage results in necrosis which leads to ischaemia (Senter & Venes, 1978). Necrosis induces the production of free radicals (Malorni *et al.,* 1993). The CNS is prone to free radical damage (LeBel & Bondy, 1991). CNS also has limited antioxidant defense mechanisms. The brain and spinal cord show evidence of low levels of catalase activity and only moderate levels of SOD and GPx under normal condition (Halliwell, 1992).

Spinal cord ischaemia leads to increase in free radicals (Cuevas *et al.,* 1989). SOD is an enzyme that revitalizes cells and reduces the rate of cell

destruction by scavenging free radicals. Our results showed a decreased expression of SOD in SCI rats when compared to control. This is indicative of increased free radicals in SCI rats which add to the neuronal damage. Decreased SOD adds to the pathogenesis in SCI (Taoka *et al.*, 1995). All treatment groups except SCI+BMC rats showed significant reversal in gene expression compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed significant reversal and up regulation when compared to SCI rats. Impaired energy metabolism in neurons induces production of increased amount of free radicals (Coyl & Puttfarcken, 1993) and initiates excitotoxic neuronal cell damage (Simon *et al*, 1984; Monyer *et al.,* 1989). The increase in free radical is not appealing in SCI. So protection of neurons from degeneration should be an effective strategy to prevent the progression of neuronal degeneration in SCI. In our experiment, administration of 5-HT and GABA along with BMC increased the expression of SOD in spinal cord region. Thus there is reduction in the free radical in spinal cord of this treatment group. Compounds that prevent oxidative damage increase the resistance of neuronal cells to degeneration.

Oxygen-derived free radical scavengers such as SOD have been shown to be important enzymes in the control of free radicals. Brain is vulnerable to oxidative stress induced by oxygen free radicals and it is due to the fact that is not particularly enriched, when compared with other organs, in any of the antioxidant enzymes. Relatively low levels of these enzymes are responsible in part for the vulnerability of this tissue (Baynes & Thrope, 1999). Our results showed an up regulated expression of SOD in cerebral cortex and corpus striatum and down regulated expression in cerebellum and brain stem of SCI rats. It was further up regulated in cerebral cortex and corpus striatum whereas it was reversed and up regulated in cerebellum and brain stem of SCI+5-HT+GABA+BMC treatment group. The comparatively decreased SOD activity in SCI suggests that the accumulation of superoxide anion radical is responsible for increased lipid peroxidation (Takenaga *et al.,* 2006). This alteration of SOD represents one of the

important factors for the vulnerability of the brain against oxygen free radicals or is relevant to the pathophysiology of SCI in Wistar rats. A study showed that SOD conjugated to polyethylene glycol can reduce ischemic brain injury in experimental animals (Liu *et al.,* 1989). Treatment with 5-HT and GABA along with BMC ameliorated the expression of enzyme and helps to control free radicals in brain regions. Our data proved 5-HT and GABA along with BMC, could exert a beneficial action against numerous morphological and functional alterations during SCI caused by the presence of free radicals in SCI condition.

GLUTATHIONE PEROXIDASE EXPRESSION IN SPINAL CORD AND BRAIN

Oxygen-derived free radicals are known to be produced in ischemic tissues and represent a potential mechanism for tissue injury and vascular dysfunction (McCord, 1985). The extent of injury is generally related to an increase or decrease of one or more free radical scavenging enzymes of which GPx is one (Pippenger *et al.,* 1998). GPx is a well-known selenoenzyme that functions as an antioxidant. GPx plays a very crucial role in our bodies as it is a free radical scavenger that protects the body from oxidation (Miyamoto *et al.,* 2003).

In the present study, gene expression of GPx mRNA showed down regulation in the spinal cord of SCI rats compared to control. All treatment groups except SCI+BMC rats showed significant reversal in gene expression compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed significant reversal and up regulation when compared to SCI rats. Oxygen derived free radicals after reperfusion of an ischemic spinal cord are partly responsible for neuronal destruction (Jon *et al.,* 1991). A decrease in GPx in SCI rats lead to neuronal destruction. A possible mechanism of neuro degeneration following SCI involves generation of toxic levels of ROS, e.g., $O_2^{-\cdot}$, H_2O_2 and $OH^{\cdot}$, which overwhelm endogenous antioxidants. Thus, the significant reduction in GPx levels promoted

by SCI leads to a reduction of effectiveness of the antioxidant enzyme defense system, sensitizing the cells to ROS. Scavenging the free radicals lessen experimental SCI (Jon *et al.,* 1991). The increase in GPx in SCI+5-HT+GABA+BMC treatment group decreases the ROS generated as a result of SCI. This shows that 5-HT and GABA along with BMC can activate an anti oxidant defence mechanism in SCI.

Gene expression of GPx mRNA showed significant up regulation in the cerebral cortex, brain stem and corpus striatum and down regulation in cerebellum of SCI rats compared to control. The SCI+5-HT+GABA+BMC treatment group showed further significant up regulation in cerebral cortex, brain stem and corpus striatum whereas it was reversed and up regulated in cerebellum when compared to control and SCI rats. Immunocytochemical studies showed localization of GPx to both brain astrocytes and neurons (Damier *et al.,* 1993; Olanow, 1993; Trepanier *et al.,* 1996). Comparatively decreased GPx activity in SCI suggests that the accumulation of free radicals which worsens SCI. Treatment with 5-HT and GABA along with BMC ameliorated the expression of enzyme and helps to control free radicals in brain regions. The antioxidant GPx, is reported to help reduce oxidative stress and increase neuroprotective factors and similar antioxidants can help promote greater recovery in SCI (Ran *et al.,* 2006; Ayromlou *et al.,* 2011). We conclude that administration of 5-HT and GABA along with BMC after spinal cord trauma greatly decreases oxidative stress and allows tissue preservation, thereby enabling otherwise monoplegic animals to locomote.

TNFα EXPRESSION IN SPINAL CORD

Posttraumatic inflammatory reaction contributes to secondary injury after traumatic SCI. Inflammatory cells that appear after SCI produce TNFα. TNFα mediates cell death (Fontaine *et al.,* 2002; Kenchappa *et al.,* 2004). TNFα exerts its effector actions, at least partially, through the activation of a pro-inflammatory

transcription factor, NF-kB, which in turn up regulates such genes as iNOS, cytokines, adhesive molecules and others. We studied the expression of TNFα in our model of SCI. Targeting of TNFα represents a potential strategy to reduce the secondary damage in SCI. Gene expression of TNFα mRNA showed up regulation in the spinal cord of SCI rats compared to control. TNFα is produced locally in the spinal cord following traumatic injury and this TNFα production is caused by the SCI (Wang *et al.,* 1996; De Leo *et al.,* 1997; Winkelstein *et al.,* 2001; Ohtori *et al.,* 2004).

In SCI, the expression of TNFα, at the site of injury regulates the precise cellular events (Streit *et al.,* 1998). After experimental SCI, the levels of TNFα is significantly increased (Hayashi *et al.,* 2000; Harrington *et al.,* 2005). This is in accordance with our result which suggests apoptosis in the spinal cord of SCI rats. The treatment groups reversed the expression of TNFα when compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed prominent reversal than all the other group of rats. Inhibition of TNFα reduces the development of inflammation and tissue injury associated with SCI (Genovese *et al.,* 2008). 5-HT and GABA along with BMC has a potential in decreasing the expression of TNFα in the injured spinal cord. 5-HT and GABA along with BMC protects the neuronal cells and promotes its survival and re-establishes the connections in the injured spinal cord through its co-mitogenic activity. Our study identifies an important role for TNFα in the pathogenesis of SCI and demonstrates that 5-HT and GABA along with BMC can inhibit TNFα and apoptosis in SCI.

NF-κB EXPRESSION IN SPINAL CORD

The nuclear transcription factor NF-κB is important in the expression of many genes whose proteins are involved in the control of apoptosis, multiple stresses and in inflammatory responses. Downstream products of NF-κB activation include inflammatory cytokines, TNFα and NOS. Cellular and molecular analyses of brain and spinal cord tissues in experimental rodent models

of stroke, epileptic seizures, and traumatic injury have begun to reveal the complex functions of NF-κB in modifying neuronal degeneration and recovery. Traumatic SCI activates NF-kB within macrophages, microglia, endothelial cells and neurons. We studied the changes in gene expression of NF-κB in our rat SCI model.

In our study, gene expression of NF-κB mRNA showed significant up regulation in the spinal cord of SCI rats compared to control. In brain injury and SCI models, NF-κB is highly activated (Bethea *et al.,* 1998; Schneider *et al.,* 1999; Nomoto *et al.,* 2001; Beni *et al.,* 2004; Rafati, 2005) and the expression of NF-κB dependent genes is up regulated (Bethea *et al.,* 1998; Siren *et al.,* 2001), indicating a critical function of this factor in CNS pathophysiology. Activation of NF-κB in neurons *ex vivo* has been shown to be linked to excitotoxic cell death in the CNS (Grilli *et al.,* 1996). NF-κB can be activated by exposure of cells to TNFα. Increased expression of TNFα followed by increase in NF-κB have been observed (Xu *et al.,* 1998). These reports are in accordance to our results. Thus it has become clear that NF-κB influences the neurodegenerative process by directly affecting gene expression in neurons themselves and by indirectly regulating gene expression in glial cells. The treatment group of rats - SCI+5-HT, SCI+GABA and SCI+BMC rats reversed these changes when compared to SCI rats. SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC treatment group showed significant reversal and down regulation when compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed prominent reversal and down regulation when compared to SCI rats. The inhibition of NF-κB activation prevented neuronal cell death (Grilli *et al.,* 1996; Xu *et al.,* 1997; Acarin *et al.,* 2001). Inhibition of NF-kB activity has been shown to reduce inflammation and improves functional recovery after SCI (Brambilla *et al.,* 2005; Xiao *et al.,* 2012). 5-HT and GABA along with BMC showed a modulatory role in the expression of NF-κB. This particular treatment decreased the expression of NF-κB, promoting neuronal protection and functional recovery in SCI rats.

BDNF EXPRESSION IN SPINAL CORD

BDNF is widely and abundantly expressed in the CNS and is available to some peripheral neurons (Murer *et al.,* 2001; Nockher *et al.,* 2005). It is a crucial neurotrophic factor and possess pro-survival and/or differentiation effects and has been implicated in the development of the nervous system (i.e., neuronal growth and differentiation) and in neuronal survival, repair and synaptic plasticity (Thoenen *et al.,* 1991; Lewin *et al.,* 1996; Thoenen, 2000; Huang & Reichardt, 2001). It also participates in axonal growth, path finding and in the modulation of dendritic growth and morphology. Changes in central or peripheral BDNF concentrations have been linked to a wide variety of conditions. We analysed the change in BDNF expression during SCI. BDNF has a neuroprotective effect in SCI (Uchida *et al.,* 2012).

Gene expression of BDNF mRNA showed was up regulated in the spinal cord of SCI rats compared to control. A decreased production of BDNF protein has been found in neurodegenerative disorders and in affective disorders (Post, 2007; Schulte-Herbruggen *et al.,* 2007). We also observed similar pattern in SCI rats. SCI+5-HT, SCI+GABA, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC group showed further significant up regulation when compared to SCI rats. SCI+BMC rats showed no significant up regulation when compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed prominent up regulation when compared to control. BDNF promote the survival and differentiation of 5-HT neurons *in vitro*. BDNF can sustain or increase the myelination of small axons *in vitro* (Chan *et al.,* 2001). BDNF has been reported to enhance ongoing adult neurogenesis (Pencea *et al.,* 2001) and contribute to hippocampal mossy fiber sprouting (Vaidya *et al.,* 1999). The increase in BDNF mRNA reduced motor functional deficits in spinal cord transaction rats (Gao *et al.,* 2012; Jin *et al.,* 2012). BDNF results in

enhanced connectivity of the peripheral motor bridge in a rodent model of SCI (Martin *et al.,* 2012). This would have resulted in improvement in locomotor function in SCI in our treatment group, SCI+5-HT+GABA+BMC. BDNF is a survival gene which contains CREB. Increases in CREB then underlie the up regulation of BDNF expression by binding to CRE-elements and enhancing transcription (Conti *et al.,* 2002). There is evidence from primary cultures of hippocampal neurons that BDNF enhances spontaneous release of glutamate and ACh, suggesting that BDNF functions there as a retrograde modulator of presynaptic transmitter release (Lessmann *et al.,* 1994). BDNF induce alterations in immediate early genes expressed in dorsal horn neurons in the spinal cord (Kerr *et al.,* 1999). In the present study we also observed an increase in CREB, ACh and BDNF when 5-HT and GABA along with BMC is administered. This points to the fact that 5-HT and GABA along with BMC promote neuronal survival and neuronal differentiation in spinal cord by modulating BDNF during SCI . BDNF also influences the survival factor CREB and the neurotransmitter release.

GDNF EXPRESSION IN SPINAL CORD

GDNF regulate survival, development and function in the nervous system. It increases axonal caliber, a known determinant of myelin thickness (Aguayo *et al.,* 1976; Weinberg & Spencer, 1976). GDNF is capable of sustaining the myelination of nerve fibers (Hoke *et al.,* 2003). GDNF protect against neurodegeneration is associated with caspase-3 suppression (Li *et al.,* 2007). Thus GDNF suppresses apoptosis. Neuronal subpopulations affected by GDNF include motor neurons, midbrain dopaminergic neurons and Purkinje cells.

In the current study, gene expression of GDNF mRNA showed down regulation in the spinal cord of SCI and rats compared to control. This is indicative of apoptosis in SCI rats as a result of activation of caspases (Li *et al.,* 2007). SCI+5-HT, SCI+GABA, SCI+BMC SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC groups showed significant reversal and up regulation when compared to SCI rats SCI+5-HT+GABA+BMC treatment group showed prominent up regulation when compared to control and SCI group. Increased GDNF increased the number of motoneurons which regenerate their axons, accompanying with an increase in axon sprouting within the distal nerve stump (Boyd & Gordon, 2003; Zhang *et al.,* 2009; *Ling-Xiao et al.,* 2012). Previous studies demonstrate significant effects of GDNF on neurite outgrowth *in vitro* and accelerated time course and increased amount of axonal regeneration and myelination *in vivo*, suggesting a direct effect of GDNF on neurons (Iannotti *et al.,* 2003; Liqun *et al.,* 2009). GDNF consistently and significantly enhanced spinal cord myelination (Liqun *et al.,* 2009). So the increase in GDNF as a result of administration of 5-HT and GABA along with BMC increases neurite outgrowth and myelination in injured spinal cord. This suggests that 5-HT and GABA along with BMC is capable of inducing neuronal regeneration by increasing the expression of neuronal regeneration markers. The neuronal regeneration is achieved through differentiation of BMC into neurons by the co-mitogenic activity of 5-HT and GABA. Co-mitogenic activity of 5-HT and GABA has earlier been established (Sudha & Paulose, 1998; Biju *et al.,* 2002; Nandhu, 2011)

IGF-1 EXPRESSION IN SPINAL CORD

IGFs are peptide hormones secreted from many different cells. IGF-1 is being found to have numerous potential, including healing and rejuvenating the nervous system. Increase in IGF-1 shows improvements with damaged nerve tissue, brain injury, seizure, stroke, neurodegenerative diseases such as

Alzheimer's disease, muscular dystrophy, multiple sclerosis, Parkinson's disease, SCI and neuropathies.

Researchers believe that IGF-1 can also stop programmed cell death. IGF-1 is required for normal growth and health maintenance. The primary purpose of IGF-1 is to stimulate cell growth. Researchers also believe that IGF-1 plays an important role in mediating various growth factors and their passage through the blood brain barrier back and forth from the brain to the periphery (Cotman *et al.,* 2002). It can be hypothesized that since BDNF is responsible for many CNS and Peripheral nervous system plastic changes, then IGF-1 is suggested to be the upstream driving force to help regulate the action of these important neurotrophin cascade events (Cotman *et al.,* 2002). IGF-1 is important for recovery following SCI because this growth factor promotes both oligodendrocyte and neuron growth and repair while also is associated with neural plasticity as it is able to be differentiated to create new pathways in the brain and spinal cord (Cotman *et al.,* 2002). So we studied the gene expression of IGF-1 in our experimental model of SCI.

Gene expression of IGF-1 mRNA showed up regulation in the spinal cord of SCI rats compared to control. SCI+5-HT, SCI+GABA, SCI+BMC, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC groups showed further significant up regulation when compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed prominent up regulation when compared to SCI rats. SCI is characterized by diminished IGF-1 (Min-Liu *et al.,* 2010). IGF-1 has been shown to support injured neurons, inhibit apoptosis of brain neurons involved in memory and axon growth and influence progenitor cells in the way neural protective proteins are expressed (Rojas *et al.,* 2008). Treatment with 5-HT and GABA along with BMC up regulated the expression of IGF-1. So it appears that IGF-1 is involved in how 5-HT and GABA along with BMC promotes functional recovery. However, other neurotrophic factors are responsible in creating an environment to potentiate beneficial effects including BDNF and

GDNF as discussed earlier. IGF-1 is suggested to be an upstream mediator for BDNF. So based on this SCI study, it is suggested recommended that 5-HT and GABA along with BMC is related to possible benefits related to IGF-1 independently and it can be that this increase will have led to further action of BDNF and its downstream neuroplastic and neuroprotective effects (Rojas *et al.*, 2008). Our data provide new experimental evidences which suggest that BDNF, GDNF and IGF exert their potential neuroprotective effects probably *via* regulation of apoptotic and cell survival pathways. Overall, this study demonstrates the efficacy of a clinically applicable pharmacological therapy for rapid initiation of neuroprotection post SCI and sheds light on the signaling involved in its action.

Akt-1 EXPRESSION IN SPINAL CORD

Akt promotes survival, proliferation and tumorigenesis. One mechanism by which Akt promote survival is through the inhibition of a component of the cell death machinery. Among molecules central to the regulation of cell death in eukaryotes are members of the Bcl-2 family of proteins. Akt pathway is involved in survival in pathological conditions, such as in the SCI model with reduced oligodendrocyte apoptosis induced by signaling through Akt (Azari *et al.*, 2006).

Gene expression of Akt was up regulated in SCI rats. In all treatment groups, the gene expression was significantly up regulated than SCI rats. SCI+5-HT+GABA+BMC treatment group showed prominent up regulation when compared to SCI rats. Survival factors can suppress apoptosis in a transcription-independent manner by activating the serine/ threonine kinase Akt, which then phosphorylates and inactivates components of the apoptotic machinery, including Bad and Caspase 9. The sequestration of Bad from mitochondria frees Bcl-xL to facilitate antiapoptotic signaling. As a consequence, the dynamic interaction between Bcl-xL and Bad represents a critical determinant of cell fate downstream of the phosphatidylinositol-3 kinase (PI3K)/Akt cascade, and represent an

alternative mechanism for cells to evade apoptosis (Zha *et al.,* 1996; Del peso *et al.,* 1997). Akt over expression leads to myelination (Ana *et al.,* 2008). Growth factor signaling through Akt has been shown to impact primarily cell proliferation and cell survival. IGF-1 signaling through Akt enhances oligodendrocyte progenitor cell survival (Ness & Wood, 2002; Ness *et al.,* 2002). Administration of 5-HT and GABA along with BMC in SCI has led to increase in Akt which indicates the combined role of these neurotransmitters in dodging apoptosis. Akt enhances recovery following SCI (Walker *et al.,* 2012). Since Akt expression is important in myelination, this affects the neuronal connectivity in a positive manner. Thus the re-establishment of neuronal network in SCI rats as a result of administration of 5-HT and GABA along with BMC has resulted in functional recovery of these SCI rats which is evident from the behavioural tests in our experiment.

Cyclin D2 EXPRESSION IN SPINAL CORD

Cell cycle proteins, namely cyclins play an important role in the response to CNS injury. These proteins are significantly increased after injury in neurons showing apoptosis and caspase activation (Di Giovanni *et al.,* 2003; De Biase *et al.,* 2005). Inhibition of cell cycle progression has been reported to provide neuroprotection in other models of CNS injury (Cernak *et al.,* 2005; Di Giovanni *et al.,* 2005; Tian *et al.,* 2006). Cyclin D2 is a member of the family of D-type cyclins that is implicated in cell cycle regulation, differentiation and oncogenic transformation. We investigated the functional role of Cyclin D2 in the injured spinal cord.

We observed an increase in the expression of cyclin D2 in SCI rats. In all the individual treatment groups the expression of cyclin D2 was reversed when compared to SCI rats. In combination treatment groups, it was reversed and down regulated. SCI+5-HT+GABA+BMC treatment group showed prominent down regulation than all the other groups. Cyclins are markers of cell cycle activation

and deactivation. Cell cycle activation has been associated with apoptotic cell death in non-proliferating cell types, such as neurons and oligodendrocytes (Di Giovanni *et al.,* 2005) and both cell types undergo apoptotic cell death after SCI (Shuman *et al.,* 1997; Grossman *et al.,* 2001; Beattie *et al.,* 2002a, b; McBride *et al.,* 2003; Stirling *et al.,* 2004; Knoblach *et al.,* 2005). This explains the up regulation of cyclin D2 in SCI rats. We had demonstrated the apoptosis in SCI rats earlier as well. *In vivo* evidence shows S phase entry in ischemic neurons (Kuan *et al.,* 2004) and even expression of cdc2 (i.e., cdk1)/cyclin B1 complex, a marker of the G2–M phase transition, in degenerating neurons in AD (Vincent *et al.,* 1997). However, cell cycle activation in neurons initiates signals for neuronal death instead of proliferation which is evident in our experiment. We observed a down regulation of cyclin D2 in treatment group of rats. Since down regulation of cyclin D2 provides neuro protection (Cernak *et al.,* 2005; Di Giovanni *et al.,* 2005; Tian *et al.,* 2006), it also suggest that 5-HT and GABA along with BMC treatment increases the expression of cyclin D 2 can promote neuroprotection. Forced maintenance of cyclin D2 favours proliferation at the expense of neuronal differentiation. This suggests that down regulation of cyclin D2 in 5-HT and GABA along with BMC administered rats promotes neuronal differentiation. This in turn leads to re-establishment of neuronal network in this group of rats. The co-mitogenic activity of 5-HT and GABA has been established before (Sudha &Paulose, 1998; Biju *et al.,* 2002). Differetiation of BMC into neurons by the co-mitogenic activity of 5-HT and GABA has also been established (Nandhu, 2011). These results contribute to our understanding of how the cell cycle control can be linked to the patterning programs to influence the balance between proliferation and neuronal differentiation in discrete progenitors domains.

SECOND MESSENGERS IN SPINAL CORD AND BRAIN REGIONS

Second messengers are molecules that relay signals received at receptors on the cell surface to target molecules in the cytosol and/or nucleus. They serve to

greatly amplify the strength of the signal. They are small, readily diffusible molecules that operate in signal transduction. Binding of a ligand to a single receptor at the cell surface end up causing massive changes in the biochemical activities within the cell. Neurons use many different second messengers as intracellular signals. These messengers differ in the mechanism by which they are produced and removed, as well as their downstream targets and effects .The major second messengers are IP_3, cAMP and cGMP. The excited muscarinic receptor activate G protein, directly affected opening and closing of ion channels, and indirectly affected ion channels *via* a second messenger system, ultimately promoting neuronal regeneration and repair (Jones, 1993; Felder 1995; Dawei *et al.,* 2010). We studied the second messenger content in the spinal cord and brain regions.

IP_3 CONTENT IN SPINAL CORD AND BRAIN REGIONS

IP_3 is a second messenger derived from the hydrolysis of PIP2 by PLC. PLC is used as effector enzyme by a particular class of G protein coupled receptors. The subunit αq of the G protein activates PLC. Muscarinic receptors belong to the class of G protein coupled receptors. Muscarinic M1 and M3 are coupled to PLC and *via* heterotrimeric G-protein (Gq /11) hydrolyze PIP2 producing IP_3 and DAG (Abe *et al.,* 1992). IP_3 activates IP_3 receptors that induce Ca^{2+} release from IP_3 sensitive ER stores. The rise in intracellular Ca^{2+} regulates a number of processes.

IP_3 content in the spinal cord was decreased in SCI rats. All treatment groups showed an increase in the IP_3 content when compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed prominent increase than all the other group of rats in the spinal cord region. In the brain regions – cerebral cortex, cerebellum, brain stem and corpus striatum as well, the IP_3 content was decreased in SCI rats. It was reversed in cerebral cortex, brain stem and corpus striatum and reversed and increased in cerebellum in SCI+5-HT+GABA+BMC treatment

group. IP_3 content in the spinal cord and brain regions were decreased in SCI rats due to the decrease in muscrainic receptors with a resultant decrease in the expression of PLC in this group of rats. The decreased expression of PLC has directly affected the IP_3 content resulting in decreased IP_3 content. IP_3 mobilizes Ca^{2+} from intracellular sources after binding with IP_3 receptors, DAG activates the phosphorylating enzyme protein kinase C (Dekker *et al.,* 1995; Berridge *et al.,* 1998). These events mediate cellular activation and subsequent biological responses such as neurotransmitter release, cell growth, differentiation, neuronal development and gene expression (Nishizuka, 1988; Berridge & Irvine, 1989). Disruptions in this second messenger pathway are implicated in neurodegenerative disorders (Abe, 1997; Mattson *et al.,* 2000; LaFerla, 2002). In the treatment rats, the IP_3 content was increased, indicative of increase in signal transduction pathway in this group of rats. Both 5-HT and GABA has a role in increasing the ACh content (Wolf *et al* 1987; Iwamoto & Marion, 1993 a,b), thereby activating muscarinic and nicotinic receptors. This shows that 5-HT and GABA along with BMC has a role in modulating second messengers through the modulation of muscarinic receptors.

cAMP CONTENT IN SPINAL CORD AND BRAIN REGIONS

cAMP is the most widely distributed second messenger both in higher organisms and in various primitive life forms. It is used as a second messenger especially by G protein coupled receptors. Inside the cell, cAMP activates the enzyme protein kinase A which in turn catalyzes the phosphorylation of various cellular proteins, ion channels and transcription factors. This phosphorylation alters the function of the target proteins leading to a specific cellular response. cAMP is synthesized from ATP by the action of the enzyme adenylyl cyclase. Activation of adenylyl cyclases elevates the intracellular level of cAMP which activates cAMP-dependent protein kinase A, PKA. PKA phosphorylates and activates CREB. CREB is involved in cell survival pathway.

cAMP content in the spinal cord was decreased in SCI rats. All treatment groups showed reversal in the cAMP content when compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed prominent increase than all the other group of rats in the spinal cord region. In the brain regions – cerebral cortex, cerebellum, brain stem and corpus striatum as well, the cAMP content was decreased in SCI rats. It was reversed in cerebral cortexand reversed and increased in cerebellum, brain stem and corpus striatum in SCI+5-HT+GABA+BMC treatment group. treatment group. cAMP stimulates the proliferation of many cell types, but in some cases cAMP inhibits cellular proliferation (Pastan *et al.*, 1975; Bokoch, 1993; Dugan *et al.*, 1999; Hagemann & Rapp, 1999; Zwartkruis & Bos, 1999; Wang *et al.*, 2000). It has also been noted that muscarinic M1, M3 and M5 receptors stimulate cAMP accumulation in intact cells (Lai *et al.*, 1992; Peralta *et al.*, 1988). cAMP also activates CREB. The cAMP/CREB signaling pathway has been strongly implicated in the regulation of a wide range of biological functions such as growth factor-dependent cell proliferation and survival. cAMP content was decreased in SCI rats in the spinal cord and brain regions which is indicative of reduced cell proliferation, leading to apoptosis. Since muscarinic receptors are involved in the activation of cAMP (Lai *et al.*, 1992; Peralta *et al.*, 1988), the increase in cAMP content in SCI rats administered with 5-HT and GABA along with BMC attributes to the increase in muscarinic receptors in this group of rats. We also observed an increase in the expression of CREB in rats administered with 5-HT and GABA along with BMC. Also, since cAMP activates CREB, administration of 5-HT and GABA along with BMC suggest its role in regulating cell survival pathway by acting through cAMP and CREB. This signifies the importance of 5-HT and GABA along with BMC in therapeautic application in SCI.

cGMP CONTENT IN SPINAL CORD AND BRAIN REGIONS

Cyclic GMP and the enzymes that synthesize this cyclic nucleotide are widely distributed in brain and other parts of the body (Goldberg & Haddox, 1973; Waldman & Murrad, 1987). cGMP is produced from GTP by the action of guanylyl cyclase. Once the intracellular concentration of cGMP is elevated, this nucleotide binds to two different classes of targets. The most common targets of cyclic nucleotide action are protein kinase, cGMP-dependent protein kinase (PKG). cGMP binds to ion channels, thereby influencing neuronal signaling. Cyclic nucleotide signals are degraded by phosphodiesterases, enzymes that cleave phosphodiester bonds and convert cGMP into GMP. cGMP formation mediated by ACh was first reported over two decades ago with rat heart (George *et al.,* 1970). Shortly thereafter, muscarinic responses in other tissues were reported.

In the current study, cGMP content in the spinal cord were decreased in SCI rats. The individual treatment groups did not show significant change in the cGMP content. All the combination treatment groups showed siginificant reversal in the cGMP content when compared to SCI rats. SCI+5-HT+GABA+BMC treatment group showed prominent increase than all the other group of rats in the spinal cord region. In the brain regions – cerebral cortex, cerebellum, brain stem and corpus striatum as well, the cGMP content was decreased in SCI rats and reversed in cerebral cortex, reversed and increased in cerebellum, brain stem and corpus striatum in SCI+5-HT+GABA+BMC treatment group. The decrease in cGMP in SCI rats is due to the decrease in muscarinic receptors in this group of rats. Since ACh directly influences the synthesis of cGMP, the increase in muscarinic receptors as a result of increase in ACh has led to an increase in cGMP content in rats administered with 5-HT and GABA along with BMC. This shows that 5-HT and GABA along with BMC can modulate the signal transduction by activating muscarinic receptors, leading to an increase in cGMP content.

Brdu AND NeuN CO-LABELING IN SPINAL CORD

BrdU and NeuN co-labelling study demonstrated the differentiation of implanted BMC to neurons by the co-mitogenic activity of 5-HT (Sudha &Paulose, 1998) and GABA (Biju *et al.,* 2002). NeuN is a mature neuronal marker and is expressed by all the viable neurons in the brain. BrdU labels the proliferating cells. In our experiment Brdu labels the BMC that has been administered into spinal cord. We observed that in the spinal cord of rats treated with BMC in combination with 5-HT and GABA, most of BrdU-positive cells were immunoreactive to NeuN, indicating that the implanted BMC have differentiated into mature neurons. 5-HT alone, GABA alone and BMC administered alone to SCI rats did not show immunoreaction to NeuN, rather the number of BrdU-positive cells was lower when compared to the groups administered along with 5-HT and GABA suggesting cell death in SCI rats in the absence of proliferative factors. 5-HT and GABA are involved in a variety of cellular processes involved in regulating metabolism, proliferation and morphology. The fine integration of these dynamic events appears to involve multiple receptor action. This points to the need for cell-based therapies using fetal tissue or stem cells in SCI patients .Our combination treatment in the present study, which is effective in restoring the altered cholinergic neurotransmission, promoting cell survival by inhibiting apoptosis and inducing neuronal differentiation into BMC, will serve as an ideal therapeutic strategy taking into account the problems associated with SCI.

Earlier studies, from our laboratory reported the functional regulation of the GABA and 5-HT receptor subtypes during SCI (Paulose *et al.,* 2009). ACh is the main neurotransmitter in the motoneurons of the spinal cord and it is important for peripheral movement. The present study demonstrated the modulation of cholinergic neurotransmission in SCI. Our behavioural and molecular results showed that 5-HT and GABA along with BMC potentiates a restorative effect by reversing the alterations in cholinergic receptor function in SCI condition. Since

the individual treatment groups – SCI+5-HT, SCI+GABA and SCI+BMC and the combination treatment of SCI+5-HT+BMC and SCI+GABA+BMC were not as effective as the final combination treatment group- SCI+5-HT+GABA+BMC, the ineffective groups were eliminated and only the combination treatment group of SCI+5-HT+GABA+BMC rat was studied in the brain regions. 5-HT and GABA combination also modulate second messenger systems, inhibit cell death, promote cell survival and induce BMC differentiation into neurons, thereby re-establishing connections in injured spinal cord. 5-HT and GABA has been reported to have co-mitogenic activity (Sudha & paulose, 1998; Biju *et al.*, 2002). 5-HT and GABA along with BMC induces the differentiation of BMC into neurons (Nandhu, 2011), which is also evident in our experiment. Thus, it is suggested that 5-HT and GABA along with BMC in SCI rats renders protection against oxidative stress and apoptosis thereby ameliorating the cholinergic alterations and motor deficits which makes them clinically significant for functional re-establishment neuronal network and recovery from SCI.

Summary

1. Spinal Cord Injured rats were used as models to study the alterations in spinal cord and brain cholinergic receptors and cholinergic enzymes; second messengers - IP_3, cAMP and cGMP; transcription factor CREB; second messenger enzyme PLC; apoptotic factors - Bax, caspase-8, TNFα and NF-κB; antioxidant enzymes- GPx and SOD; neuronal survival factors - BDNF, GDNF, IGF-1,Akt-1, and cyclin D2 and their regulation by 5-HT, GABA and BMC individually and in combinations.

2. The examination of body weight showed a significant decrease in SCI rats compared to control. The treatment combinations with 5-HT, GABA and BMC regained the body weight when compared to SCI rats whereas 5-HT, GABA and BMC supplemented individually showed no significant reversal in the body weight.

3. Behavioural studies: rotarod, grid walk and narrow beam tests were conducted to assess the motor control and co-ordination in control and experimental rats. SCI rats showed a significant deficit in motor control and co-ordination. Rats treated with 5-HT, GABA and BMC in combinations reversed the behavioural response when compared to SCI rats. 5-HT, GABA and BMC supplemented individually showed no significant improvement in locomotor functions when compared to SCI rats.

4. Acetylcholine esterase expression was analyzed in the spinal cord and brain regions. In SCI rats, the expression was up regulated in the spinal cord and brain regions- cerebral cortex, cerebellum, brain stem and corpus striatum region. Treatment with 5-HT and GABA individually and SCI +

5-HT+ BMC and SCI + GABA + BMC showed a reversal in the acetylcholine esterase expression in the spinal cord region, whereas SCI+5-HT+GABA+BMC reversed the altered expression in spinal cord and different brain regions. SCI+BMC group did not show any significant reversal in the spinal cord and brain regions.

5. Choline acetyl transferase expression level is used as a marker for acetylcholine synthesis. In SCI rats, ChAT expression was down regulated in the spinal cord and cerebellum whereas it was up regulated in cerebral cortex, brain stem and corpus striatum when compared to control. In the spinal cord region, all the treatment groups except SCI + BMC reversed the changes with BMC combination groups showing a reversal with up regulation. In the brain regions, SCI+5-HT+GABA+BMC group reversed the ChAT expression when compared to SCI rats.

6. Total muscarinic receptor binding was analysed in the spinal cord and brain regions of control and experimental rats. Total muscarinic receptor binding parameter B_{max} was decreased in spinal cord region and brain regions and K_d showed a decrease in spinal cord, cerebral cortex, cerebellum and brain stem. All treatment groups except SCI + BMC reversed the altered parameters in the spinal cord region. B_{max} showed a reversal in the cerebral cortex, cerebellum, brain stem and corpus striatum of SCI+5-HT+GABA+BMC with a significant reversal in K_d in the cerebral cortex and brain stem.

7. The Scatchard analysis and gene expression studies of muscarinic M1 receptor was analysed in the spinal cord and brain regions. The Scatchard analysis revealed a decrease in B_{max} in the spinal cord and brain regions in SCI rats with a decreased K_d in the spinal cord, cerebellum and brain stem. In the cortex and striatum of SCI rats, K_d was significantly

increased. B_{max} showed a significant reversal in all the treatment groups in spinal cord region whereas in the brain regions only SCI+5-HT+GABA+BMC group showed reversal. In the spinal cord region, K_d showed a significant reversal in all treatment groups except SCI+BMC. In the brain regions of SCI+5-HT+GABA+BMC group, K_d showed a significant reversal in cerebral cortex and brain stem. The gene expression of muscarinic M1 receptor mRNA was down regulated in SCI rats in the spinal cord and brain regions. In the spinal cord region, the gene expression was reversed in all treatment groups. The muscarinic M1 receptor mRNA expression was reversed in cerebellum and reversed with up regulation in cerebral cortex, brain stem and corpus striatum of SCI+5-HT+GABA+BMC group. Immunohistochemistry studies using specific antibodies confirmed the scatchard analysis and Real Time PCR analysis of muscarinic M1 receptor expression at protein level in control and experimental rats in the spinal cord and brain regions.

8. Muscarinic M3 receptor binding and gene expression were studied in spinal cord and brain regions. In SCI rats, B_{max} was decreased significantly in the spinal cord region and brain stem whereas it showed an increase in cortex, cerebellum and corpus striatum. The K_d in SCI rats showed no significant change in the spinal cord region whereas it was significantly increased in cortical and cerebellar regions and significantly decreased in brain stem and corpus striatum. In the spinal cord region, all treatment groups showed a reversal in B_{max} with an increased K_d in SCI + GABA group. K_d showed no significant change in any other group. In the brain regions of SCI+5-HT+GABA+BMC group, both B_{max} and K_d were reversed significantly compared to SCI group. Immunohistochemistry studies using specific antibodies confirmed the scatchard analysis and

Real Time PCR analysis of muscarinic M3 receptor expression at protein level in control and experimental rats in the spinal cord and brain regions.

9. α7 nicotinic acetylcholine receptor gene expression was studied in spinal cord and brain regions of control and experimental rats. In SCI rats, α7 nicotinic acetylcholine receptor was down regulated in spinal cord region, cerebellum, brain stem and corpus striatum when compared to control. In the spinal cord region, the expression showed a significant reversal in SCI+5-HT and SCI+GABA groups when compared to SCI rats whereas SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC showed a reversal with up regulation. SCI+BMC rats showed no significant reversal when compared to SCI rats. The gene expression was significantly reversed in the cerebellum, brain stem and corpus striatum of SCI+5-HT+GABA+BMC treated groups when compared to SCI rats. Immunohistochemistry studies using specific antibodies confirmed the gene expression of α7 nicotinic acetylcholine receptor expression at protein level in control and experimental rats in the spinal cord and brain regions.

10. Second messenger enzyme - PLC mRNA expression showed down regulation in SCI rats in the spinal cord region, cerebellum and brain stem when compared to control. In the cortex and corpus striatum of SCI rats the gene expression was significantly up regulated compared to control. In the spinal cord region, all the treatment groups significantly reversed and up regulated the gene expression when compared to SCI rats. In the cerebellum and brain stem of SCI+5-HT+GABA+BMC treated groups the altered PLC expression was significantly reversed and up regulated, but in the cortex and corpus striatum the expression was further up regulated.

11. Transcription factor, CREB was analyzed in the spinal cord and brain regions. CREB gene expression was down regulated in SCI rats when compared to control in spinal cord and brain regions. In the spinal cord region of SCI+5-HT, SCI+GABA, SCI+5-HT+BMC and SCI+GABA+BMC groups the gene expression was significantly reversed and SCI + BMC showed no significant change when compared to SCI rats. In SCI+5-HT+GABA+BMC rats, the gene expression was reversed in the cerebellum and reversed with an up regulation in the spinal cord, cerebral cortex, brain stem and corpus striatum when compared to SCI rats.

12. Apoptotic factor Bax was up regulated in SCI rats when compared to control in spinal cord and brain regions. In the spinal cord region, 5- HT and GABA treatment alone and SCI+5-HT+BMC and SCI+GABA+BMC groups showed significant reversal in the Bax gene expression whereas BMC alone showed no significant reversal when compared to SCI rats. In SCI+5-HT+GABA+BMC group, the expression was significantly reversed in corpus striatum and significantly reversed with a down regulation in spinal cord, cerebral cortex, cerebellum and brain stem when compared to SCI rats.

13. Caspase-8 mRNA gene expression was studied in the spinal cord and brain regions to study apoptosis and its gene expression was up regulated in SCI rats when compared to control in spinal cord and brain regions. All groups except SCI+BMC showed significant reversal in the gene expression of caspase-8 when compared to SCI rats in the spinal cord region. It was significantly reversed in corpus striatum and reversed with down regulation in cerebral cortex, cerebellum and brain stem of SCI+5-HT+GABA+BMC treated groups.

14. Antioxidant enzyme, SOD gene expression was studied in spinal cord and brain regions of control and experimental rats. In SCI rats, the gene expression of SOD was down regulated in spinal cord, cerebellum and brain stem whereas it was up regulated in the cerebral cortex and corpus striatum when compared to control. In the spinal cord region, SCI+5-HT, SCI+GABA, SCI+5-HT+BMC and SCI+GABA+BMC treated groups significantly reversed the gene expression when compared to SCI rats. In SCI+5-HT+GABA+BMC treated groups, the SOD expression was reversed with an up regulation in the spinal cord, cerebellum and brain stem when compared to SCI rats. In the cerebral cortex and corpus striatum of SCI+5-HT+GABA+BMC rats the expression was further up regulated when compared to SCI rats.

15. Gene expression of GPx was analysed to study the oxidative stress in spinal cord and brain regions. GPx expression was down regulated in SCI rats when compared to control in spinal cord and cerebellum and up regulated in cerebral cortex, brain stem and corpus striatum of SCI rats when compared to control. In the spinal cord region, SCI+5-HT, SCI+GABA, SCI+5-HT+BMC and SCI+GABA+BMC groups significantly reversed the gene expression where as SCI+BMC showed no significant reversal when compared to SCI rats. In SCI+5-HT+GABA+BMC treated group, the GPx gene expression was reversed with an up regulation in the spinal cord and cerebellum when compared to SCI rats. In SCI+5-HT+GABA+BMC rats, a further significant up regulation was observed in cerebral cortex, brain stem and corpus striatum when compared to SCI rats.

16. Pro-apoptotic factor TNFα showed up regulation in its gene expression in spinal cord of SCI rats when compared to control. SCI+5-HT, SCI+GABA, SCI+BMC, SCI+5-HT+BMC and SCI+GABA+BMC treated groups reversed these changes when compared to SCI rats in the spinal cord region.

17. Gene expression of NF-κB mRNA showed up regulation in the spinal cord of SCI rats when compared to control. SCI+5-HT, SCI+GABA and SCI+BMC rats reversed these changes when compared to SCI rats. SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats showed significant reversal with down regulation when compared to SCI rats.

18. Cell survival factor BDNF was up regulated in spinal cord of SCI rats when compared to control. SCI+5-HT, SCI+GABA, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC rats showed significant up regulation when compared to SCI rats. SCI+BMC rats showed no significant change when compared to SCI rats.

19. Gene expression of GDNF mRNA showed down regulation in the spinal cord of SCI and rats compared to control. SCI+5-HT, SCI+GABA, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC treated groups showed significant reversal and up regulation when compared to SCI rats. SCI+BMC rats showed no significant change when compared to SCI rats.

20. IGF-1 gene expression was also studied in spinal cord region. Gene expression of IGF mRNA showed up regulation in the spinal cord of SCI rats compared to control. SCI+5-HT, SCI+GABA, SCI+BMC, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC treated

groups showed further significant up regulation when compared to SCI rats.

21. Akt-1, involved in cell survival was analyzed in the spinal cord of control and experimental rats. Akt-1 gene expression showed up regulation in the spinal cord of SCI rats compared to control. SCI+5-HT, SCI+GABA, SCI+BMC SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC treated groups showed further significant up regulation when compared to SCI rats.

22. Gene expression of cyclin D2 mRNA showed up regulation in the spinal cord of SCI rats compared to control. SCI+5-HT, SCI+GABA and SCI+BMC treated groups showed a significant reversal when compared to SCI rats. The combination treatment group of rats, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC showed significant reversal and down regulation in the gene expression of cyclin D2 mRNA when compared to SCI rats.

23. IP3 content was measured in spinal cord and brain regions of control and experimental rats. IP3 content was decreased in spinal cord, cerebral cortex, cerebellum, brain stem and corpus striatum of SCI rats when compared to control. In the spinal cord region, SCI+5-HT, SCI+GABA, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC groups showed significant reversal in the IP3 content whereas SCI+BMC showed no significant change when compared to SCI rats. It was significantly reversed in cerebral cortex, brain stem and corpus striatum of SCI+5-HT+GABA+BMC when compared to SCI rats. It was significantly reversed and increased in cerebellum of SCI+5-HT+GABA+BMC when compared to SCI rats.

24. cAMP content was analyzed in spinal cord and brain regions of control and experimental rats. cAMP content was decreased in spinal cord, cerebral cortex, cerebellum, brain stem and corpus striatum of SCI rats when compared to control. In the spinal cord region, all groups except SCI+BMC showed a significant reversal in the cAMP content when compared to SCI rats. In SCI+5-HT+GABA+BMC treated group, the cAMP content was significantly reversed in cerebral cortex and brain stem and significantly reversed and increased in cerebellum and corpus striatum when compared to SCI rats

25. cGMP content in the spinal cord, cerebral cortex, cerebellum, brain stem and corpus striatum was significantly decreased in SCI rats when compared to control. In the spinal cord region, the treatment groups with 5-HT, GABA and BMC alone showed no significant change whereas the combination treatment group of rats, SCI+5-HT+BMC, SCI+GABA+BMC and SCI+5-HT+GABA+BMC showed a significant reversal in the cGMP when compared to SCI rats. In SCI+5-HT+GABA+BMC treated group, the cGMP content was significantly reversed in cerebral cortex and reversed and increased in cerebellum, brain stem and corpus striatum when compared to SCI rats.

26. We demonstrated the autologous differentiation of BMC to neurons using BrdU-NeuN co-labelling studies. BMC injected into the spinal cord were tagged by proliferative marker BrdU and was seen to express NeuN which indicated neuronal cells. The BMC division and differentiation was increased when it was infused along with 5-HT and GABA. The most prominent expression was seen in rats treated with 5-HT, GABA and BMC in combination. However, BMC injected alone did not express any NeuN.

In the present study, we summarize that the cholinergic transmission was decreased in the spinal cord and brain regions of SCI rats and it has a significant role in locomotor function. The alterations in cholinergic enzymes in spinal cord and brain regions of SCI rats suggested a dysfunction of cholinergic neurotransmission in these regions. PLC, CREB, second messengers, antioxidant enzymes, apoptotic factors and cell survival factors were also altered in SCI rats. Treatment with 5-HT and GABA along with BMC effectively restored the altered cholinergic signaling. Treatment with 5-HT and GABA along with BMC also ameliorate the oxidative stress and apoptosis and induced neuronal cell differentiation and survival. Our results showed the autologous BMC differentiation to neurons in spinal cord when administered along with 5-HT and GABA. The findings from this study give an insight on the molecular mechanisms underlying SCI and the therapeutic role of 5-HT and GABA along with BMC in the functional recovery of SCI.

Conclusion

Spinal cord injury causes permanent and irrevocable motor deficits and neurodegeneration. Disruption of the spinal cord leads to diminished transmission of descending control from the brain to motor neurons and ascending sensory information. Behavioural studies showed deficits in motor control and co-ordination in SCI rats. Cholinergic system plays an important role in SCI, the evaluation of which provides valuable insight on the underlying mechanisms of motor deficit that occur during SCI. The cholinergic transmission was studied by assessing the muscarinic and nicotinic receptors; cholinergic enzymes- ChAT and AChE; second messenger enzyme PLC; transcription factor CREB and second messengers - IP_3, cAMP and cGMP. We observed a decrease in the cholinergic transmission in the brain and spinal cord of SCI rats. The disrupted cholinergic system is the indicative of motor deficit and neuronal degeneration in the spinal cord and brain regions. SCI mediated oxidative stress and apoptosis leads to neuronal degeneration in SCI rats. The decreased expression of anti oxidant enzymes – SOD, GPx and neuronal cell survival factors - BDNF, GDNF, IGF-1, Akt and cyclin D2 along with increased expression of apoptotic factors - Bax, caspase-8, TNFα and NF-κB augmented the neuronal degeneration in SCI condition. BMC administration in combination with 5-HT and GABA in SCI rats showed a reversal in the impaired cholinergic neurotransmission and reduced the oxidative stress and apoptosis. It also enhanced the expression of cell survival factors in the spinal cord region. In SCI rats treated with 5-HT and GABA, the transplanted BMC expressed NeuN confirming that 5-HT and GABA induced the differentiation and proliferation of BMC to neurons in the spinal cord. Neurotrophic factors and anti-apoptotic elements in SCI rats treated with 5-HT and GABA along with BMC rendered neuroprotective effects accompanied by

improvement in behavioural deficits. This resulted in a significant reversal of altered cholinergic neurotransmission in SCI. The restorative and neuro protective effects of BMC in combination with 5-HT and GABA are of immense therapeutic significance in the clinical management of SCI.

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PUBLICATIONS

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ABSTRACTS/ SCIENTIFIC PRESENTATIONS

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1. Young investigator award. Indian Ageing congress, Banaras Hindu University, Varanasi, India. (2010)

Figure Legends

Figure - 32 (a) & (b)

Confocal image of muscarinic M1 receptor expression in the spinal cord of control and experimental rats. → in white shows muscarinic M1 receptor. Muscarinic M1 receptor subunit antibody staining in the spinal cord showed a significant decrease in the mean pixel value in SCI rats compared to control which was significantly reversed in SCI+5-HT+GABA+BMC rats.

Figure-33 (a) & (b)

Confocal image of muscarinic M3 receptor expression in the spinal cord of control and experimental rats. → in white shows muscarinic M3 receptor. Muscarinic M3 receptor subunit antibody staining in the spinal cord showed a significant decrease in the mean pixel value in SCI rats compared to control which was significantly reversed in SCI+5-HT+GABA+BMC rats.

Figure-34 (a) & (b)

Confocal image of α7 nicotinic acetylcholine receptor expression in the spinal cord of control and experimental rats. → in white shows α7 nicotinic acetylcholine receptor. α7 nicotinic acetylcholine receptor subunit antibody staining in the spinal cord showed a significant decrease in the mean pixel value in SCI rats compared to control which was significantly reversed in SCI+5-HT+GABA+BMC rats.

Figure-35 (a), (b), (c) & (d)

Brdu-NeuN co-labelling studies in the spinal cord of control and experimental rats using immunofluorescent BrdU and NeuN primary antibodies and secondary antibodies of Alexa Fluor 594 and Alexa Fluor 488 respectively. → in white shows NeuN, BrdU and NeuN-BrdU co-labelled cells. BrdU positive BMC are red, NeuN labelled neurons are green and BrdU-NeuN co-labelled cells are yellow in colour.

Figure-54

Confocal image of mucsarinic M1 receptor expression in the cerebral cortex of control and experimental rats. → in white shows muscarinic M1

receptor. Muscarinic M1 receptor subunit antibody staining in the cerebral cortex showed a significant decrease in the mean pixel value in SCI rats compared to control which was significantly reversed in SCI+5-HT+GABA+BMC rats.

Figure-55

Confocal image of mucsarinic M3 receptor expression in the cerebral cortex of control and experimental rats. → in white shows muscarinic M3 receptor. Muscarinic M3 receptor subunit antibody staining in the cerebral cortex showed a significant increase in the mean pixel value in SCI rats compared to control which was significantly reversed in SCI+5-HT+GABA+BMC rats.

Figure-56

Confocal image of α7 nicotinic acetylcholine receptor expression in the cerebral cortex of control and experimental rats. → in white shows α7 nicotinic acetylcholine receptor. α7 nicotinic acetylcholine receptor subunit antibody staining in the cerebral cortex showed a significant decrease in the mean pixel value in SCI rats compared to control which was significantly reversed in SCI+5-HT+GABA+BMC rats.

Figure-75

Confocal image of mucsarinic M1 receptor expression in the cerebellum of control and experimental rats. → in white shows muscarinic M1 receptor. Muscarinic M1 receptor subunit antibody staining in the cerebellum showed a significant decrease in the mean pixel value in SCI rats compared to control which was significantly reversed in SCI+5-HT+GABA+BMC rats.

Figure-76

Confocal image of mucsarinic M3 receptor expression in the cerebellum of control and experimental rats. → in white shows muscarinic M3 receptor. Muscarinic M3 receptor subunit antibody staining in the cerebellum showed a significant increase in the mean pixel value in SCI rats compared to control which was significantly reversed in SCI+5-HT+GABA+BMC rats.

Figure-77

Confocal image of α7 nicotinic acetylcholine receptor expression in the cerebellum of control and experimental rats. → in white shows α7 nicotinic acetylcholine receptor. α7 nicotinic acetylcholine receptor subunit antibody staining in the cerebellum showed a significant decrease in the mean pixel value in SCI rats compared to control which was significantly reversed in SCI+5-HT+GABA+BMC rats.

Figure-96

Confocal image of mucsarinic M1 receptor expression in the brain stem of control and experimental rats. → in white shows muscarinic M1 receptor. Muscarinic M1 receptor subunit antibody staining in the brain stem showed a significant decrease in the mean pixel value in SCI rats compared to control which was significantly reversed in SCI+5-HT+GABA+BMC rats.

Figure-97

Confocal image of mucsarinic M3 receptor expression in the brain stem of control and experimental rats. → in white shows muscarinic M3 receptor. Muscarinic M3 receptor subunit antibody staining in the brain stem showed a significant decrease in the mean pixel value in SCI rats compared to control which was significantly reversed in SCI+5-HT+GABA+BMC rats.

Figure-98

Confocal image of α7 nicotinic acetylcholine receptor expression in the brain stem of control and experimental rats. → in white shows α7 nicotinic acetylcholine receptor. α7 nicotinic acetylcholine receptor subunit antibody staining in the brain stem showed a significant decrease in the mean pixel value in SCI rats compared to control which was significantly reversed in SCI+5-HT+GABA+BMC rats.

Figure-117

Confocal image of mucsarinic M1 receptor expression in the corpus striatum of control and experimental rats. → in white shows muscarinic M1

receptor. Muscarinic M3 receptor subunit antibody staining in the corpus striatum showed a significant decrease in the mean pixel value in SCI rats compared to control which was significantly reversed in SCI+5-HT+GABA+BMC rats.

Figure-118

Confocal image of mucsarinic M3 receptor expression in the corpus striatum of control and experimental rats. → in white shows muscarinic M3 receptor. Muscarinic M3 receptor subunit antibody staining in the copus striatum showed a significant increase in the mean pixel value in SCI rats compared to control which was significantly reversed in SCI+5-HT+GABA+BMC rats.

Figure-119

Confocal image of α7 nicotinic acetylcholine receptor expression in the corpus striatum of control and experimental rats. → in white shows α7 nicotinic acetylcholine receptor. α7 nicotinic acetylcholine receptor subunit antibody staining in the corpus striatum showed a significant decrease in the mean pixel value in SCI rats compared to control which was significantly reversed in SCI+5-HT+GABA+BMC rats.