EFFECT OF AGMATINE IN SPINAL CORD INJURY MODULATION BY IMIDAZOLINE RECEPTORS

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ABSTRACT

Background: Spinal cord injury often result in disability or loss of movement and sensation below the site of injury. Systematically administered agmatine significantly reduces the mechanical and thermal hyperalgesia as well as allodynia in neuropathic mice caused by spinal cord injury. However exact mechanism is still unclear. The present study examined the involvement of imidazoline receptor on functional recovery exhibited by agmatine following spinal cord injury.

Method: Compression spinal cord injury was developed by placing 5g weight for 30 sec at thoracic vertebra 10-12 segment. Animal were injected with agmatine (2.5,5,10 mg/kg,i.p.), clonidine(0.1mg/kg), moxonidine(0.5mg/kg), efaroxan (1mg/kg), idazoxan (3mg/kg) and their combination observed for locomotor recovery.

RESULT: Experimental spinal cord injury resulted in complete loss of movement of hindlimb in exposed animal. Agmatine treatment significantly improved locomotor recovery of the animals subjected to SCI. Imidazoline agonist clonidine moxonidine potentiated while, imadazoline antagonist idazoxan and efaroxan blocked effect of agmatine in SCI.

Conclusion: Chronic agmatine treatment showed effect of locomotor recovery in SCI animal and evidences suggest that this effect was possibly mediated imidazoline receptors.

KEYWORDS: Spinal cord injury, agmatine, imidazoline receptor, locomotor recovery, mice.

1. INTRODUCTION

1.1. NEURAL MECHANISMS OF PAIN

Pain occurs whenever any tissues are being damaged, and it causes the individual to react to remove the pain stimulus as after spinal cord injury.
Pain is a subjective experience, hard to define exactly, even though we all know what we mean by it. Typically, it is a direct response to an untoward event associated with tissue damage, such as injury, inflammation or cancer, but severe pain can arise independently of any obvious predisposing cause (e.g. trigeminal neuralgia), or persist long after the precipitating injury has healed (e.g. phantom limb pain). It can also occur as a consequence of brain or nerve injury (e.g. following a stroke or herpes infection). Painful conditions of the latter kind, not directly linked to tissue injury, are very common and a major cause of disability and distress, and in general they respond less well to conventional analgesic drugs than do conditions where the immediate cause is clear. In these cases, we need to think of pain in terms of disordered neural function, comparable with schizophrenia or epilepsy, rather than simply as a 'normal' response to tissue injury. Therefore it is useful to distinguish two components, either or both of which may be involved in pathological pain states:

- The peripheral nociceptive afferent neuron, which is activated by noxious stimuli.
- The central mechanisms by which the afferent input generates a pain sensation.

Good accounts of the neural basis of pain can be found in (McMahon & Koltzenburg, 2006).

1.2. NOCICEPTIVE AFFERENT NEURONS

Under normal conditions, pain is associated with impulse activity in small-diameter primary afferent fibres of peripheral nerves (Raja et al.1999). These nerves have sensory endings in peripheral tissues and are activated by stimuli of various kinds (mechanical, thermal, chemical) (Julius & Basbaum, 2001; Julius & McCleskey, 2006). They are distinguished from other sorts of mechanical and thermal receptors by their higher threshold, because they are normally activated only by stimuli of noxious intensity-sufficient to cause some degree of tissue damage. Recordings of activity in single afferent fibres in human subjects have shown that stimuli sufficient to excite these small afferent fibres also evoke a painful sensation. Many of these fibres are non-myelinated C fibres with low conduction velocities (< 1 m/s); this group is known as C polygonal nociceptors. Others are fine myelinated (Aδ) fibres, which conduct more rapidly but respond to similar peripheral stimuli. Although there are some species differences, the majority of the C fibres are associated with polygonal nociceptive endings. Afferents from muscle and viscera also convey nociceptive information. In the nerves from these tissues, the small myelinated Aδ fibres are connected to high-threshold mechanoreceptors, while the non-myelinated C fibres are connected to polygonal nociceptors, as in the skin.
Experiments on human subjects, in which recording or stimulating electrodes are applied to cutaneous sensory nerves, have shown that activity in the Aδ fibres causes a sensation of sharp, well-localised pain, whereas C fibre activity causes a dull, diffuse, burning pain.

With many pathological conditions, tissue injury is the immediate cause of the pain and results in the local release of a variety of chemicals that act on the nerve terminals, either activating them directly or enhancing their sensitivity to other forms of stimulation. The pharmacological properties of nociceptive nerve terminals are discussed in more detail below.

The cell bodies of spinal nociceptive afferent fibres lie in dorsal root ganglia; fibres enter the spinal cord via the dorsal roots, ending in the grey matter of the dorsal horn. Most of the nociceptive afferents terminate in the superficial region of the dorsal horn, the C fibres and some Aδ fibres innervating cell bodies in laminae I and II, while other A fibres penetrate deeper into the dorsal horn (lamina V). Cells in laminae I and V give rise to the main projection pathways from the dorsal horn to the thalamus.

The non-myelinated afferent neurons contain several neuropeptides, particularly substance P and calcitonin gene-related peptide (CGRP). These are released as mediators at both the central and the peripheral terminals, and play an important role in the pathology of pain.

1.3. MODULATION IN THE NOCICEPTIVE PATHWAY
Acute pain is generally well accounted for in terms of nociception an excessive noxious stimulus giving rise to an intense and unpleasant sensation. In contrast, most chronic pain states are associated with aberrations of the normal physiological pathway, giving rise to hyperalgesia (an increased amount of pain associated with a mild noxious stimulus), allodynia (pain evoked by a non-noxious stimulus) or spontaneous pain without any precipitating stimulus. An analogy is with an old radio set that plays uncontrollably loudly (hyperalgesia), receives two stations at once (allodynia), or produces random shrieks and whistles (spontaneous pain spasms). These distortions in the transmission line are beginning to be understood in terms of various types of positive and negative modulation in the nociceptive pathway, discussed in more detail below. Some of the main mechanisms are summarised in Figure 2.

1.4. HYPERALGESIA AND ALLODYNIA
- Anyone who has suffered a burn or sprained ankle has experienced hyperalgesia and allodynia. Hyperalgesia involves both sensitisation of peripheral nociceptive nerve
terminals and central facilitation of transmission at the level of the dorsal horn and thalamus—changes defined by the term neuroplasticity. The peripheral component is due to the action of mediators such as bradykinin and prostaglandins acting on the nerve terminals. The central component reflects facilitation of synaptic transmission.

Fig. 1 The termination of afferent fibres in the six laminae of the dorsal horn of the spinal cord.

Defined as pain that outlasts the precipitating tissue injury. Many clinical pain states fall into this category. The dissociation of pain from noxious input is most evident in ‘phantom limb’ pain, which occurs after amputations and may be very severe. The pain is usually not relieved by local anaesthetic injections, implying that electrical activity in afferent fibres is not an essential component. At the other extreme, noxious input with no pain, there are many well-documented reports of mystics and showmen who subject themselves to horrifying ordeals with knives, burnings embers, nails and hooks (undoubtedly causing massive afferent input) without apparently suffering pain.

1.5. NEUROPATHIC PAIN

Neurological disease affecting the sensory pathway can produce severe chronic pain—termed neuropathic pain—unrelated to any peripheral tissue injury. This occurs with central nervous system (CNS) disorders such as stroke and multiple sclerosis, or with conditions associated with peripheral nerve damage, such as mechanical injury, diabetic neuropathy or herpes zoster infection (shingles). The pathophysiological mechanisms underlying this kind of pain are poorly understood, although spontaneous activity in damaged sensory neurons, due to over expression or redistribution of voltage-gated sodium channels, is thought to be a factor
(Chahine et al., 2005). The sympathetic nervous system also plays a part, because damaged sensory neurons can express α adrenoceptors and develop sensitivity to noradrenaline (norepinephrine) that they do not possess under normal conditions. Thus physiological stimuli that evoke sympathetic responses can produce severe pain, a phenomenon described clinically as sympathetically mediated pain. Neuropathic pain, which appears to be a component of many types of clinical pain (including common conditions such as back pain and cancer pain, as well as amputation pain), is generally difficult to control with conventional analgesic drugs.

Fig. 3. The descending control system, showing the main sites of action of opioids on pain transmission.

Opioids excite neurons in the periaqueductal grey matter (PAG) and in the nucleus reticularis paragigantocellularis (NRPG), which in turn project to the rostroventral medulla, which includes the nucleus raphe magnus (NRM). From the NRM, 5-hydroxytryptamine (5-HT) and enkephalin-containing neurons run to the substantia gelatinosa of the dorsal horn, and exert an inhibitory influence on transmission. Opioids also act directly on the dorsal horn, as well as on the peripheral terminals of nociceptive afferent neurons. The locus coeruleus (LC) sends noradrenergic neurons to the dorsal horn, which also inhibit transmission. The pathways shown in this diagram represent a considerable oversimplification but depict the general organisation of the supraspinal control mechanisms. Shaded boxes represent areas rich in opioid peptides. (Fields & Basbaum, 1994) DLF, dorsolateral funiculus.
1.6. NEUROPATHIC PAIN IN SPINAL CORD INJURY

Neuropathic pain, resulting from injury, significantly impairs quality of life in people living with spinal cord injury (SCI) (Cairns et al., 1996; Celik et al., 2012; Harden and Cohen, 2003; Wetering et al., 2010). Unfortunately, however, approximately one-third of people with a spinal cord injury will experience this severe or excruciating pain within 5 years of injury (Siddall et al., 2003), compared to an estimated 1% of people in the general population experiencing the same pain characteristics. Moreover, this aberrant pain is very difficult to treat (Heutink et al., 2012). Clinicians are currently faced with a trial-and-error approach to pain management after SCI.

Opioids are considered to be among the most effective treatments for neuropathic pain, and are commonly trailed for analgesic efficacy. In the long term, approximately 20% of people will discontinue opioid treatment (Moore and McQuay, 2005), because of significant side effects (reviewed in Dellemijn, 1999; Cruccu, 2007; Dworkin et al., 2007), but even short-term trials may interact with spinal injury and impact recovery. For example, there is novel, experimental evidence showing that the therapeutic use of opioids in the acute phase of SCI (Day 1–7 post injury) can inhibit locomotor recovery (Hook et al., 2009, 2011; Woller et al., 2012). Yet, there are currently no guidelines for opioid administration, regarding timing and duration of use, following injury. As opioids are administered immediately for the treatment of pain resulting from SCI, this issue must be further explored. Based on a comprehensive review of the literature, we propose that opioids and SCI may have synergistic effects on neuronal and glial function that adversely affect locomotor recovery, the development of pathological pain, and general health. Evidence from the literature suggests that excitotoxicity and glial activation are exacerbated by opioid administration, which can negatively affect the vulnerable cellular environment of the injured spinal cord to increase cell death and reduce recovery of function. Aberrant glial activation and hyperexcitability of dorsal horn neurons (the development of central sensitization) have also been implicated in the development of pain after spinal injury (Gwak and Hulsebosch, 2011a, 2011b; Gwak et al., 2012; Hulsebosch, 2008).

The molecular changes associated with both SCI and opioid administration, highlighting the characteristics that are common to both phenomena. We first outline changes induced by SCI, focusing on neuronal and glial function. Using the same strategy, we review opioids and molecular changes underlying opioid induced analgesia, as well as pathologies associated
with repeated opioid use. Finally, we review literature suggesting that administration of opioids after a spinal cord injury can contribute to the pathology of SCI. Throughout this discussion; we emphasize the need to better understand how opioids affect the cellular and molecular environment of the injured cord.

Indeed, the data suggest that opioid treatment in the acute phase of injury might lead to augmented pain and loss of locomotor function after SCI, as well as concerns for overall health.

1.7. SPINAL CORD INJURY
SCI results in a number of consequences that can lead to cell death, excitotoxicity, and central sensitization. Each of these consequences contributes to decreased locomotor function and the development of pain following the initial trauma. As these are immediate consequences of SCI, this review focuses primarily on the acute phase of SCI; defined here, for the rodent model, as days 1–7 immediately following the injury.

1.8. NEURONAL EFFECTS OF SPINAL CORD INJURY
A) Excitotoxicity
Excitotoxicity refers to the death of cells resulting from an excessive exposure to glutamate, a major excitatory neurotransmitter in the CNS, or overstimulation of glutamate receptors (Olney, 1969; Olney and Ho, 1970). In SCI, cell death resulting from trauma induces the release of glutamate from primary afferent and injured dorsal horn neurons into the dorsal horn of the spinal cord. Glutamate levels peak 15 min after injury, remain elevated for an hour, and return to normal over a period of 1.5 h (Vera-Portocarrero et al., 2002; Liu et al. 1991; McAdoo et al., 1999; Xu et al., 1998). Studies have shown that the extracellular concentrations of glutamate reached post injury are capable of inducing functional impairments when administered to intact animals (Xu et al., 2005). In the intact animal, however, glutamate is typically regulated by neurons and astrocytes (Matos et al. 2012; Tsai et al., 2012) with excess levels being removed from the synaptic cleft in a matter of milliseconds (Clements et al., 1992). As a result of trauma, release of glutamate into the dorsal horn causes increased activation of NMDA receptors (NMDARs) and AMPA receptors (AMPARs), allowing an influx of calcium ions to the postsynaptic cell. This increased activation of NMDARs has been implicated in excitotoxic cell death following experimental injury. Indeed, administration of an NMDAR antagonist, or other agents that block glutamate receptors, soon after injury improves functional outcome following SCI.

B) Central sensitization

Increased extracellular glutamate levels, and the subsequent NMDAR activation, can lead to the induction of central sensitization, one mechanism thought to underlie the development of neuropathic pain, in the spinal cord (Artola and Singer, 1987; Woolf and Thompson, 1991). Central sensitization is a phenomenon in which neurons of the spinal cord dorsal horn become hypersensitive following peripheral tissue damage, inflammation, or injury to the CNS. This hypersensitivity continues even in the absence of the triggering stimulus (Woolf, 1983, 2007, 2011), and shares many of the molecular changes that have been described for long-term potentiation (e.g. Ji et al., 2003). Briefly, the release of glutamate resulting from SCI activates NMDARs, and subsequently allows for the influx of Ca2+, which then activates downstream intracellular kinases. This includes activation of adenyl cyclase (AC), protein kinase A (PKA), protein kinase C (PKC), and/or calcium/calmodulin-dependent kinase II (CaMKII). Through these cascades, mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK are phosphorylated. The phosphorylation of CREB (cyclic adenosine monophosphate (cAMP) response element-binding), a downstream target of ERK1/2, p38 MAPK, and CaMKII, is important in regulating transcription and maintaining central sensitization. Connecting this molecular pathway with functional implications, (Crown et al. 2005) showed that animals with signs of neuropathic pain following SCI had an increased expression of pCREB. This increase was not seen in injured animals free of neuropathic symptoms, or in control animals (Crown et al., 2005). Furthermore, inhibition of p38MAPK at 35 days post injury reversed established mechanical allodynia and decreased hyperexcitability in dorsal horn neurons in contused SCI subjects (Crown et al., 2008). These behavioral studies implicate central sensitization in the development and maintenance of pain following SCI.

C) Glial effects of spinal cord injury

Endothelial cells are damaged as a result of the initial trauma, producing hemorrhage, initially in the gray matter, which spreads over time to the surrounding white matter. The damage compromises the blood–spinal cord barrier allowing for an infiltration of inflammatory cells (Bareyre and Schwab, 2003) and disruption in blood flow to the
surrounding areas, which results in oxygen and nutrient deprivation. The hemorrhage and resulting edema also contribute to secondary damage in the spinal cord, a process further exacerbated by the infiltration of immune cells. The immune response begins with the infiltration of neutrophils, monocytes, macrophages, microglia, and lymphocytes. This immune activation, while necessary following injury, is often detrimental (Schwartz, 2003). The effects of immune activation will be discussed in this section, with a focus on microglia and astrocytes.

D) Aberrant glial activation

In the CNS, glia is present in numbers greater than neurons. In addition, astrocytes and microglia express many of the same receptors and release many of the same factors (transmitters, reactive oxygen species, etc.) as neurons, marking them as prime candidates in the development of neuropathic pain and neuronal hyperexcitability following injury (Gwak and Hulsebosch, 2011a, 2011b; Jarvis, 2010; Pineau and Lacroix, 2007; Porter and McCarthy, 1997; Wang et al., 2009). In fact, a persistent, dysfunctional glial reaction, termed “gliopathy” (Hulsebosch, 2008), is thought to contribute to central pain following injury (Costigan et al., 2009).

Normally, microglia are present in a resting state, but the presence of an activating factor (e.g. interleukin (IL)-6, adenosine triphosphate (ATP), substance P, fractalkine) results in a morphological and functional change (Gwak et al., 2012; Hulsebosch et al., 2009; Soulet and Rivest, 2008). Microglia are activated within minutes of CNS injury and initially function to remove debris and damaged cells (Avellino et al., 1995; Carlson et al., 1998; David et al., 1990; Fleming et al., 2006; Kreutzberg, 1996). However, these cells can remain activated for weeks to months following injury and contribute to continued damage and cell death via the release of glutamate, ATP, calcitonin gene-related peptide (CGRP), pro-inflammatory cytokines, reactive oxygen species (ROS), nitric oxide (NO, and proteases (Aloisi, 2001; Carlson et al., 1998; Chao et al., 1995; Dong and Benveniste, 2001; Fleming et al., 2006; Kreutzberg, 1996; Lieberman et al., 1989; Priller et al., 1995; Rischke and Krieglstein, 1991; Stanley et al., 1994; Svensson et al., 1993). Several of these factors (e.g. increased extracellular glutamate, pro-inflammatory cytokines) have been associated with the development of pain via sensitization of sensory circuits (Bennett et al., 2000a, 2000b; Detloff et al., 2008).
Astrocytes also play a dual role in SCI, becoming activated within 24 h of SCI, and remaining active for months to years (Popovich et al., 1997; Schnell et al., 1999) following injury. In the initial stages of injury, astrocytes play a crucial role in restricting inflammation and protecting neurons (Bradbury et al., 2002; Hu et al., 2010). Moreover, astrocytes are responsible for glutamate homeostasis, thus preventing excitotoxicity under normal circumstances (Lepore et al. 2011). However, astrocytes migrate to the lesion area and form a scar, which is thought to impair axonal regeneration (Bradbury et al., 2002; Hu et al., 2010). Activation of astrocytes also leads to the activation of MAPK pathways. These pathways can activate the nuclear transcription factor, NF-κB (nuclear factor κB), which subsequently results in increased production of pro-inflammatory cytokines, chemokines, prostaglandins, NO, free radicals, neurotoxins, and excitatory amino acids (Hameed et al., 2010). These substances, as with those released from microglia, are pain-mediating and, in a cyclic manner, contribute to continued neuronal hyperexcitability following injury (Detloff et al., 2008; Hulsebosch et al., 2009; Keane et al., 2006; Scholz and Woolf, 2007; Vallejo et al., 2010).

The adverse consequences of glial activation include the development of neuropathic pain and cell death following SCI. Following injury, p38 MAPK is phosphorylated in microglia, astrocytes, and neurons (Crown et al., 2008). Activation of p38 MAPK, can phosphorylate NMDARs, leading to maintained hyperexcitability (Hulsebosch, 2008), and subsequently neuronal and glial death (Crown et al., 2006). Depending on the numbers of NMDARs being activated, this hyperexcitability can also lead to a loss of GABAergic cells, contributing to a loss of inhibitory tone and the development of neuropathic pain. Indeed, an increased expression of p-p38 is seen in animals experiencing neuropathic pain resulting from SCI, but not in their pain-free counterparts (Crown et al., 2006). Moreover, inhibiting the action of p38 MAPK reduces neuropathic pain symptoms in a contusion model (Crown et al., 2008). Similarly, administration of propentofylline (PPF), which blocks activation of both microglial and astrocytes, for the first 7 days after injury decreased dorsal horn neuron hyperexcitability and mechanical allodynia following a hemisection of the spinal cord (Gwak et al., 2008). Together, these results demonstrate a role of glia in the development of pain following SCI. Aberrant glial activation may also contribute to the loss of locomotor function after SCI. Liu et al. (2008) found that administration of IL-1β on days 1–3 following SCI leads to impaired locomotor recovery. IL-1β is expressed within 15 min following injury in a rodent model of SCI, and is one of the first cytokines released by activated microglia (Kim et al., 2006;
Experimental evidence indicates that IL-1β can induce apoptosis by phosphorylation of p38 MAPK, activating the pro-apoptotic caspase-3 cascade (Mika, 2008; Springer et al. 1999). Apoptosis, a programmed cell death, leads to the release of pro-inflammatory cytokines and induces migration of immune cells to the injury site (Desbarats et al., 2003; Kang et al., 1997; Letellier et al. 2010; Seino et al., 1997). Furthermore, cytokines, such as IL-1β, can activate both cytochrome c and caspase-9, which, together, lead to the activation of caspase-3 (Sekhon and Fehlings, 2001; Springer et al., 1999). Cell death initiates the release of pro-inflammatory cytokines, NO, and reactive oxygen species (Block and Hong, 2005; Cho et al., 2011; Min et al., 2003, 2004), causing further, sustained activation of microglia, enhancing the pro-inflammatory environment of the injured spinal cord, and affecting locomotor recovery after SCI.

1.9. AGMATINE

Agmatine, a novel neurotransmitter and endogenous ligand of imidazoline receptors, is formed by decarboxylation of L-arginine by arginine decarboxylase (ADC). It is stored in synaptic vesicles in a large number of neurons with selective distribution in the central nervous system (CNS). It is accumulated by uptake, released by depolarization, and its action is terminated by selective reuptake or enzymatic degradation by agmatinase (Reis and Regunathan, 2000). Based on several preclinical evidences, agmatine appears to be an endogenous neurotransmitter/neuromodulator in brain, involved in several physiological functions and has potential for new drug development.

1.10. METABOLIC PATHWAY

Two main branches of L-arginine metabolism are well established, one yielding citrulline and nitric oxide (NO), while the other yielding ornithine and polyamines (Fig. 4). A third branch of arginine metabolism, which produces agmatine (decarboxylated arginine), attracted the attention in several CNS disorder.

Agmatine had long been known to exist in bacteria and plants as a metabolic intermediate in the biosynthesis of polyamines. For many years, it was believed that ADC did not exist in higher organisms and, therefore, ornithine decarboxylase (ODC) provided the only enzyme for mammals to synthesize polyamines. In 1994, enzymatic decarboxylation of arginine was demonstrated to also occur in bovine brain to form agmatine; human form of ADC has now been cloned and characterized (Iyo et al., 2006; Zhu et al., 2004; Li et al., 1994).
In central nervous system, agmatine is catabolized to form putrescine by an enzyme agmatinase, (Iyer et al., 2002). In peripheral tissues agmatine is alternatively oxidized by diamine oxidase to form guanido-butanoic acid, which is readily excreted from the body.

Two brain-enriched enzymes, ADC and agmatinase, are the molecular engines that drive the newly named ‘agmatine pathway’ of polyamine biosynthesis agmatine pathway and the ODC pathway.

Fig.4. Synthesis and Metabolism of Agmatine (Halaris and plietz, 2007).

1.11. DISTRIBUTION OF AGMATINE

Agmatine is widely distributed in mammalian brain. In rat brain, agmatine has been detected by High performance liquid chromatography (HPLC) in concentrations (2.5–15.5ng mg-1) (Li et al., 1994; Raasch et al., 1995; Feng et al., 1997), which are comparable to those of the classic monoamine transmitters. The concentration of agmatine varies regionally, in stomach with the highest concentration followed by the aorta, small and large intestine, and spleen; it is found in lower concentrations in the lungs, vas deferens, adrenals, kidneys, heart, liver, skeletal muscle, brain and testes (Raasch et al., 1995).

In CNS the highest numbers of neurons that exhibit agmatine like immunoreactivity are present in the hypothalamus, rostral midbrain, amygdala and periventricular areas including the laterodorsal nucleus, locus coeruleus, and raphe nucleus dorsally, and the periaqueductal grey. In the lower brainstem, immunoreactivity is localized selectively to visceral relay nuclei, the nucleus tractus solitarii and the pontine parabrachial complex (Otake et al., 1998). Thus, the distribution of agmatine-containing neurons is concentrated in regions of the brain.
that subserve visceral and neuroendocrine control, processing of emotions, pain perception and cognition.

Fig.5. Distribution of agmatinergic cell bodies within the rodent brain (Otake et al., 1998).


1.12. REUPTAKE OF AGMATINE
Agmatine can be inactivated biologically in mammalian brain by uptake into synaptosomes (Sastre et al., 1997). However, unlike uptake of monoamines (Gilad and Gilad, 1991), uptake of agmatine is unaffected by blockade of the Na+-K+-ATPase pump or replacement of extracellular Na+. Competition analysis indicates that agmatine uptake is not mediated by transporters for noradrenaline, dopamine or 5-HT or by transporters for structurally related amino acids. Although agmatine is an amine and a precursor of polyamines, its uptake differs from that of polyamines (Gilad and Gilad, 1991). In synaptosomes, agmatine transport appears to be intimately related to Ca2+ fluxes, which is not surprising because at physiological pH, agmatine is positively charged and behaves as an organic cation. Uptake of agmatine is diminished by the nonselective Ca2+-channel blockers CoCl2, CdCl2 and verapamil, and is facilitated in a Ca2+-free buffer. If indeed there is transport through a Ca2+ channel its identity is not known. Uptake of agmatine is not blocked by nifedipine, ω-conotoxin, which indicates that blockade of uptake, does not involve L or N-type Ca2+ channels. Passage through ligand-gated Ca2+ channels might be of importance because agmatine can enter parasympathetic or invertebrate neurons via a nicotinic acetylcholine
receptor (Kuzirian, 1986) (although nicotine does not block uptake in rat brain synaptosomes). Conceivably, entry could be via other voltage and or ligand-gated Ca2+ channels such as the NMDA receptor (Yang and Reis, 1999). Thus, it is possible that agmatine might enter neurons by several routes, perhaps influencing different cellular compartments and functions, although this requires clarification.

1.13. RELEASE OF AGMATINE
Like other transmitters, agmatine is released from synaptosomes by depolarization in a Ca2+-dependent manner. This has been demonstrated following pre-incubation of rat brain slices or synaptosomes with guanido-[14C] agmatine or [3H]-putrescine, and exposure to 55 mM KCl. Such depolarization results in a significant Ca2+-dependent release of guanido-[14C] agmatine but not [3H] putrescine, which indicates that radioactive putrescine formed from agmatine by the action of agmatinase is not released (Reis, 1998).

1.14. MOLECULAR TARGETS / RECEPTORS OF AGMATINE

![Diagram of a purported synapse of an agmatine-containing neuron](image)

Fig.6. Schematic diagram of a purported synapse of an agmatine-containing neuron (Reis and Regunathan 2000).

Agmatine has a variable receptor affinity in the central nervous system (Fig. 6). It binds with high affinity to α2 adrenergic, imidazoline and inhibits NMDA receptors (Yang and Reis, 1999; Olmos et al., 1999; Li et al., 1994). In addition to these receptors, agmatine is irreversibly inhibits neuronal nitric oxide synthase (nNOS) and down regulates the inducible nitric oxide synthase (iNOS) (Demady et al., 2001; Galea et al., 1996). These interactions
might have important consequences with respect to the action of agmatine in brain (Halaris and Plietz, 2007).

1.15. PAIN
Agmatine dose dependently attenuated neuropathic pain in rodents (Onal et al., 2001). It also reversed the allodynia and hyperalgesia in the spinal and sciatic nerve ligation. The decrease in neuropathic pain by agmatine involves the attenuation of NMDA mediated Ca\(^{2+}\) release which results in hyperpolarisation and a reduction in neuronal excitability.

However, the exact mechanism is still uncertain agmatine is an endogenous ligand to Imidazoline receptors and several of its pharmacological effects are mediated through its interaction with imidazoline receptors.

1.16. IMIDAZOLINE RECEPTOR
Based on their binding properties, biochemical and functional effects this novel class of binding site has been subdivided into three distinct subtypes, the imidazoline I1, imidazoline I2 and imidazoline I3 binding sites (Eglen et al., 1998). The imidazoline I2 binding site has been further subdivided into the imidazoline I2A and imidazoline I2B subtypes based on the ability of amiloride to differentially displace [3H] idazoxan from different tissue types and in different species (Parini et al., 1996). In the last few years, highly selective imidazoline I2 binding site ligands have been developed including 2-BFI (2-(2-benzofuranyl)-2-imidazoline) and RS- 45041-190 (4-chloro-2-(imidazolin-2-yl)- isoindolene).

Accumulating evidence suggests that I2 receptors are involved in pain modulation. Ligands acting at I2 receptors are effective for tonic inflammatory and neuropathic pain but are much less effective for acute phasic pain. When studied in combination, I2 receptor ligands enhance the analgesic effects of opioids in both acute phasic and chronic tonic pain. Imidazoline I2 receptor ligands can attenuate the development of tolerance to opioid analgesia and inhibit drug withdrawal or antagonist precipitation induced abstinence syndrome in animals. Taken together, drugs acting on I2 receptors may be useful as a monotherapy or combined with opioids as an adjuvant for treating pain. Future studies should focus on understanding the relative efficacy of I2 receptor ligands and developing new compounds to fill the gap in intrinsic efficacy continuum of I2 receptors.
Selective imidazoline receptor agonists exhibit antinociceptive activity in animals (Paalzow 1974; Browning et al. 1982; Ossipov et al. 1989; Fairbanks et al. 2000; Dogrul and Uzbay, 2004). Antinociceptive activity from agmatine treatment could be expected because it binds to imidazoline. Although these preclinical evidences clearly demonstrated the antinociceptive potential of agmatine and imidazoline receptor. Their mutual interaction is not clearly investigated.

This study was planned to explore possible involvement of imidazoline receptor in action of agmatine in mice subjected to SCI.

2. Review of literature

Spinal cord injury often results in disability or loss of movement and sensation below the site of injury. Presently few treatments for spinal cord injury are available, however with less significant functional improvement.

Agmatine an endogenous amine that exists in mammalian brain and has been proposed as a novel neurotransmitter/neuromodulator (Reis and Regunathan, 2000). The distribution of agmatine-containing neurons is concentrated in regions of the brain that subserve visceral and neuroendocrine control, processing of emotions, pain perception and cognition and memory. Agmatine has been implicated in several biological processes like neuroprotection (Olmos et al., 1999), antinociception (Onal et al., 2004), convulsions (Bence et al., 2003), stress (Zhu et al., 2008), depression (Zomkowski et al., 2002) and anxiety (Lavinsky et al., 2003). It is interesting to note that agmatine also dose dependently attenuates neuropathic pain in rodents (Onal et al., 2002). Its intraperitoneal administration reversed long-lasting hypersensitivity, hyperalgesia and allodynia induced by neuropathic pain (Laughlin et al., 1997; Vanderah et al., 1996; Fairbanks et al., 2000). Further, agmatine also attenuated the pain associated with diabetic neuropathy (Fairbanks et al., 2000; Karadag et al., 2003; Onal et al., 2003; Wu et al 2003). Its peripheral administration enhanced morphine analgesia dose-dependently in neuropathic rats (Kolesnikov et al., 1996; Yes-ilyurt and Uzbay, 2001). Moreover, systemically administered agmatine significantly reduces the mechanical and thermal hyperalgesia as well as allodynia in neuropathic mice caused by spinal cord injury.

Agmatine binds to several target receptors such as imidazoline, N-methyl-D-aspartate (NMDA), nicotinic cholinergic (NIC), α2-adrenergic, serotonin receptors and inhibits nitric oxide synthase. Agmatine is co-localised with imidazoline receptor in several brain areas.
Moreover, several pharmacological effect of agmatine in mediated through imidazoline receptors. The role of imidazoline receptor in nociception is fairly well established. Imidazoline binding sites have currently attracted attention in nociception as well as drug addiction (Lewis et al., 2000, Lewis et al., 2007). Moreover, the brain structures that is involved in the drug abuse and pain perception including hypothalamus, hippocampus, amygdala, etc., are rich in imidazoline binding sites and its endogenous ligands (Ruggiero et al., 1998). Imidazoline binding sites are a family of unique non-adrenergic high-affinity binding sites that exist in three major subclasses (I1, I2, and I3) based upon their ligand selectivity, subcellular distribution, and physiological functions (Michel et al, 1989., Eglen et al, 1989).

We found that, several imidazoline receptor agonists including moxonidine, clonidine, and antagonist idazoxan, efaroxan possess antinociceptive activity (Diaz et al, 1997, Fairbanks and Posthumus, 2000).

In view, of these preclinical evidences we hypothesized that agmatine induced functional recovery of spinal cord injury might be mediated through imidazoline receptors.

3. Objective
Agmatine, a putative neurotransmitter have been demonstrated to modulate the nociceptive behaviour in rodents. In addition, it also improves the functional recovery in animal subjected to SCI however mechanism is not clearly understood. The objective of present study was, to investigate the possible involvement of imidazoline receptors in agmatine induced functional recovery in animals subjected to spinal cord injury.

Plan of Work
The study was planned to be carried out on following lines:
1. Dose specific study of agmatine in SCI.
2. Effect of imidazoline agonist and antagonist on agmatine in SCI.

4. MATERIAL AND METHOD
4.1 Animals
Adult male Swiss-albino mice (22–27 g) were grouped house and given free access to food (Trimurty Feeds, Nagpur, India), and drinking water. They were maintained on a 12 h light/dark cycle, in controlled temperature (25 ± 2°C) and relative humidity (50–70%). All
experimental procedures were approved and carried out under strict compliance with Institutional animal and ethical committee according to guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests; Government of India; New Delhi.

### 4.2 Drugs

Following drugs were used.

1. Agmatine Sulphate
2. Clonodine (I₁ Imidazoline agonist)
3. Efaroxan (I₁ Imidazoline antagonist)
4. Moxonidine (I₂ Imidazoline agonist)
5. Idazoxan (I₂ Imidazoline antagonist)

Agmatine, moxonidine and efaroxan, idazoxan were obtained from Sigma chemicals, St. Louise, USA. All drugs were dissolved in saline just before the experiments and administered via intraperitoneal route (i.p.) in a volume of 1 ml/kg. Normal saline (0.9 % NaCl) was used as control.

### 4.3 Surgery procedure for ESCI

The method described and validated by Farooque (2000), was employed for producing ESCI in mice. Mice were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) injected i.p. The thoraco-lumbar vertebral region was located and using the intrascapular space as a reference point, the skin and subcutaneous tissues in the thoracic T10–12 region were incised. The paravertebral muscle fascia was penetrated, and muscles were peeled laterally using blunt dissection forceps.

The T10–12 lamina was exposed, and a total laminectomy was performed without damaging the dura mater. SCI was achieved in each mouse by compressing the exposed spinal cord with a 5 g weight for 30 s. In sham-operated mice, the above-mentioned procedure was carried out except that spinal cord compression was not performed. The incision was sutured layer to layer using chromic catgut sutures.

In the post-operative period, mice were treated with gentamicin (40 mg/kg) twice daily during the first 3 days as prophylaxis against urinary tract infection. Mice were also injected daily with 1 ml lactated ringer subcutaneously for a period of 10 days. Drinking water,
softened chow and regular pellets were provided ad libitum in the cages. Bladders were emptied manually twice a day until bladder function returned to normal.

4.4 Assessment of locomotor recovery by Hindlimb motor function scoring system for mouse

“Hind limb motor function scoring system” was employed in the present study (Farooque, 2000; Basso et al., 2006, Pajoohesh-Ganji et al., 2010, Ung et al., 2007). This test includes monitoring the ability of mice to walk on bars of different widths. It permits detection of minor deficits that may be otherwise missed in open field and other test methods. The test is easy to perform and reproducible in our laboratory conditions. Individual animals were allowed to freely explore in open and well illuminated arena (0.7 - 0.9 m), and observed for 1 min. Parameters like the movements in the hip, knee, and ankle joints, plantar placement, coordination between forelimbs and hindlimbs as well as weight bearing capacity were carefully observed and the performance of the mouse was scored accordingly (Table 1). Briefly, the score 0 was given to the animals not showing any noticeable movement. The scores 1, 2 or 3 were given to the animals showing barely visible movement at any hindlimb joint (hip, knee or ankle), movement of one or more hindlimb joints in one or both limbs, or animals showing alternate stepping and forward propulsive movements of the hind limbs, but no weight bearing, respectively. Scores 4 or 5 were given to the animals showing ability to bear weight on their hind limbs and could walk with some deficit, or no deficit, respectively. The animals were scored 6, 7, 8, 9 or 10 if they were able to walk on bars of width 2, 1.5, 1, 0.7 or 0.5 cm, respectively.

During the study, mortality was observed in some mice (<9%) across the different groups, data from such animals were not considered for the statistical purpose.

Table No: 1 Hindlimb motor function scoring system for mouse.

<table>
<thead>
<tr>
<th>Characteristics feature</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No movement of the hindlimbs</td>
<td>0</td>
</tr>
<tr>
<td>Barely perceptible movement of any hindlimb joints like hip, knee or ankle</td>
<td>1</td>
</tr>
<tr>
<td>Brisk movements at one or more hind limb joints like hip, knee, or ankle in one or both limbs but no co-ordination</td>
<td>2</td>
</tr>
<tr>
<td>Alternate stepping and propulsive movements of hind limbs but no weight bearing</td>
<td>3</td>
</tr>
<tr>
<td>Weight bearing and can walk with some deficit</td>
<td>4</td>
</tr>
<tr>
<td>Normal walking</td>
<td>5</td>
</tr>
<tr>
<td>Normal walking and can walk on a 2 cm wide bar</td>
<td>6</td>
</tr>
</tbody>
</table>
Can walk on a 1.5 cm wide bar | 7
Can walk on a 1 cm wide bar | 8
Can walk on a 0.7 cm wide bar | 9
Can walk on 0.5 cm wide bar | 10

Locomotor recovery was assessed using “Hindlimb motor function scoring system” proposed by Farooque (2000). Individual animals were allowed to explore freely in open and well-illuminated arena for 1 min. The above characteristic features were observed and scored accordingly from 0 to 10.

4.5. Treatment Protocol

1. Effect of agmatine on SCI

After spinal cord injury animal were injected with different doses of agmatine (2.5, 5, 10 mg/kg, i.p.) daily for 14 days between 9.00 hrs to 12.00 hrs. Animals were observed for motor hindlimb score on day 14 on post injury.

Depending upon the results of this experiment effective and subeffective dose of agmatine were find out and used for combined studies.

2. Effect of imidazoline receptors agonist on effect of agmatine in SCI

In separate group, animal exposed to SCI were injected with imidazoline receptor agonist clonidine (0.1 mg/kg) or moxonidine (0.5 mg/kg) and subeffective dose of agmatine daily for 14 days between 9.00 hrs to 12 hrs and observed for motor hindlimb score on day 14 of post surgery.

3. Effect of imidazoline receptors antagonist on effect of agmatine in SCI

In separate group, animal exposed to SCI were injected with imidazoline receptor antagonist efaroxan (1 mg/kg) or idazoxan (3 mg/kg) daily for 14 days 15 min. before agmatine between 9.00 hrs to 12 hrs and observed for motor hindlimb score on day 14 of post surgery.

The doses of agmatine and imidazoline receptor agonist or antagonist were selected on the basis of available literature and confirms in our preliminary findings.

4.6. Statistical analysis

All data were presented as the mean ± SEM. The results of locomotor recovery in spinal cord injured mice were analyzed by one-way ANOVA followed by Dunnett's test. Effects of
combinations were analyzed by a one-way ANOVA followed by post-hoc Newman’s–Keuls test. Results of statistical tests with P < 0.0001 were considered significant.

5. RESULTS

1) Effect of ESCI on locomotor function:
Experimental spinal cord injury resulted in complete loss of movement of hindlimb in exposed animal. The data of 24 hrs post surgery showed significant decreased in the locomotor score (2.5 + 5) as compared to saline treated animals (10+2).

The locomotor score was slightly improved on day 14 of experimental spinal cord injury compared to day 1 (F (2, 14) = 41.09, P<0.05) but was significantly less as compared to normal animals.

![Graph showing locomotor function score](image)

**Fig.7.** Effect of ESCI on locomotor function in mice. The locomotor recovery was monitored by motor function score (MFS) of mice on day 14 of ESCI.

Data were represented as mean of MFS+ SEM of 5 mice in each group. Data was analysed by one-way ANOVA followed by Dunnett’s multiple comparison test as a post hoc analysis, *p<0.05 vs control.

2) Effect of agmatine on spinal cord injury
Chronic treatment of agmatine (2.5, 5, 10 mg/kg, i.p.) starting from day 1 following ESCI progressively improved the locomotor score in mice compared to saline treated animal.

Application of Dunnett’s multiple comparison test revealed significant recovery of motor function on day 14 of post-surgery by agmatine treatment (5, 10 mg/kg, i.p.). However, its lower dose (2.5 mg/kg, i.p.) was ineffective (F (4, 20) = 191.7, P <0.05).
Fig. 8. Effect of agmatine treatment on locomotor score in spinal cord injured mice.

The locomotor recovery was monitored by motor function score (MFS) on day 14 of ESCI. Data were represented as mean of MFS + SEM of 5 mice in each group. Data was analysed by one-way ANOVA followed by Dunnett’s multiple comparison test as a post hoc analysis, *p<0.05 vs control.

3) Effect of I₁ agonist clonidine and agmatine combination in SCI

Daily administration of subeffective dose combination of agmatine (2.5 mg/kg, i.p.) and I₁ agonist clonidine (0.1 mg/kg, i.p.) significantly improved the motor score as compared to their individual effect. The doses of agmatine and clonidine did not have effect on functional recovery of animal subjected to ESCI (F (4, 20) = 282.6, P<0.05).

Fig. 9. Effect of agmatine (2.5 mg/kg, i.p.) and clonidine (0.1 mg/kg, i.p.) and their combination on locomotor recovery in spinal cord injured mice. Each mouse was subjected to the motor function score (MFS) test on day 14.
Data were represented as mean of MFS+ SEM for 5 mice in each group. Data were analysed by one-way ANOVA followed by Newman’s-Keuls multiple comparison test, *p<0.05 vs control.

4) Effect of I$_2$ agonist moxonidine and agmatine combination in SCI

Chronic administration of subeffective dose combination of agmatine (2.5 mg/kg, i.p.) and I$_1$ agonist moxonidine (0.5 mg/kg, i.p.) significantly improved the motor score as compared to their individual effect. The doses of agmatine and moxonidine did not have any effect on functional recovery of ESCI mice (F (4, 20) = 288.5, P<0.05).

![Graph showing effect of agmatine and moxonidine combination](image1.png)

Fig.10. Effect of agmatine (2.5 mg/kg, i.p.) and moxonidine (0.5 mg/kg, i.p.) and their combination on locomotor recovery in spinal cord injured mice. Each mouse was subjected to the motor function score (MFS) test on 14 day.

5) Effect of I$_1$ antagonist efaroxan on agmatine induced functional recovery in SCI

Pretreatment of animal with I$_1$ antagonist efaroxan (1 mg/kg, i.p.) before agmatine (10 mg/kg, i.p.) for day 14 significantly blocked the effect of agmatine on locomotor recovery in animal subjected to ESCI. The dose of efaroxan did not have any effect on ESCI (F (4, 20) = 41.76, P<0.05).
Fig.11. Effect of agmatine (10 mg/kg, i.p.) and efaroxan (1 mg/kg, i.p.) and their combination on locomotor recovery in spinal cord injured mice. Each mouse was subjected to the motor function score (MFS) test on 14 day.

Data were represented as mean of MFS+ SEM for 5 mice in each group. Data were analysed by one-way ANOVA followed by Newman’s-Keuls multiple comparison test, *p<0.05, **p<0.01 vs control.

6) Effect of I₁ antagonist idazoxan on agmatine induced functional recovery in SCI

Treatment of animal with I₂ antagonist idazoxan (3 mg/kg, i.p.) before agmatine (10 mg/kg, i.p.) for day 14 significantly attenuated the effect of agmatine on locomotor recovery in animal subjected to ESCI. The dose of idazoxan used in the present study did not have any effect on ESCI (F (4, 20) = 410.8, P<0.05).

Fig.12. Effect of agmatine (10 mg/kg, i.p.) and idazoxan (3 mg/kg, i.p.) and their combination on locomotor recovery in spinal cord injured mice. Each mouse was subjected to the motor function score (MFS) test on day 14.
Data were represented as mean of MFS+ SEM for 5 mice in each group. Data were analysed by one-way ANOVA followed by Newman’s-Keuls multiple comparison test, *p<0.05, **p<0.01 vs control.

6. DISCUSSION

In the present study, we employed compression method for inflicting SCI since it mimics the typical human injury, wherein compression is caused by bony fragments or extruded disc material (Farooque, 2000). While experimental injury inflicted at the T10–12 level resulted in hindlimb muscle paralysis, considerable recovery was noticed over a period of 14 days.

In the present study, we employed compression method for inflicting SCI since it mimics the typical human injury, wherein compression is caused by bony fragments or extruded disc material (Farooque, 2000). While experimental injury inflicted at the T10–12 level resulted in hindlimb muscle paralysis, considerable recovery was noticed over a period of 14 days. In the present study, we employed compression method for inflicting SCI since it mimics the typical human injury, wherein compression is caused by bony fragments or extruded disc material (Farooque, 2000). While experimental injury inflicted at the T10–12 level resulted in hindlimb muscle paralysis, considerable recovery was noticed over a period of 14 days.

In the present study, the motor function score scale suggested by (Farooque, 2000) was used to study the walking pattern of SCI in mice. The walking activity of each mouse was graded on the scale of 0–10. Since the test consists of observing the rat walking on the horizontal bars, minor deficits that are not easily detected in open field test can be readily revealed. In the present study, mice subjected to ESCI showed significant locomotor recovery within 14 days. aCSF or saline did not show any effect as compared to that of non-treated SCI mice. However, the observed change in the vehicle treated mice is because of natural healing process and not due to vehicles (aCSF or saline). The improvement in the hindlimb function was observed with respect to movements of hindlimb joints and weight bearing. These results are in accordance with the previous findings where improved motor function was noticed in vehicle-treated SCI mice in similar time frame (Farooque, 2000; Isaksson et al., 2005).

It is now well accepted that imidazoline receptors plays a potential role in mechanism and modulation of neuropathic pain signalling. Since, agmatine exhibits antinociceptive action against neuropathic pain and shows affinity for imidazoline receptors we investigated the involvement of imidazoline receptors in agmatine induced functional recovery in SCI.

We found that, the effect of agmatine on spinal cord injury was significantly potentiated by I₁ agonist clonidine and moxonidine. In contrast, it was completely blocked by pre-treatment of animals with I₁ antagonist efaroxan and I₂ antagonist idazoxan. These results confirm our hypothesis that, the beneficial effect of agmatine was mediated at least partly through imidazoline receptors.

Imidazoline binding sites have currently attracted attention in nociception. Moreover, the brain structures involved in the drug abuse and pain perception including hypothalamus,
hippocampus, amygdala, etc., are rich in imidazoline binding sites and its endogenous ligands.

Imidazoline binding sites were a family of unique non-adrenergic high-affinity binding sites that exist in three major subclasses (I1, I2, and I3) based upon their ligand selectivity, sub cellular distribution, and physiological functions. The I2 binding sites (I2A and I2B) are allosteric and were located on monoamine oxidases. Furthermore, the involvement of imidazoline I1/I2 endogenous ligands like agmatine and B-carboline in nociception is now fairly well established.

It is important to note that most of the agents used in present study shows considerable affinity towards α2-adrenergic receptors. Agmatine is a neurotransmitter with multireceptive affinity. It acts as antagonist of NMDA and NOS inhibitors. Thus their involvement in observed effect of agmatine cannot completely ruled out.

7. SUMMARY AND CONCLUSION
Agmatine, a putative neurotransmitter has been demonstrated to modulate the nociceptive behavior in rodents. In addition, it also improves the functional recovery in animal in animal subjected to SCI however mechanism is not clearly understood. Agmatine is co-localized with imidazoline receptor in several brains areas. The present study examined the involvement of imidazoline receptor on functional recovery exhibited by agmatine following spinal cord injury. Compression spinal cord injury was developed by placing 5 g weight for 30 sec at thoracic vertebra 10-12 segment. Experimental spinal cord injury resulted in complete loss of movement of hindlimb in exposed animal. Animal treatment significantly improved locomotor recovery of the animals subjected to SCI. Imidazoline agonist clonidine and moxonidine potentiated while, Imidazoline antagonist idazoxan and efaroxan blocked effect of agmatine in SCI.

In conclusion, the present study suggests that, chronic agmatine treatment showed locomotor recovery in SCI animal and this effect was possibly mediated through imidazoline receptors.

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