Scuola Internazionale Superiore di Studi Avanzati - Trieste

ROLE OF ENDOCANNABINOID SYSTEM AND ACID SENSING ION CHANNELS IN SPINAL LOCOMOTOR CIRCUITS DURING PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

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DECLARATION

During my course tenure between November, 2012 and December, 2016 at International School for Advanced Studies, Trieste, Italy, I have carried out the following studies as a part of my thesis:

- Veeraraghavan P, Nistri A. (2015). Modulatory effects by CB1 receptors on rat spinal locomotor networks after sustained application of agonists or antagonists. Neuroscience. 303, 16-33.

- Veeraraghavan P, Dekanic A, Nistri A. (2016). A study of cannabinoid-1 receptors during the early phase of excitotoxic damage to rat spinal locomotor networks in vitro. Neuroscience. 333, 214-228

- Mazzone GL, Veeraraghavan P, Gonzalez-Inchauspe C, Nistri A, Uchitel OD. (2017). ASIC channel inhibition enhances excitotoxic neuronal death in an in vitro model of spinal cord injury. Neuroscience. 343, 398-410 (in this study my contribution was collecting electrophysiological data, analyzing them and manuscript writing)

I have also contributed electrophysiological recording and data analysis to the following study that is not included in the current thesis:

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1. LIST OF ABBREVIATIONS

Δ^9 THC	Δ^9Tetrahydrocannabinol
2AG	2-Arachidonoyl glycerol
5HT	5-Hydroxytryptamine
ABHD6	Serine hydrolase α-β-hydrolase domain 6
ABHD12	Serine hydrolase α-β-hydrolase domain 12
AEA	Anandamide
APV	Aminophosphonovalerate
ASIA	American Spinal Injury Association
ASIC	Acid sensing ion channel
CaMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CB1R	Cannabinoid 1 receptor
CB2R	Cannabinoid 2 receptor
cDNA	Complementary DNA
CNS	Central nervous system
CNQX	6-Cyano-7-nitroquinoxaline-2, 3-dione
CPG	Central pattern generator
CSF	Cerebrospinal fluid
DR	Dorsal root
EAE	Experimental autoimmune encephalomyelitis
ECB	Endocannabinoids
FAAH	Fatty acid amide hydrolase
FL	Fictive locomotion
GABA	Gamma-aminobutyric acid
GPCR	G-protein coupled receptor
KA	Kainate
LTD	Long term synaptic depression
LTP	Long term synaptic potentiation
MAGL	Monacylglycerol lipase

MAP	Mitogen-activated protein
MCAO	Middle cerebral artery occlusion
MS	Multiple sclerosis
mGluR1	Metabotropic glutamate receptor-1
MPG	Motor pattern generator
mRNA	Messenger RNA
NADA	N-arachidonoyldopamine
NAT	N-acyltransferase
NMDA	N-methyl D-aspartate
NO	Nitric oxide
NTSCI	Non-traumatic spinal cord injury
NAc	Nucleus accumbens
PARP1	Poly ADP-ribose polymerase enzyme 1
PcTx1	Psalmotoxin
PD	Parkinson's disease
PE	Phosphatidylethanolamine
PFC	Prefrontal cortex
PLC	Phospholipase C
PLD	Phopholipase D
PM	Pathological medium
PKA	Protein kinase A
PKD2L1	Polycystin 2 like 1, transient receptor potential cation channel
PNS	Peripheral nervous system
ROI	Region of interest
SAMHSA	Substance abuse and mental health services administration
SCI	Spinal cord injury
SEM	Standard error mean
SN	Sensory neurons
TRPV1	Transient receptor potential cation channel subfamily V member 1
TSCI	Traumatic spinal cord injury
VR	Ventral root

2. ABSTRACT

Background: Mammalian spinal cord can generate well-coordinated locomotor activity called fictive locomotion in the absence of any higher brain center input or of rhythmic sensory feedback. This activity clearly provides evidence for the central pattern generator (CPG) that produces the locomotor rhythm. Such CPG consists of glutamatergic excitatory and glycinergic and GABAergic inhibitory interneuronal connections that finally excite or inhibit the motoneuronal pools. Many factors including fast (ion channels) and slow (modulatory G-protein coupled receptors; GPCRs) processes control these neuronal pools to act rhythmically. These factors are perturbed following spinal cord injury (SCI) in the early and late phases. The present study addresses the role of a few modulatory processes, namely acid sensing ion channels (ASICs) and cannabinoid 1 receptors (CB1Rs) at both initial and the late phases of injury.

Objectives: Recent evidence has shown that the deletion of ASICs slows down the progression of disease in ischemic conditions, whereas the same protocol increases seizure severity. CB1R activation or deletion also results in neuroprotective or toxic mechanisms. In order to understand the importance of ASICs and CB1Rs in the spinal locomotor circuits, it is crucial to analyze them in physiological and pathological conditions. To investigate this issue, both organotypic slice culture and an in vitro rat spinal cord model were used. With the latter, fictive locomotion can be recorded from the ventral roots of the lumbar region for a time window of 24 h. Network parameters like synaptic transmission, fictive locomotion and disinhibited bursting provide information to explain the physiological modifications and pathological severity after excitotoxicity caused by transient kainate (KA; glutamate analog) application. Drugs that modulate CB1Rs and ASICs may supply evidence for the role of these processes in fictive locomotion.

Results and conclusion: Our results show that the CB1R activation or block for 24 h diminished the locomotor rhythm. In particular, CB1R pharmacological block completely depressed both dorsal root (DR) and chemically evoked fictive locomotion. This depression was amplified following KA treatment. Furthermore, a limited

neuroprotection was observed after CB1R agonists (anandamide; AEA or 2-arachidanoyl glycerol; 2AG) and an endogenous cannabinoid uptake inhibitor. These results allow us to propose the innate activity of CB1R (that is well preserved) to be important after KA mediated excitotoxicity, while any neuroprotective role might come in later phases after injury.

A low concentration of KA that can induce a borderline injury elicited rapid glutamate release combined with proton discharge (acidification) in the organotypic SCI model. In response to this challenge, the ASIC subtypes (1a, 1b, 2a and 3) mRNA levels were found to be elevated after 24 h. Both neuronal numbers and network activity were highly depressed after application of ASIC pharmacological blockers that intensified the consequences of KA treatment. These results indicate that moderate acidification might be beneficial for the recovery (or limitation) of KA mediated excitotoxicity. Hence, this study demonstrates that both ASICs and CB1Rs activity are important in the early phase of experimental SCI in vitro. Their pharmacological modulation can outline future strategies for neuroprotection.

3. MAMMALIAN SPINAL LOCOMOTOR NETWORK

Locomotion is an inherent and coordinated motor activity that is the result of coupling between the neural dynamics (sensory feedback and rhythm control) and the body dynamics (musculoskeletal function). It is neither a simple reflex activity which is stereotypically driven by sensory stimulation in a repeated manner, nor a skilled activity, like playing any sport, that requires learning and complex coordination of muscles in a non-repeated fashion (Grillner and Wallen, 1985; Marder and Bucher, 2001; McLean and Dougherty, 2015).

Locomotion is a fundamental skill that includes gait transition, control of speed and direction by the nervous and musculoskeletal systems. Animal locomotion is characterized by rhythmic activity that includes various forms such as swimming, crawling, walking, flight, hopping, brachiation and burrowing (Ijspeert, 2002). Despite the diversity in types, the general organization of vertebrate locomotor circuit is well conserved. The basic rhythmic activity is controlled by the interaction of three components, sometimes called the motor pattern generator (MPG): (1) spinal central pattern generators (CPGs), (2) sensory feedback and (3) descending supraspinal control (Frigon, 2012; McLean and Dougherty, 2015).

Simplest movements are involuntary reflexes (knee jerk, pupil dilation), that are stereotyped and graded responses to sensory input, and have no threshold except that the stimulus must be large enough to activate the relevant sensory input pathway. Fixed action patterns are also involuntary and stereotyped, but they typically have a stimulus threshold that must be reached before the action could be triggered, and are less graded and more complex than reflexes. Rhythmic motor patterns like walking, scratching, or breathing are stereotyped and complex, but these rhythms are subjected to continuous voluntary control by higher and lower locomotor centers. Directed movements like reaching are voluntary and complex, but generally they are neither stereotyped nor repetitive. Rhythmic motor patterns which are complex (unlike reflexes) yet stereotyped (unlike directed movements) and, by definition, repetitive (unlike fixed action patterns)

comprise a large part of basic animal behavior. Due to its behavioral importance and experimental advantage, rhythmic motor pattern generation has been studied extensively in a wide range of animals (Hooper, 2001).

3.1. Anatomical view – a general outline

Spinal cord is a long and relatively thin neural structure. It is enclosed in the vertebral column (a protective bone structure). It is comprised of the white (ascending and descending myelinated fibers) and the butterfly-shaped gray matter (unmyelinated cells that are involved in reflex and network activity) tissues. In the mid, there is a central canal that contains cerebrospinal fluid (CSF) circulating to and from the ventricles in brain. In the periphery, three layers (dura, arachnoid, and pia) surround the entire cord for protection (Kandel et al., 2000).

In humans, the length of spinal cord varies between 43 and 45 cm comprising 31 different segments (differing between species) that are grouped as 8 cervical, 12 thoracic, 5 lumbar, 5 sacral, and 1 coccygeal segments (Netter, 2006). In rats there are 8 cervical, 13 thoracic, 6 lumbar, 4 sacral, and 3 coccygeal segments (Molander et al., 1984; Molander et al., 1989). From every segment a pair (right and left) of spinal nerves are projected. And it is supplied by a single ventral spinal artery and two dorsal spinal arteries. Spinal nerves comprise two different types: 1. sensory nerve roots (a group of afferent 6–8 rootlets), and 2. motor roots (a group of efferent 6–8 rootlets; Netter, 2006; Nógrádi and Vrbová, 2006).

As mentioned before, each spinal cord segment sends out a pair of nerves that innervates different skin areas and sets of muscles. In brief, cervical segments provide innervations to and from muscles involved in respiration, head, neck, and arm movements. Thoracic segments provide motor control of the paws, chest, back, and abdominal muscles whereas the lumbar and sacral segments are generally associated with the control of muscles involved in locomotion, micturition, bowel, and reproductive functions (Guertin, 2013).

3.1.1. Afferent and efferent pathways

Afferent pathways carry information from sensory receptors to the brain regions. The primary afferent neurons synapse on projection neurons (located in spinal gray matter) which join the ascending tracts to the brain. The function of a particular pathway is determined by the type of primary afferent neurons and the type of projection neurons that synapse in the brain. In general, these pathways can be categorized into three broad functional types: 1. Conscious discrimination/localization (e.g. pain, temperature, discriminative touch) requires a specific ascending spinal pathway to the contralateral thalamus which, in turn, sends an axonal projection to the cerebral cortex, 2. Affective related (emotional & alerting behavior) information involves ascending spinal pathways directed to the brainstem, 3. Subconscious sensory feedback for posture/movement control involves ascending spinal pathways principally to the cerebellum or brainstem nuclei that project to the cerebellum (Drake et al., 2009).

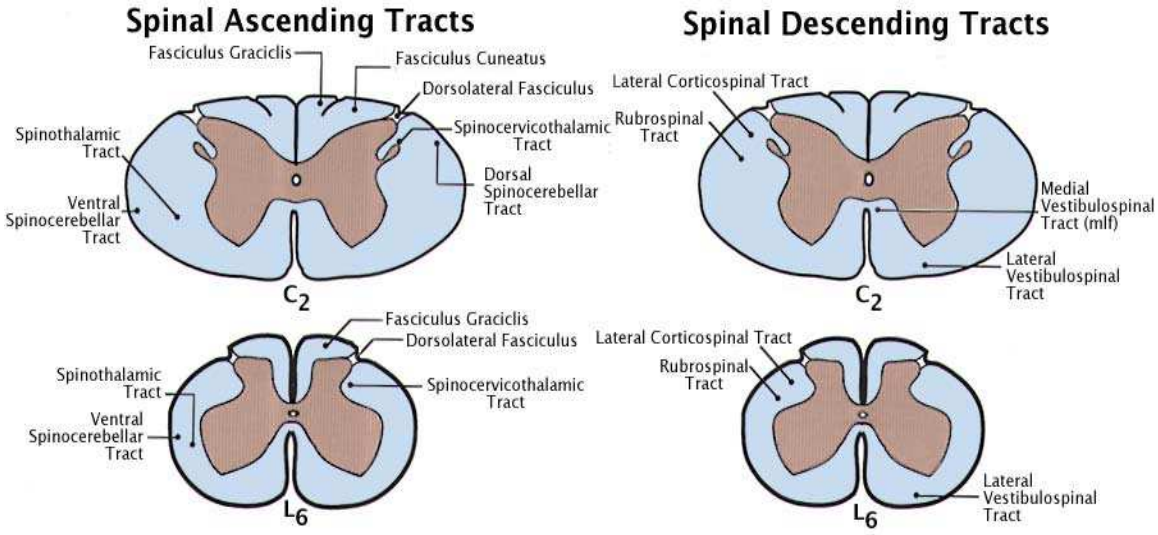


Fig 1: Spinal afferent and efferent tracts (modified from Fletcher, 2006)

Brain axons travel in descending tracts of the spinal white matter. They arise from various locations in the brain and synapse primarily on interneurons. By synapsing on interneurons, descending tracts regulate spinal reflexes, excitability of efferent neurons (for posture and movement) and spinal projection neurons (Knierim, 1999). In certain conditions, they also affect axon terminals of primary afferent neurons, blocking release of neurotransmitter (presynaptic inhibition; Johnson, 2006).

3.1.2. Cytoarchitecture of rat spinal cord

The structural organization of rat spinal cord is similar to the feline one. There is no much difference between adult and newborn rats except less distinct borders of laminar regions. The distribution of cells and fibers within the gray matter of the spinal cord exhibits a pattern of lamination. The cellular pattern of each lamina is composed of various sizes or shapes of neurons classified into 10 layers by Rexed. Laminae I to IV is in the dorsal horn and are concerned with exteroceptive, whereas laminae V and VI are concerned primarily with proprioceptive sensations and act as a relay between the periphery to the midbrain and the cerebellum. Lamina VII is an intermediate zone and acts as a relay between muscle spindles to midbrain and cerebellum which innervate neurons in autonomic ganglia and laminae VIII-IX comprise the ventral horn and contain mainly motor neurons. The axons of these neurons innervate mainly skeletal muscle. Lamina X surrounds the central canal and contains preganglionic neurons and neuroglia (Dafny, 1999).

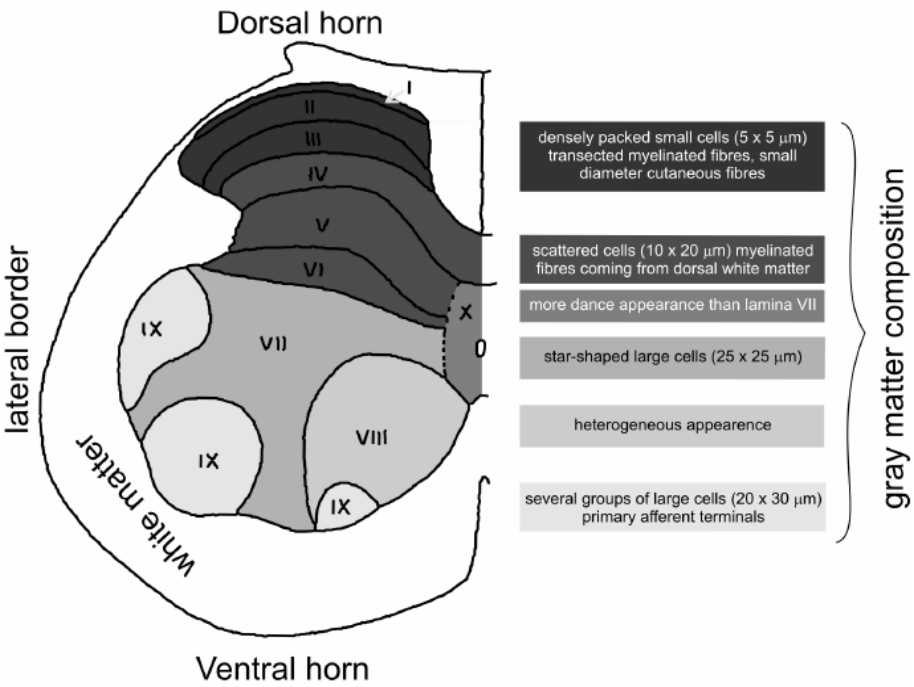


Fig 2: Cross sectional view of lumbar 5 region (modified from Ostroumov, 2006)

3.2. Functional organization

As mentioned before, white matter tracts essentially serve as 'relay region' for signals that carry descending motor control and ascending sensory-related information between the brain and spinal cord. The gray matter, however is an 'action execution' region, where neurons for local reflex arcs and complex circuits are present (Guertin and Steuer, 2009).

3.2.1. Spinal reflex

Classical reflexes involve simple reflex arcs or pathways. A simple reflex typically leads to a rapid, predictable, repeatable, stereotyped, and involuntary motor reaction in response to the given stimulus. Mostly, reflexes are mediated within one or two spinal segments, by relatively minimal neural pathways (interneurons and motoneurons) that generally involve either one (monosynaptic), two (disynaptic), or more (polysynaptic) synapses according to the stimulus threshold. These reflexes are either autonomic (related with internal organs) or somatic (related with skeletal muscle responses) origin.

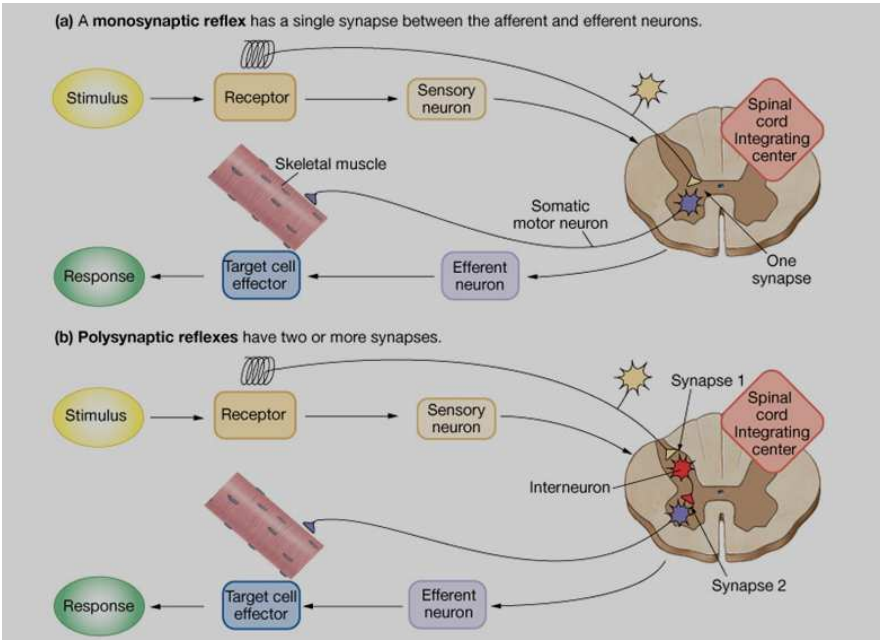


Fig 3: A simple illustration of reflex activity (modified from http://www.easynotecards.com/notecard_set/21865)

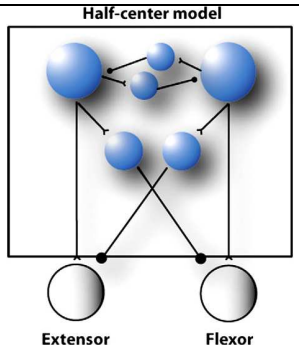
Monosynaptic reflex arc (Ia) is one of the somatic reflex pathways. This is a simple and fast reflex mediated by primary afferent (Ia) inputs from muscle spindles typically

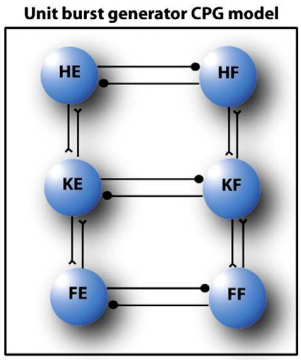
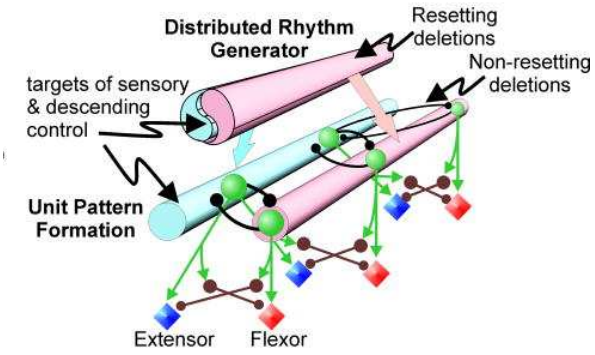
activated by muscle stretch (for example: patellar reflex). The Ia afferent input enters the dorsal spinal cord and establishes monosynaptic connections with homonymous alpha-motoneurons in the ventral horn area. A polysynaptic reflex has more than one synapse and one or more interneurons in its reflex arc. All reflexes except stretch reflex are polysynaptic (for example: flexor and plantar reflex; Lloyd and McIntyre, 1955; Perl, 1962).

3.2.2. Central pattern generator

Extensive work by Sherrington and Graham Brown initiated the idea of potential existence of a spinal neuronal network for locomotion. Then many studies have emerged proposing various model of network operation. Table 1 lists the major models of CPG (data extracted from Guertin, 2009).

Table 1: Different conceptual models of CPG

Model	Highlights
Half center model (Brown, 1911, 1914)	 Rhythm is generated by reciprocal activation and inhibition of motoneurons.
Miller and Scott model (Miller and Scott, 1977)	Renshaw cells rather than fatigue are responsible for the alternation between flexion and extension.
Ring model (Székely et al., 1969; Gurfinkel and Shik, 1973)	CPG contains a closed chain of at least five groups of neurons (2 pure extensors, 2 pure flexors and 1 bifunctional) that project to the motoneurons either directly or through specific interneurons to produce rhythms.

Flexor burst generator model (Pearson and Duysens, 1976)	It is a asymmetrical model proposing that the rhythmic excitatory drive is from the flexor burst generator to populations of flexor motoneurons. Then the burst generator would inhibit, via an inhibitory interneuron, resulting in the extensor movement.
Unit burst generator model (Grillner and Zangger, 1974; 1979)	 A symmetrically organized generator in the spinal cord that can produce basic pattern of motor commands in the absence of any peripheral input.
Two-level (multi level) half center model (Perret and Cabelguen, 1980; McCrea and Rybak, 2008)	 Two half-centers (extensor and flexor ones) would send motor commands to bifunctional motoneurons. A rhythm generator and a pattern generator are functionally separated since they are two distinct entities that can be independently and spontaneously changing. Later the model has evolved by adding more such half centers throughout the spinal segments.

Initially there were two hypotheses for the generation of rhythmic and alternating movements: 1. The reflex chain model which explains that sensory neurons excite interneurons that activate motor neurons to the muscle, 2. The CPG model which explains that a central circuit generates rhythmic patterns of activity in the motor neurons to antagonist muscles. Later, the CPG model was proved by different groups showing that completely isolated and deafferented spinal cords could generate patterns that are

very similar to those recorded during intact locomotion (Grillner and Zangger, 1974; Wilson, 1966).

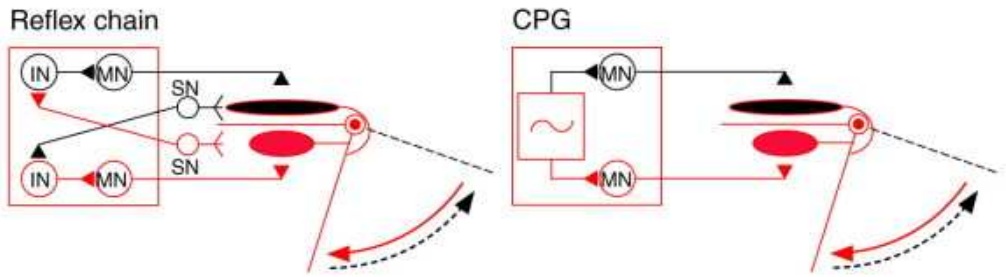


Fig 4: Hypotheses of rhythmic movements. (a) Reflex model (b) CPG model (Marder and Bucher, 2001)

The cellular organization of neural circuits that results in flexor–extensor and left–right coordination remains incompletely understood even at the present time (Kiehn 2016). Detailed studies using neuronal pool specific deletions and computational modeling of circuits indicate the plausible wiring diagram of the neurons including interneurons that contribute to rhythm generation (Grillner 2006; McCrea and Rybak, 2008). Fig 5 shows a currently-popular model of integrated pattern formation that is compatible with experimental data and computational simulations (Rybak et al., 2015; Zhang et al., 2014a).

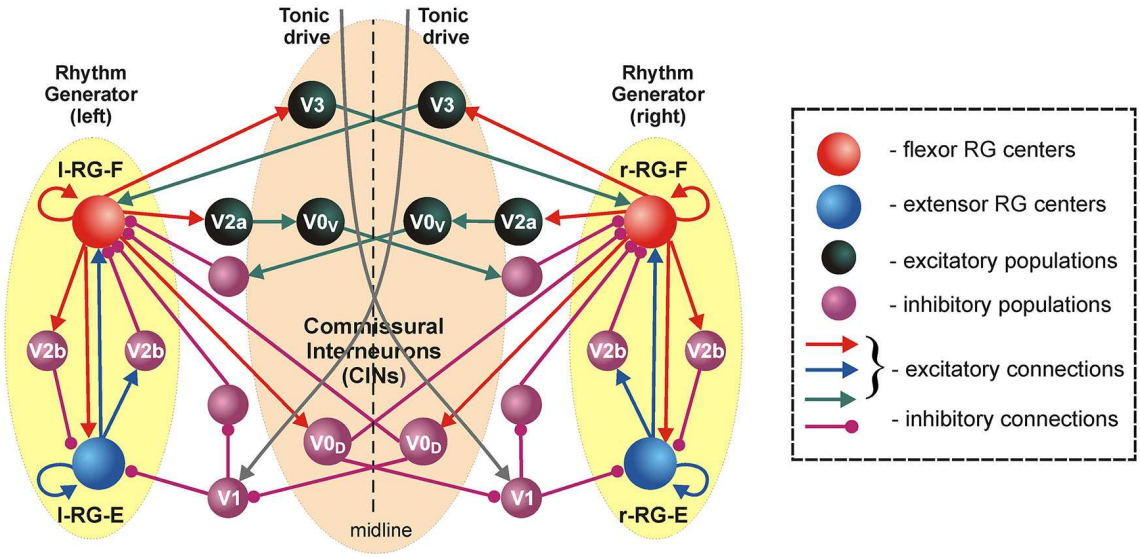


Fig 5: Schematic representation of bilaterally interacting rhythm generators in the spinal cord illustrating the role of interneurons (Rybak et al., 2015).

Based on these animal CPG models, some studies have developed a simple mathematical model to prototype that can control human motor system and generates periodic motions in a musculoskeletal arm based on the CPG network (Nandi et al., 2008; Razavian et al., 2015). This computational model generates a motion similar to real human based on a simple descending command (i.e. the desired frequency of motion; Razavian et al., 2015). This provides the evidence that using animal locomotion inputs and electromyography data from humans, human motion prosthetics can be developed.

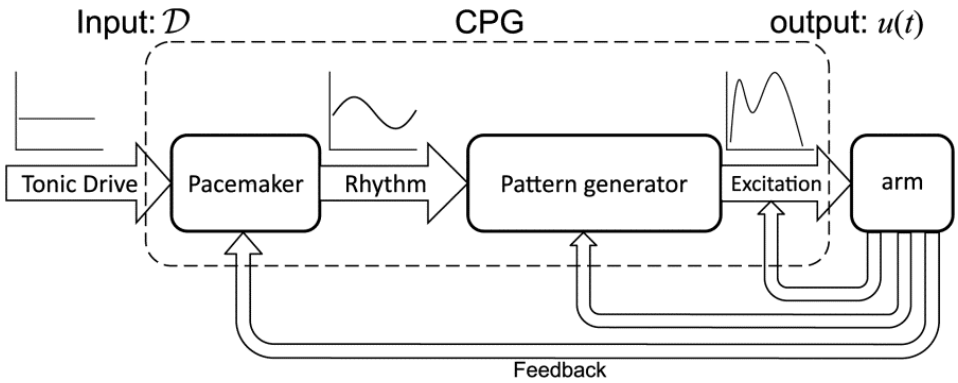


Fig 6: The layered structure of the CPG controller that can generate a motion similar to human limb movement (Razavian et al., 2015)

4. SPINAL CORD INJURY

Acute spinal cord injury (SCI) is an insult that results in temporary or permanent sensory, motor, or autonomic function disturbances. Traumatic SCI can be divided into penetrative (damage to the spinal cord by any sharp object or even the vertebra) and non-penetrative injuries (contusion or compression developed by any kind of accident). SCI can be complete or incomplete. With a complete SCI, the cord can't send signals below or above the level of the injury which leads to paralysis. With an incomplete injury, there is some movement and sensation below the injury (Kalsi-Ryan et al., 2014).

Upon various radiological evaluation and diagnostic tests (for muscle contraction and sensation), the extent of injury is graded by the American Spinal Injury Association (ASIA) Impairment Scale, using the following categories (ASIA, 2000; Ditunno et al., 1994):

1. A (Complete) - No sensory or motor function is preserved in below the injury (including sacral segments S4-S5; Waters et al., 1991)

2. B (Motor complete and sensory incomplete) - Sensory, but not motor, function is partially preserved below the neurologic level and extends through sacral segments S4-S5

3. C (Motor and sensory incomplete) - Motor and sensory functions are partially preserved below the neurologic level, and most key muscles below the neurologic level have a muscle grade of less than 3

4. D (Motor and sensory incomplete) - Motor and sensory functions are partially preserved below the neurologic level, and most key muscles below the neurologic level have a muscle grade that is greater than or equal to 3

5. E (Normal) - Sensory and motor functions are normal

Respiratory dysfunction following SCI at various level of spinal segments can result in loss of ventilatory muscle function, pneumothorax, hemothorax, pulmonary contusion or

decreased central ventilatory drive. A direct relationship exists between the level of injury and the degree of respiratory dysfunction (Zimmer et al., 2007):

1. Lesions at C1 or C2 - Vital capacity is only 5-10% of normal

2. Lesions at C3 through C6 - Vital capacity is 20% of normal, and cough is weak and ineffective

3. High thoracic cord injuries (T2 through T4) - Vital capacity is 30-50% of normal, and cough is weak

4. Lower cord injuries - Respiratory function improves

5. Injuries at T11 - Respiratory dysfunction is minimal; vital capacity is essentially normal, and cough is strong.

There are no established ways to reverse damage to the spinal cord. But there are new treatments, including prostheses and medications that may promote nerve cell regeneration or improve the function that remains after a SCI. First, SCI treatment focuses on preventing any further damage to the spinal cord. This includes emergency actions such as immobilization of the spine (to reduce any further trauma), maintenance of breathing ability, shock prevention and avoidance of possible complications, such as stool or urine retention, respiratory or cardiovascular distress and formation of deep vein blood clots in the extremities. Intravenous administration of methylprednisolone is one treatment option for an acute SCI. If methylprednisolone is given within eight hours of injury, some people experience mild improvement. Often surgery is necessary to remove fragments of bones, foreign objects, herniated disks or fractured vertebrae to avoid any further deformity. Once the initial injury or disease stabilizes, secondary problems such as deconditioning, muscle contractures, pressure ulcers, bowel and bladder issues, respiratory infections and blood clots may arise. Once the initial injury and the secondary phase is stabilized by surgery and medications, rehabilitation is required for recovery. Drugs can be administered to control pain, muscle spasticity, and bladder and bowel functions. There are many pilot studies for recovering the locomotor (and standing) function back such as stem cell therapy, computer devices (for someone with limited

hand function), electronic aids to daily living, electrical stimulation devices, robotic gait training (for retraining walking ability after SCI) (Evaniew et al., 2015; Zimmer et al., 2007). However, none of these treatments and recovery processes completely restores the function of spinal cord.

4.1. Epidemiology

SCI occurs with an annual incidence of 12.1–57.8 cases per million (van den Berg et al., 2010a) with lower survival of non-traumatic SCI compared with traumatic SCI (van den Berg et al., 2010b). In most developing countries, the majority of patients are young adults (20–40 years of age), thereby causing a high burden to these countries (Ackery et al., 2004). The rates of SCIs vary across countries, regions, and cities. SCI can occur accidentally (traumatic) or due to any disease (non traumatic). Studies show a high male-to-female ratio of traumatic SCI (TSCI) and peak incidence is seen in younger than 30 years old. It has been reported that 40 cases per million in United States suffer from TSCI (0.4% of the total population), in Germany it is 66 cases per million population, in France 12.7 cases per million population and in Canada 46 cases per million population (Guertin, 2013). Majority (40-50%) of SCI cases is due to motor vehicle accidents and then to sports related injuries (24%), violence (7-23%) followed by falls in the elderly population (12-20%; Norenberg et al., 2004; Sekhon and Fehlings, 2001). Non-traumatic SCI (NTSCI) is mainly due to cancer, infection, vascular problems and nerological diseases. Higher risks of TSCI are reported during adolescence and older adults. NTSCI again can be complete or incomplete. In Canada, no complete SCI was seen in NTSCI patients, whereas in India 36% of NTSCI were complete (Agarwal et al., 2007)

4.2. Pathophysiology

Shortly after SCI many clinical changes occur including airway block, hemorrhage, neurogenic shock, ileus etc. Despite injury level, spinal or neurogenic shock generally occurs immediately after trauma. The injury can be divided into primary lesion and secondary lesion. The primary lesion is further classified into concussion (transitory spinal cord dysfunction in the absence of structural damage), contusion (like craniocerebral injuries, the damage is due to any vertebral disc displacement), laceration

(injury due to vertebral facture) and compression (is again like a contusion but the continuity of the columns are immediately restored completely without any intervention; Bailey 1971; Jellinger, 1976). This primary injury can cause numerous changes in morphology and physiology of the cord.

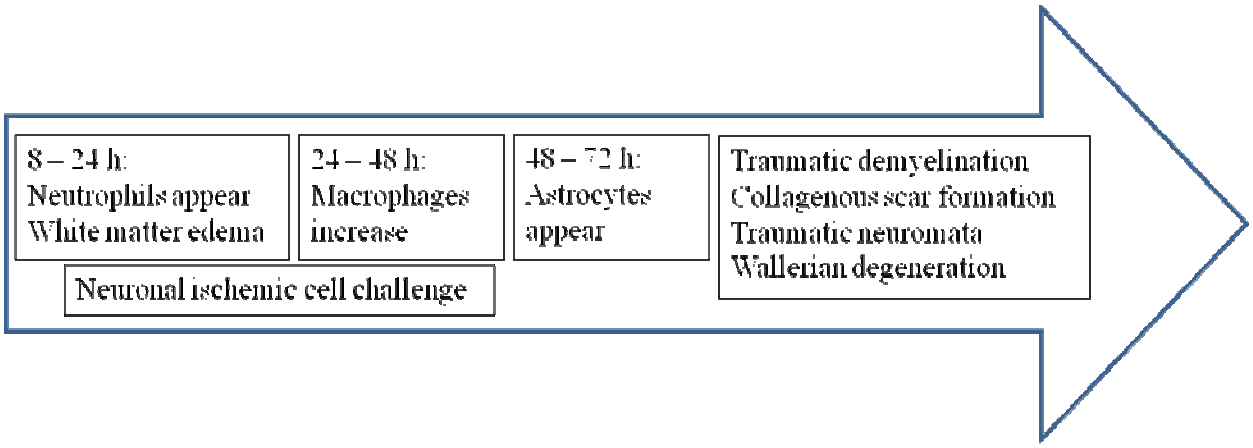


Fig 7: Progression of spinal cord injury after initial trauma

After the initial injury the presence of any pericapillary extravasation of erythrocytes and serum constituents are clinically observed (Kakulas, 1984). At cellular level, the initial injury can cause spinal cord deformation, laceration, crush and compression shortly after any trauma. And this leads to immediate cell death, axonal disruption and retraction, vascular and metabolic changes, which leads to secondary injury processes that can occur hours and days after injury. They are mainly associated with glutamate excitotoxicity, higher release of inflammatory mediators, free radical generation which results in local hypoxic condition and finally leads to scar formation (Park et al., 2004).

4.3. Kainate-induced neonatal rat SCI model

Because there is no curative treatment for SCI, establishing an ideal animal model is important to identify injury mechanisms and develop therapies for individuals suffering from spinal injuries. Some animal models are used for investigating pathophysiological mechanisms and others for investigating the mechanisms underlying tissue engineering and spinal cord regeneration (treatment). Over the past three decades, there has been substantial research for establishing animal models, and a large number of therapeutic strategies have been developed (Kwon et al., 2011; Tetzlaff et al., 2011).

The pathology of human SCI is not greatly different from that of experimental animal models, except for some specific differences. Allen (1911) first created SCI models using a weight drop technique and this was later considered as a standard experimental spinal cord contusion injury model (Koozekanani et al., 1976). Experimental animal models for SCI include the spinal cord ischemia-reperfusion injury model (Cheriyan et al., 2014, Lafci et al., 2013), spinal cord traumatic injury model (Koozekanani et al., 1976), photochemical-induced SCI model (Piao et al., 2009), spinal cord transection model (Min et al., 2011), and bidirectional distraction SCI model (Seifert et al., 2011; Zhang et al., 2014b).

There are many limitations in experimental animal models including complex pathology (surrounding the injury site), difficulty in studying specific injury processes, restriction in identifying affected cells without anesthesia (which can cause interference in results; Bajrektarevic and Nistri, 2016; Kaur et al., 2016; Shabbir et al., 2015a). Hence, to provide a more complete understanding of the mechanisms (biochemical or physiological parameters) of SCI, numerous in vitro systems may be used. These comprise primary neuronal cultures for studying cellular changes (Kehl et al., 1997), co-cultures of neurons and glia to study scar formation (Skaper and Facci, 2012), organotypic and isolated spinal cord preparations to study the entire network changes after injury (Cifra et al., 2012; Taccola et al., 2008).

Isolated spinal preparations from rodents are widely used to study the integrated sensory and motor pathways since their functional circuitry remains undamaged (Smith and Feldman, 1987; Cazalets et al., 1995). The neonatal rat in vitro preparation (which is used in our entire study) contains all the network components for rhythmic locomotion which can be recorded through ventral roots (VRs) (Cazalets et al., 1995). Excitatory chemicals can be directly applied to the preparation to evoke the CPG and the output which is similar to the real locomotion (fictive locomotion) can be recorded from VRs in both flexor and extensor pools of lumbar region (L2 and L5 respectively). Electrical stimulation can be given to the dorsal root (DR) and either reflex or rhythm can be recorded from VRs. This provides a clear ground to study entire locomotor network including sensory pathways. A fictive locomotion through DR stimuli can only be evoked

if the dorsal sensory neurons, interneurons and the motoneurons are integrated well. These phenomena provide a better understanding of entire system. To study any injury process, a wide time window is required and this is feasible with the neonatal (P0-P2 rats) preparations. Their survival up to 24 h in physiological solution is a greater advantage. In our lab we use kainic acid (KA, glutamate analog) for 1 h to evoke the primary injury and the preparation is kept for 24 h to study the secondary injury events. Despite the KA washout, the secondary damage progresses and peaks within the first few hours (Kuzhandaivel et al., 2011). During this time frame, the present model enables repeated testing of locomotor network activity and of pharmacological treatments on injury.

5. ENDOCANNABINOID SYSTEM

For at least 8000 years, humans have used *Cannabis sativa* for recreational and therapeutic purposes. The prevalence estimates of cannabis use are in the range of 10-30% of the adult population. In 2010, the Substance Abuse and Mental Health Services Administration (SAMHSA) estimated a lifetime prevalence of cannabis use of 52.1%. Marijuana and many of its constituents influence the CNS in a complex and dose-dependent manner (Zuardi, 2006). The chief psychoactive component, Δ_9-tetrahydrocannabinol (Δ_9-THC) was successfully isolated and chemically characterized by Mechoulam and Gaoni in 1965 (Mechoulam and Gaoni, 1965 a, b). The endocannabinoid (ECB) system includes endogenous ligands, their receptors and the enzymes that are responsible for their synthesis and degradation (Pertwee, 2009). The entire ECB system regulates pain, memory, learning and synaptic plasticity, appetite, immune function, lipid metabolism and many other physiological mechanisms (Hohmann et al., 1995; Di Marzo et al., 1998; Kishimoto and Kano, 2006).

5.1. Cannabinoid receptors

Howlett (1984) have first shown the existence of THC-sensitive CB receptors that decreased cAMP production in neuroblastoma cell cultures mediated by a $G_{i/o}$-coupled pathway and it was then characterized by Devane et al (1988) from brain using immunohistochemical and radioligand binding methods (Howlett, 1985; Howlett and Fleming, 1984; Howlett et al., 1986; Devane et al., 1988).

5.1.1. Receptor types and distribution

The dominant types of cannabinoid receptors are CB1R, CB2R and non CB1/CB2R (GPR18, GPR55, GPR119 and TRPV1) (Devane et al., 1988; Howlett, 1995; Howlett et al., 2002a, b; Pertwee 2007a, b, 2008; Pertwee et al., 2007; Ross, 2003). Most of the endogenous cannabinoid signals are mediated through guanine-nucleotide binding proteins-coupled heptahelical CB1 and CB2 receptors (receptor nomenclature follows Alexander et al., 2013; Console-Bram et al., 2012).

CB1Rs are widely distributed in the nervous system and in peripheral tissues. A dense staining of CB1R is seen in mammalian hippocampus, cerebral cortex, brainstem, cerebellum and in the areas that are involved in pain modulation, including the periaqueductal gray and the dorsal horn of the spinal cord (Svìzenskà et al., 2008). In spinal cord, CB1Rs are densely located in the regions that processes pain signals. Superficial dorsal horn and the dorsolateral funiculus are highly stained structures for CB1R (Farquhar-Smith et al., 2000; Herkenham et al., 1991; Sañudo-Peña et al., 1999). Immunoreactivity of CB1R in dorsal horn is predominantly found in interneurons, particularly in laminae I, II, and inner II/III transition, and in lamina X (Farquhar-Smith et al., 2000; Tsou et al., 1998). The majority of CB1Rs are found on axons of larger diameter neurons with myelinated A fibres (Bridges et al., 2003). The peripheral system that expresses CB1Rs includes skeletal muscle, liver, pancreas and adipose tissue that are involved in energy and cellular metabolism (Herkenham, 1990; Kaur et al., 2016; Piomelli, 2005). CB2Rs are widely distributed in peripheral tissues, and in immune tissues. Expression of CB2R gene transcripts are found in the spleen, tonsils, thymus, mast cells and blood cells (Atwood and Mackie K, 2010; Cabral and Griffin-Thomas, 2009). Their expression is also found in microglia, retina and cerebellar granule cells (Svíženská et al., 2008).

The intensity of CBRs decreases in neurodegenerative diseases, such as Parkinson's and Huntington's disease (Svìzenskà et al., 2008). Callen et al have shown that even at lower expression levels, CB1R and CB2R forms heteromers in the following brain regions: pineal gland, nucleus accumbens and globus pallidus. CB1R-CB2R heteromers exhibited bidirectional cross-antagonism and agonist co-activation (Callen et al., 2012).

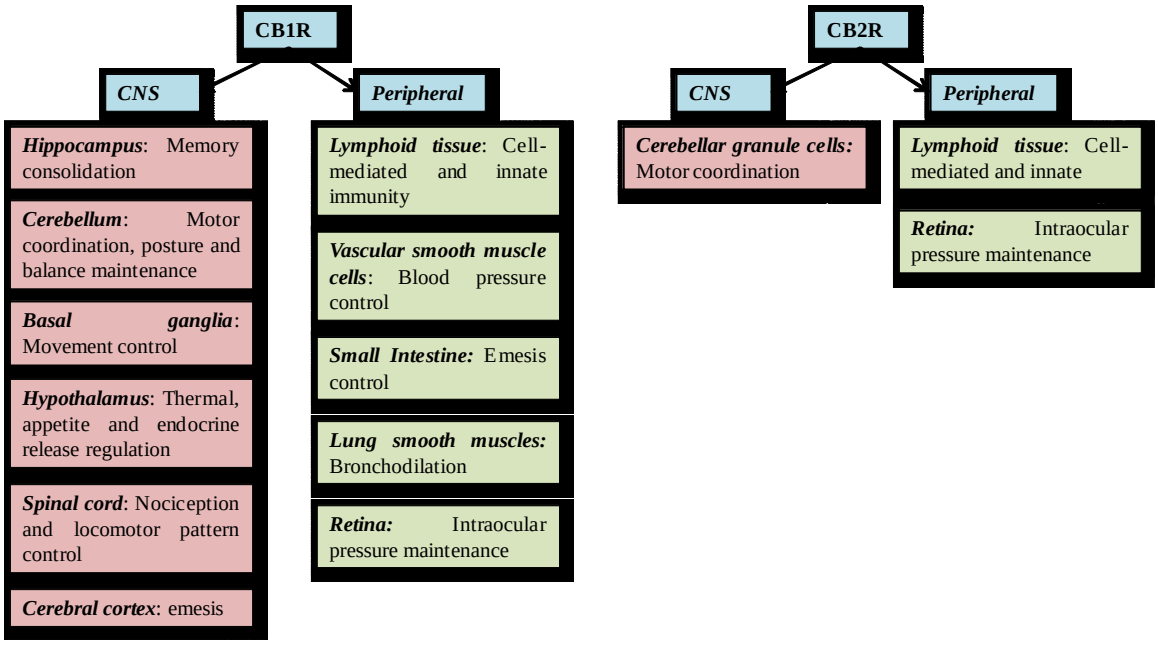


Fig 8: Expression CB1R and CB2Rs and its function in CNS and in peripheral organs.

5.1.2. Structure of cannabinoid receptors

The CB1R gene is polymorphic, signifying its various role in the biological system. Using an oligonucleotide probe derived from a member of GPCRs, cDNA of CB1R was first isolated from a rat cerebral cortex library (Matsuda et al., 1990). The human CB1R gene locus is on chromosome 6, position: 6q14–q15 (Caenazzo et al., 1991; Hoehe et al., 1991). The human CB2R gene is located in chromosome 1 p36 (Onaivi et al., 2006). Both CB1 and CB2 receptors share 44% amino acid sequence identity throughout the total protein (Munro et al., 1993).

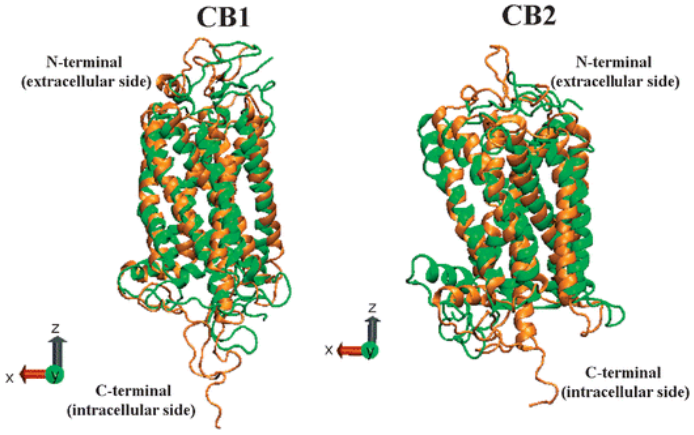


Fig 9: Structure of CB1R and CB2R (Ramos et al., 2011)

The CB1R gene is intronless and its structure is similar in rodents and human. The CB2R gene is intronless only in its coding region (Onaivi et al., 2002). The CB1R gene is highly conserved across humans and rodents, whereas the CB2R gene is more divergent across different species (Griffin et al., 2000). Like other GPCRs, cannabinoid receptors contain C-terminal intracellular domain coupled to G protein subunits, an N-terminal extracellular domain that possesses glycosylation site and seven hydrophobic stretches of 20–25 amino acids that form transmembrane α helices, connected by alternating extracellular and intracellular loops. CB1 and CB2 receptor three-dimensional models have been constructed and compared between human, rat and mouse (Bramblett et al., 1995; Ramos et al., 2011). Unlike most GPCRs where the cysteines occur in the second and third extracellular domains, both CB1R and CB2Rs have no cysteines within the second extracellular domain, but the third contains two or more cysteines residues. Cysteines stabilize the tertiary structure of the receptor by forming intra-molecular disulfide bridges. One other variation from most other GPCRs is that the CB1R and CB2Rs lack a highly conserved proline residue in fifth hydrophobic domain. The clusters for cAMP-dependent protein kinase and Ca^{2+}-calmodulin-dependent protein kinase sites are conserved across the human, rat, and mouse receptor proteins. There is a single potential protein kinase C site which is also conserved in CB1Rs (Matsuda, 1997). Hua *et al.* have recently shown the crystal structure of human CB1R in complex with a tight binding antagonist AM6538 and also the same group have provided information about binding modes of cannabinoid agonists using molecular docking experiments (Hua et al., 2016)

5.2. Endogenous and synthetic ligands

Cannabinoids are diverse chemical compounds that acts on CBRs in brains and in other peripheral tissues. These compounds include, the endocannabinoids (endogenous ligands from body), the phytocannabinoids (like Δ_9-THC and cannabidiol from plant sources), and synthetic cannabinoids (cannabimimetics including the aminoalkylindoles, 1,5-diarylpyrazoles, quinolines, and arylsulfonamides, as well as eicosanoids related to endocannabinoids). At least five different endogenous compounds in human brain are known to have affinity towards cannabinoid receptors, namely, N-

arachidonoylethanolamide (anandamide; AEA), 2-arachidonoylglycerol (2-AG), noladin ether, virodhamine and N-arachidonoyldopamine (NADA).

AEA was the first endogenous cannabinoid receptor agonist to be identified and well documented (Devane et al., 1992). The second major ECB, 2-AG was primarily isolated from canine intestine (Mechoulam et al., 1995) and rat brain (Sugiura et al., 1995). This lipid messenger has been reported in inflammatory responses, immune function, cell proliferation, embryo development and long-term potentiation in the hippocampus (Sugiura and Waku, 2000). 2-AG is present in the brain at higher levels than AEA. Therefore, 2-AG is considered the dominant endogenous cannabinoid in the brain (Childers and Breivogel, 1998). Another endocannabinoid, Noladin ether was in the thalamus and hippocampus and at much lower amounts in the spinal cord (Fezza et al., 2002). AEA and 2-AG were found in areas of the brain with high densities of CBRs (Devane et al., 1992; Egertova and Elphick, 2000; Piomelli, 2005; Pertwee, 2009). AEA binds to both CB1 and CB2 receptors (Glass and Northup, 1999), but its affinity for the CB2 receptor is approximately four-fold less than for CB1 receptors (Felder et al., 1995). 2-AG exhibits a lower affinity for CB1 than anandamide and it is also a natural ligand for CB2R (Suigiura and Waku, 2000).

The first selective CB1 receptor antagonist synthesized was, the diarylpyrazole SR141716A (Rimonabant; Rinaldi-Carmona et al., 1994). In vitro and in vivo studies have shown the reversal effects of rimonabant on actions induced by cannabinoids at CB1Rs (Howlett et al., 2002a; Pertwee, 1997). Although rimonabant is CB1R-selective, it is not CB1R-specific as it also binds with CB2Rs but with lower affinity (Hirst et al., 1996). Other studied CB1R-selective antagonists are LY320135, AM251 and AM281 that also produce inverse cannabimimetic effects (Pertwee, 2006).

5.2.1. Structure of ligands

Cannabinoid term is used for the typical C-21 compounds present in Cannabis saliva and includes their analogs and transformation products and they often possess chiral centers. Two different numbering systems depending upon the structure namely, dibenzopyran and monoterpenoid, are generally used for identifying the cannabinoid

related compounds. The most important pharmacologically active member of the phytocannabinoids family is (-) delta-9-THC,6a,10a-trans-tetrahydrocannabinol (THC; Gaoni and Mechoulam, 1964a). The three-dimensional shape of AEA strongly resembles THC. AEA has a long hydrocarbon tail which makes it soluble in fat and allows it to easily slip across the blood-brain barrier (Pertwee, 2005). 2-AG is a unique molecular species of monoacylglycerol having arachidonic acid at the second position of the glycerol backbone (Sugiura et al., 2002)

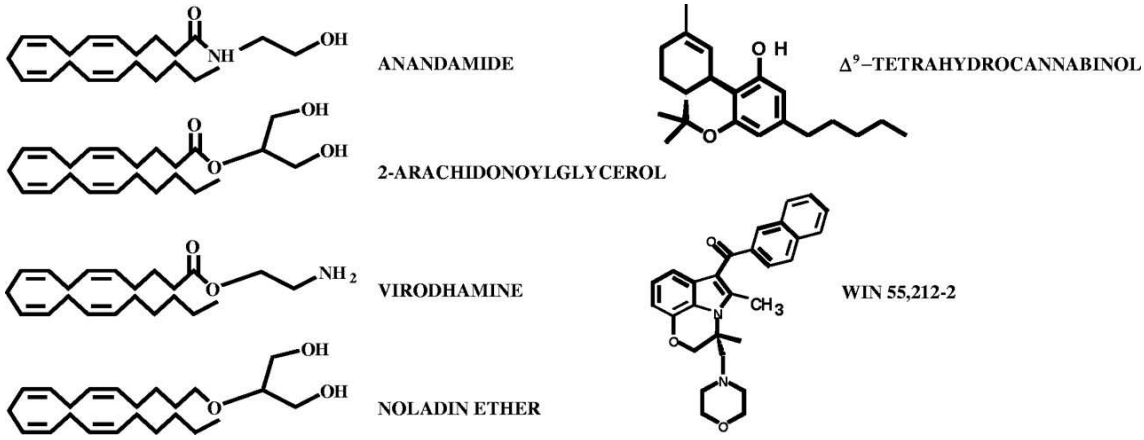


Fig 10: Chemical structures of cannabinoid receptor agonists (Freund et al., 2003; Rodríguez et al., 2005)

The first synthesized, CB1R inverse agonist rimonabant was in the market for anti-obesity treatment. The structure of this diarylpyrazole derivative is shown in the Fig 9. AM251 is a close analogue of rimonabant, only differing by replacing an I-atom for a Cl-atom on the 5-phenyl ring (Gatley et al., 1996; Lan et al., 1999; Rinaldi-Carmona et al., 1994 and 1995).

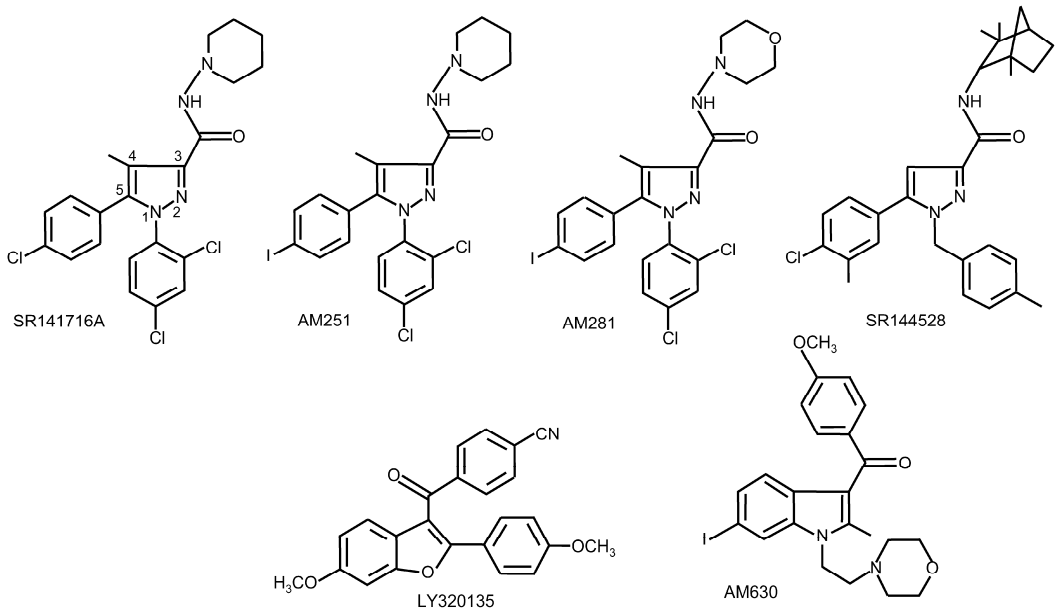


Fig 11: Chemical structures of cannabinoid receptor antagonists / inverses agonists (Pertwee, 2005)

5.3. Endogenous cannabinoid synthesis and degradation

Synthesis:

Both the eicosanoids, AEA and 2-AG are synthesized 'on demand' from the hydrolysis of a pre-formed membrane phospholipid precursor through a common two-step pathway, termed ‘the transacylation-phosphodiesterase pathway’. They are produced following the enhancement of intracellular Ca^{2+} concentrations, either by cell depolarization, or by mobilization of intracellular Ca^{2+} stores subsequent to stimulation of $G_{q/11}$ protein-coupled receptors. The enzymes catalyzing the final step of both AEA and 2-AG biosynthesis are all Ca^{2+}-sensitive (Childers and Breivogel, 1998; Di Marzo, 2006). AEA is formed from phosphatidylethanolamine (PE) in which an acyl group is transferred from the glycerophospholipid molecule to the amino group of PE by the enzyme Ca^{2+}-dependent N-acyltransferase (NAT). The second reaction is phopholipase D (PLD)-type mediated hydrolysis leading to the formation of N-arachidonoylethanolamide (Anandamide, AEA) and phosphatidic acid (Wang and Ueda, 2009). 2-AG is formed from arachidonic acid-enriched membrane phospholipids such as inositol phospholipids

through phospholipase C and diacylglycerol lipase or through actions of phospholipase A_1 and phospholipase C (Sugiura et al., 1995).

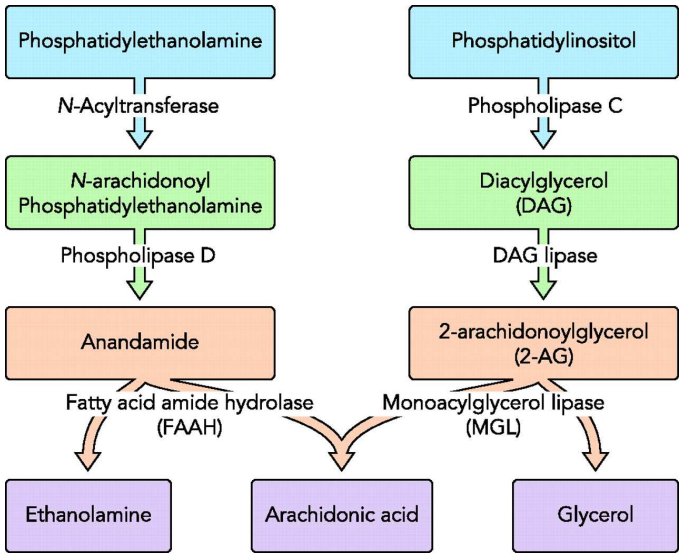


Fig 12: Synthesis and degradation of AEA and 2-AG (El Manira and Kyriakatos, 2010)

Degradation:

AEA is a highly unstable compound, and get rapidly hydrolysed by amidase like fatty acid amide hydrolase (FAAH) yielding ethanolamine and arachidonic acid (Deutsch and Chin, 1993). AEA inactivation mainly involves two mechanisms: 1. Intracellular hydrolysis by the membrane associated enzyme FAAH (Di Marzo et al., 1999); 2. Uptake by rapid, saturable, presynaptic transmembrane transporter which delivers the compound to FAAH (Beltramo et al., 1997; Piomelli et al., 1999). Monoacylglycerol lipase (MAGL) is the main 2-AG degrading enzyme; three other serine hydrolases also contribute, namely FAAH, serine hydrolase α-β-hydrolase domain 6 (ABHD6), and serine hydrolase α-β-hydrolase domain (ABHD12) (Blankman et al., 2007). These pathways lead to two major 2-AG breakdown products: arachidonic acid and glycerol (Freund et al., 2003). There are many synthetic inhibitors of these degrading enzymes that are used to pharmacologically elevate the levels of endogenous cannabinoid levels, for ex. FAAH inhibitors like LY 2183240 and URB 597, and MAGL inhibitors like JZL 195 and Pristimerin (Blankman and Cravatt, 2013).

5.4. Signaling

Endocannabinoids act retrogradly to modulate neurotransmitter release from CBR-expressing presynaptic terminals (Egertovà and Elphick, 2000). CB1 and CB2 receptors in presynaptic nerve terminals are coupled with $G_{i/o}$ subunits that lead to negative regulation of adenylyl cyclase (attenuating the production of cAMP) and positive regulation of mitogen-activated protein (MAP) kinase. Decrease in cAMP accumulation inhibits cAMP-dependent protein kinases (PKA). In the absence of cannabinoids, PKA phosphorylates the potassium channel protein, thereby decreasing outward potassium current. In the presence of cannabinoids, the phosphorylation of the channel by PKA is reduced, which leads to an enhanced outward potassium current. CB1Rs are also coupled positively to A-type and inwardly rectifying potassium channels, and negatively to N-type and P/Q-type calcium channels and to D-type potassium channels (Howlett and Mukhopadhyay, 2000; Howlett et al., 2002a; Pertwee, 1997). Hence, in CB1R expressing presynaptic terminals, cannabinoids are able to inhibit glutamate (Shen et al., 1996), acetylcholine (Gifford et al., 1997a) and noradrenaline release (Schlicker et al., 1997) by inhibiting presynaptic calcium channels. These transduction pathways mediate long term synaptic depression (LTD) in homoneuronal synapses. Studies have also shown that endocannabinoid-CB1R mediated signaling leads to long term potentiation (LTP) through stimulation of astrocyte-neuron signaling in heteroneuronal synapses (Gómez-Gonzalo et al., 2015)

CB2Rs are primarily located on immune cells (among them leucocytes and lymphocytes of the spleen and tonsils) and modulate cytokine release (Pertwee, 2001). Activation of B- and T-cell CB2Rs by cannabinoids leads to inhibition of adenylyl cyclase activity in these cells and to a reduced response for any immune challenge (Condie et al., 1996).

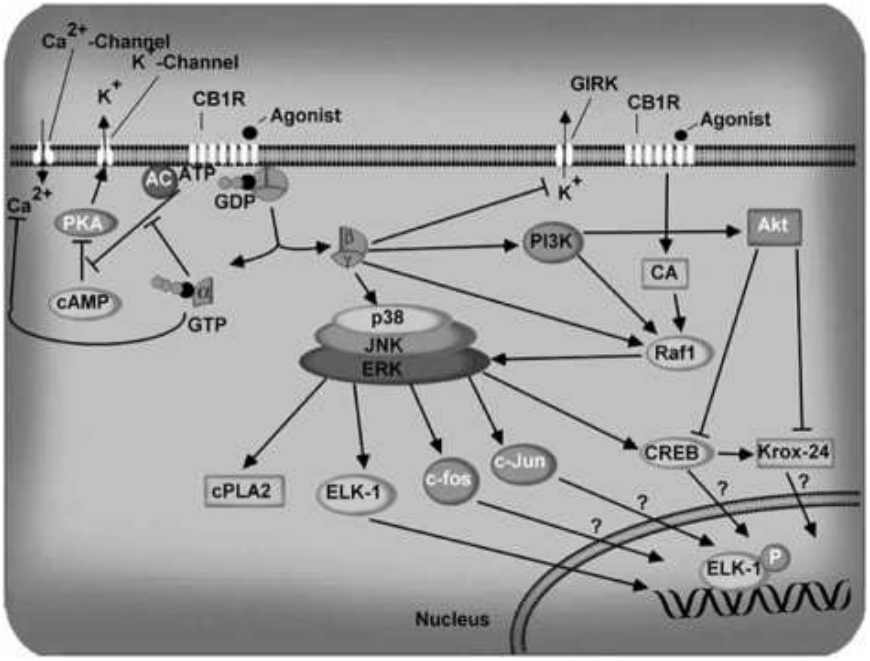


Fig 13: CB1R signaling pathways: CB1Rs inhibit N-, P/Q- and L-type Ca^{2+} channels and adenylyl cyclase by the α subunits of the G-protein. Adenylyl cyclase inhibition subsequently reduces cAMP production that decreases activation of cAMP-dependent protein kinase A (PKA), which finally leads to decreased phosphorylation of K^+ channels. Action on GIRK and PI3K are through αβ subunits. Inducing responses of CB1Rs are shown by a (→) sign and inhibitory effects by a (⊥) sign (Basavarajappa, 2007).

5.5. Cannabinoids in locomotor networks

Cannabinoids have been shown to increase dopamine in the prefrontal cortex (PFC; Pistis et al., 2002). In vivo studies have demonstrated the increase in extracellular dopamine in nucleus accumbens (NAc) after Δ_9-THC administration (Chen et al., 1990a; 1991; Tanda et al., 1997). In addition, dose-dependent increase in dopamine release has been observed in striatum and PFC (Chen et al., 1990a, b; Malone & Taylor, 1999). In this context, there is a strong correlation between cannabinoid-induced behavioral effects and dopamine alterations in higher brain centers (Polissidis et al. 2009, 2010). However, CB1Rs are not reported to be located at dopaminergic terminals of these main regions; thus, the effects of cannabinoids on dopaminergic neurotransmission are mainly indirect and modulated via other neurotransmitter activity such as glutamate (Fernandez-Ruiz et al., 2010).

A few studies have shown that Δ_9-THC and other cannabinoid synthetic agonists, such as WIN55 212-2, induce a decrease in glutamatergic neurotransmission in the hippocampus, striatum and NAc (Adermark et al., 2009; Cannizzaro et al., 2006; Fujiwara and Egashira,

2004; Pintor et al., 2006; Sano et al., 2008). On the other hand, other studies have shown that CB1R activation with Δ9-THC or WIN55,212-2 increases extracellular glutamate levels in the cortex (Ferraro et al., 2001; Pistis et al., 2002). The biphasic cannabinoid-induced actions on glutamatergic neurotransmission in multiple higher brain centers cause effects on motor cognition, activity, reward function and neuroprotection or neurotoxicity. Pharmacological treatments with CB1R agonists exert biphasic effects also in a dose-dependent manner on motor activity, with low doses inducing motor activation and high doses producing suppressed motor activity or catalepsy (Drews et al., 2005; Polissidis et al., 2010; Rodvelt et al., 2007; Sanudo-Pena et al., 2000; Sulcova et al., 1998).

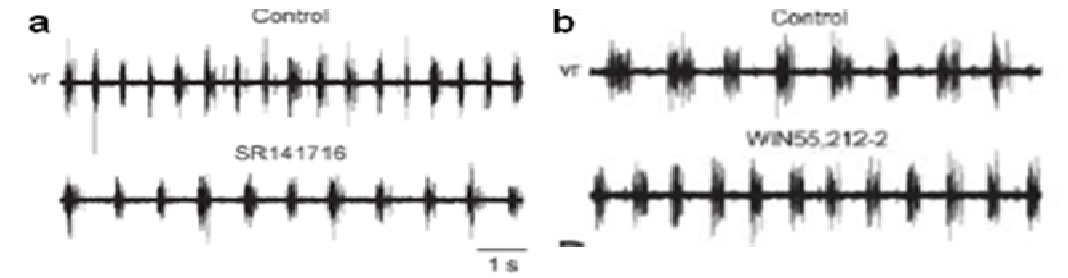


Fig 14: Locomotor rhythm induced by NMDA in the lamprey spinal cord *in vitro.* Subsequent application of (a) antagonist SR141716 (5 µM) decreased burst frequency (b) agonist WIN55,212-2 (5 µM) increased the burst frequency (El Manira et al., 2008)

In lower vertebrate locomotor networks, experiments on the isolated lamprey spinal cord show that endocannabinoids are released during ongoing rhythmic activity in spinal cord and participate in setting the baseline rhythm (See Fig 13). Endocannabinoids are found to be released in response to metabotropic glutamate receptor-1 (mGluR1) activation. This conditional release of endocannabinoids allow motoneurons and midline crossing interneurons to be modulatory neurons by enabling them to regulate their inhibitory synaptic inputs and, thus, to contribute to the locomotor burst frequency. These results provide evidence that endocannabinoid signaling in the lower vertebrate locomotor circuit contributes to motor pattern generation and regulation (El Manira et al., 2008). Thus, understanding endocannabinoid signaling is pivotal to understanding the complex networks that are regulating the basic locomotion.

5.6. Endocannabinoid system in neuroprotection

CB1R activation influences intra-cellular homeostasis, intercellular communication, and the modulation of neuro-transmitter release. Cannabinoids are anti-excitotoxic substances as they are able to maintain the glutamate homeostasis (Grundy et al., 2001; Romero et al., 2002; Van der stelt et al., 2001). Studies both in vitro (Shen et al., 1996; Shen and Thayer, 1999) and in vivo (Schlicker and Kathman, 2001) experimental models have demonstrated that cannabinoid agonists inhibit glutamate release via binding to CB1Rs located presynaptically on glutamatergic terminals, an effect was reversed by selective CB1 antagonists (Grundy et al., 2001; Van der stelt et al., 2001). Cannabinoids have also been shown to inhibit GABAergic transmission in several brain areas (Katona et al., 1999; Szabo et al., 1998). Release of nitric oxide (NO), which plays a key role in neurodegenerative processes, is controlled by the cannabinoid system, that inhibits the production of NO by microglia (Waksman et al., 1999), astrocytes (Molina-Holgado et al., 1997), neurones (Hillard et al., 1999), and macrophages (Coffey et al., 1996). In addition, CB1R activation influences dopaminergic signal transmission in striatal slices (Cadogan et al., 1997) and in vivo (Gessa et al., 1998), inhibits noradrenaline release in isolated rat atria and vasa deferentia (Ishac et al., 1996), inhibits acetylcholine release in vivo (Carta et al., 1998) and inhibits serotonin release from mouse brain cortical slices (Nakazi et al., 2000). Besides their role in controlling neurotransmitter release, it has been shown that CB1R knockouts are more susceptible to experimental autoimmune encephalomyelitis (EAE), neurodegeneration and show increase in caspase 3 activation (Pryce and Baker, 2007). Likewise, in conditional mutant mice that lack expression of the CB1R in principal forebrain neurons, the excitotoxic agent kainic acid induces stronger seizures in vivo (Marsicano et al., 2003).

It should be noted, that in some experimental models, endocannabinoids do not provide any protection to neurons or produce neurotoxic effects. AEA was shown to be ineffective against prolonged exposure to toxic levels of glutamate (Andersson et al., 2000; Skaper et al., 1996). The CB1R antagonist rimonabant evokes a neuroprotective response in unilateral intrastriatal NMDA microinjected neonatal rats by reducing the ipsilateral infarct area and the number of degenerating cortical neurons. This protective

effect is abolished by co-injection of the cannabinoid agonist WIN55,212-2, indicating that block of cannabinoid receptor activity inhibits caudal propagation of the excitotoxic response (Hansen et al., 2002). Furthermore, genetically engineered mice lacking FAAH (in which brain AEA levels are permanently elevated) appear to be more susceptible toward kainate-induced seizures (Clement et al., 2003). In summary, studies so far show a mixed view of cannabinoid agonists and CB1R on neuroprotection. The protective or toxic role is also further dependent on dose and time of application on the system. Hence, it is very important to precisely study the role of these ligands in any experimental model.

6. ACID SENSING ION CHANNELS

Under physiological conditions, intra- and extra-cellular pH is maintained between 7.0 and 7.4. Increased neuronal excitability alters pH to activate a wide range of receptors and ion channels in cell membranes, including a variety of voltage- and ligand-gated ion channels. Waldmann and his colleagues have first cloned acid-sensing ion channels (ASICs) that are activated in response to diminished extracellular pH (Waldmann et al., 1997). Acid sensing ion channels (ASICs) are members of Na^+ channel superfamily that are permeable to cations and are activated by extracellular acidosis. They are modulated by extracellular alkalosis (Benson et al., 1999; Delaunay et al., 2012), intracellular pH (Wang et al., 2006) and various other factors like neuropeptides, arachidonic acid, nitric oxide, ATP, serotonin and polyamines (Wemmie et al., 2013). In mammals, there are four genes (ASIC 1-4) encoding at least six subunits of these channels (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4; Wemmie et al., 2006). Each subunit has two hydrophobic transmembrane regions flanking with a large extracellular loop and short intracellular N- and C-termini (Gonzales et al., 2009; Jasti et al., 2007). The subunit composition of ASIC channel determines its properties like pH sensitivity, ion selectivity, activation kinetics and desensitization kinetics. Each subunit possesses unique property. For example, ASIC1a and ASIC3 channels are activated only if the extracellular pH decreases to ≤ 7 (Benson et al., 2002; Hesselager et al., 2004), whereas ASIC2a channels are not activated until the pH is less than 6 (Askwith et al., 2004; Benson et al., 2002).

Table 2: pH sensitivity data of homomeric and heteromeric channels (adapted from Huang et al., 2015)

Subunit composition	1a	1b	2a	2b	3
1a	5.8-6.8	5.8-6.3	5.4-6.1	6.2-6.4	6.3-6.7
1b		5.8-6.1	4.9	N/A	6.3-6.7
2a			3.8-4.5	4.8	5.6-6.1
2b				N/A	6.5
3					6.3-6.7

ASIC subunits can assemble in homo or hetero-tetrameric forms. The heteromers have distinct activation and desensitization characteristics, pharmacological properties, and different abilities to interact with intra and extracellular factors. For example, the desensitization rate of ASIC1a/ASIC2a heteromeric channels is more rapid than that of ASIC1a or ASIC2a homomeric channels (Askwith et al., 2004).

Table 3: Characteristics of ASIC channels. SN - sensory neurons. (Table modified from Bronstein-Sitton, 2004)

Protein name	*Isoforms*	*Alternative Names*	*Expression Pattern*	*Function*
ASIC1	ASIC1a	ASICα, BNaC2α	CNS, SN	Synaptic plasticity, learning, memory
	ASIC1b	ASICβ, BNaC2β	SN	Channel modulation?
ASIC2	ASIC2a	MDEG, BNaC1α	CNS, SN (+), taste buds	Sour taste sensing, Mechanosensation, Brain tumor suppression
	ASIC2b	MDEG2, BNaC1β	CNS, SN (+++), taste buds	Sour taste sensing
ASIC3		DRASIC	SN	Pain modulation, ischemic pain, mechanosensation
ASIC4		SPASIC	CNS, inner ear	Channel modulation?

ASIC isoforms in the rat have amino acid sequence similarity of 45-80%. The primary structure of ASIC channels is quite conserved for a variety of mammalian species (rats, mice, and humans). For example, the mouse ASIC1a and the human ASIC1a share over 99% of their amino acid sequence identity. ASIC1a, ASIC2a and ASIC2b are widely expressed in the CNS, while almost all subunits (except ASIC4) are present in sensory neurons of the PNS (Deval and Lingueglia, 2015; Lin et al., 2015). Activation of ASICs induce depolarization, associated with direct and indirect Ca^{2+} entry that will finally modulate the number of downstream signaling cascades. ASICs have been involved in several physiological and pathological processes such as nociception and pain, neuronal acidotoxicity, synaptic function and plasticity, and other conditions.

6.1. Expression and role of ASIC channels in spinal cord

ASICs are widely expressed throughout the central and peripheral nervous systems and play a role in nociception, learning and memory, and fear conditioning. ASIC1a is a key subunit in the modulation of nociceptive signals in the CNS as it is broadly expressed in all the laminas of the adult spinal cord (Duan et al., 2007; Mazzuca et al., 2007). The homomeric ASIC1a is the major channel underlying ASIC currents in rat spinal dorsal

horn neurons (Duan et al., 2007). Significant expression of ASIC2a and ASIC2b is also seen in the dorsal region of spinal cord (Wu et al., 2004). ASIC2a is broadly expressed in all the laminae of the adult spinal cord, where it is often co-localized with ASIC1a and plays a significant modulatory role (Baron et al., 2008; Jasti et al., 2007). The role of ASICs in the spinal cord is probably complex, because these channels could be involved at different levels of nociception. ASIC1a homomeric channels in the CNS are involved in the control of the endogenous opioid system, thereby having analgesic role (Mazzuca et al., 2007).

A recent study by Jalalvand and colleagues (2016) has demonstrated that increase or decrease in pH reduces the locomotor burst rate in the lamprey isolated spinal cord. They have confirmed that this suppressive effect is caused by central canal neurons (CSF-c). Furthermore, it is also shown that the CSF-c neurons possess a novel innate homeostatic mechanism to sense any change in pH (regardless of acidic or alkaline condition) and to respond by causing a depression of the motor activity. The CSF-c neurons express acid-sensing (ASIC3) and alkaline-sensing (PKD2L1) channels. Since CSF-c neurons are found in all vertebrates, their pH-sensing and locomotor modulatory function is most likely to be conserved (Jalalvand et al., 2016). However, there are very sporadic studies of the expression of ASICs in the spinal cord and its role in reflex and complex rhythmic activities.

6.2. Role of ASICs in synaptic plasticity, neurotoxicity and protection

Synaptic plasticity

H^+ activated inward current results in excitatory post synaptic potentials (EPSCs) that are 15-20 times lower than the current generated from glutamate receptors (Du et al., 2014; Highstein et al., 2014; Kreple et al., 2014) which makes it very hard to detect any specific proton-activated component during neurotransmission (Alvarez de la Rosa et al., 2003). However, it is now shown that this small component is one of the boosting mechanisms for neurotransmission, and is important for the generation of long-term potentiation (LTP) in amygdala (Du et al., 2014) and in hippocampal slices (Wemmie et al., 2002). Protons inhibit NMDA receptors directly. However, the LTP effect is due to the fact that

activation of ASIC1a results in membrane depolarization which helps relieving voltage-dependent blockade of NMDA receptors by Mg^{2+}, thus facilitating the activation of NMDA receptors and the expression of LTP (Huang et al., 2015). In contrast, one study using ASIC1a knockout have shown no difference in hippocampal LTP compared to wildtype (Wu et al., 2013) suggesting that ASICs can mediate proton signaling at synaptic sites, but the exact contribution to synaptic plasticity may vary, depending on the system and condition.

Neurotoxicity and protection

Following brain ischemia, there is a large increase in reactive oxygen species accompanied by a large reduction in brain pH down to 6.0 in severe conditions (Hertz, 2008; Xiong et al., 2007). This pH reduction is large enough for robust activation of ASIC1a channels. Studies have shown that ASIC1a gene deletion protects mice from brain injury induced by middle cerebral artery occlusion (MCAO) (Pignataro et al., 2007; Xiong et al., 2007). Amiloride, a non-specific ASIC inhibitor, has a similar protective effect on MCAO (Pignataro et al., 2007). Activation of NMDA receptors, through the recruitment of calcium/calmodulin-dependent protein kinase II (CaMKII) signaling, increased ASIC1a current and potentiates ischemia-induced neuronal injury (Gao et al., 2005). Consistent with these studies, an additive protection was observed when ASIC channels are inhibited along with NMDA receptors inhibition (Mishra et al., 2011; Pignataro et al., 2007).

In normal conditions, ASIC1a expression in axons is relatively low (Vergo et al., 2011; Zha et al., 2006; Zha et al., 2009). However, in the animal EAE model (where pH was about 6.0-6.5; Friese et al., 2007) and human multiple sclerosis (MS) patients, an increase in ASIC1a expression or trafficking to axons, as well as an upregulation of ASIC1a in oligodendrocytes (Arun et al., 2013; Vergo et al., 2011) is observed. ASIC1a gene deletion or inhibition reduces EAE-induced axonal injury and improves the clinical score (Friese et al., 2007). In the same way, amiloride alleviates the severity of the disease (Vergo et al., 2011). In a recent clinical study, where MS patients were orally treated with amiloride for a period of one year, has shown significant reduction in brain atrophy rate,

axonal damage and myelin loss (Arun et al., 2013). Studies have demonstrated that substantia nigra neurons express ASICs and typical ASIC-type currents (Arias et al., 2008; Pidoplichko and Dani, 2006). In a mouse Parkinson's disease (PD) model, ASIC inhibition with amiloride or Psalmotoxin (PcTx1) attenuates the reduction of immunoreactivity to tyrosine hydroxylase and dopamine transporters (markers for dopaminergic neurons; Arias et al., 2008).

Extracellular pH decreases after spinal cord or traumatic brain injury (TBI; Gupta et al., 2004; Vink et al., 1987; Yin et al., 2013). In TBI patients, the degree of pH reduction is correlated with the injury condition, with lower pH associated with higher lethality (Gupta et al., 2004; Timofeev et al., 2011). In rodents, inhibiting ASIC1a with amiloride or PcTx1 alleviates the severity of TBI (Hu et al., 2011; Yin et al., 2013). Thus, targeting ASICs, especially when combined with other therapeutic interventions, may be an effective therapeutic strategy for alleviating neuronal injuries.

7. IMPORTANCE OF TREATMENT DURING EARLY PHASE OF SCI

Subsequent to an initial insult, there is a cascade of downstream events termed 'secondary injury', which culminate in degenerative events in the structure and function of the spinal cord (Borgens and Liu-Snyder, 2012; Hilton et al., 2016; Oyinbo, 2011; Park et al., 2004). These secondary injury mechanisms include, but are not limited to, ischemia, inflammation, free radical-induced cell death, glutamate excitotoxicity, cytoskeletal degradation, and induction of extrinsic and intrinsic apoptotic pathways (Borgens and Liu-Snyder, 2012; Hilton et al., 2016). Research from our group has shown both positive and negative effects of drugs in early stages of SCI (see Table 3). Our previous study on methoxyflurane (a volatile anesthetic) showed complete recovery of structure and function of the KA treated spinal cord after 24 h (Shabbir et al., 2015a). Furthermore, the anesthetic propofol protected from KA mediated injury organotypic cultures (Bajrektarevic and Nistri, 2016). These data strongly support that excitotoxicity (a main secondary injury mechanism; Park et al., 2004) can be reversed within a time window of 24 h from the initial insult.

Table 4: List of studies from our group on SCI models (★Protection; ★Toxic)

Compounds and study	Protection	Toxicity
6-Cyano-7-nitroquinoxaline-2, 3-dione (CNQX) and aminophosphonovalerate (APV), glutamate antagonists on KA or pathological medium (PM) treated in vitro rat spinal cord injury (SCI) model (Margaryan et al., 2010)	★Partial recovery after CNQX and APV treatment in both KA and PM treated preparations	
PJ 34 (poly ADP-ribose polymerase enzyme 1; PARP1 inhibitor) on excitoxic damage evoked by KA on rat organotypic cultures (Mazzone and Nistri, 2011) and on in vitro rat SCI model (Nasrabady et al., 2011)	★Protection in histology of both organotypic and in vitro SCI model. Limited functional recovery in the in vitro model	
Riluzole, an inhibitor of glutamate release, on KA mediated in vitro SCI model (Samano et al., 2012)	No functional and histological protection	

Methoxyflurane (volatile anesthetic) on KA in vitro SCI (Shabbir et al., 2015a)	★Complete protection of neurons and function	
VER155008 (HSP 70 inhibitor) on KA induced in vitro SCI model (Shabbir et al., 2015b)		★
Methylprednisolone sodium succinate (MPSS; glucocorticosteroid) on either KA or PM treated in vitro rat SCI model (Samano et al., 2016)	★Partial (white matter recovery)	
Propofol (anesthetic) on KA treated organotypic culture SCI model (Bajrektarevic and Nistri, 2016)	★Complete	
Propofol (anesthetic) on in vitro KA mediated SCI model (Kaur et al., 2016)	★ Partial (no functional recovery)	

An abnormal acid-base balance is encountered in many traumatic brain injury (TBI) patients on the first day of injury (Clausen et al., 2005; Taha and Ammar, 2015), among them acidosis is the commonest factor (Taha and Ammar, 2015). This change persists for four days post injury in patients (Clausen et al., 2005; Taha and Ammar, 2015). Similarly, Arevalo-Martin et al. have shown that endogenous cannabinoids are modulated within few hours post-SCI (Arevalo-Martin et al., 2012). These studies prompted our interest in the potential role of ASICs and CB1R s in KA mediated SCI during the first 24 h and to investigate further their impact on the locomotor rhythm.

8. THESIS AIMS

To understand how spinal CPGs produce locomotor movements and to assist the recovery of function after SCI, it is necessary to functionally dissect out the network connectivity, the intrinsic properties of the constituent neurons and their neuromodulatory mechanisms. It is well known that in spinal locomotor networks concerted operation of ion channels and transmitter receptors is required for initiating, setting and fine-tuning the frequency and amplitude of ongoing locomotor activity (El Manira et al., 2002; Grillner et al., 2001; Katz and Clemens, 2001; Harris-Warrick, 2002).

Hence, this thesis focuses on:

- How endocannabinoid system modulation can contribute to set the baseline locomotor activity in mammalian spinal cord
    - Importance of CB1R in the fictive locomotion
    - CB1R activity in the isolated neonatal rat spinal cord
    - Cannabinoid ligands and uptake inhibitors mediated modulation in the rhythm
- How endocannabinoid system can modulate the impaired rhythmicity very early after SCI
    - Expression and activity of CB1R after experimental SCI
    - Importance of CB1R after SCI
- How ASICs can modulate the locomotor rhythm after SCI
    - pH modulation after KA mediated injury and changes in ASIC expression
    - Blockade of ASIC channels and its importance to SCI

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MODULATORY EFFECTS BY CB1 RECEPTORS ON RAT SPINAL LOCOMOTOR NETWORKS AFTER SUSTAINED APPLICATION OF AGONISTS OR ANTAGONISTS

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Abstract—Sustained administration of cannabinoid agonists acting on neuronal CB1 receptors (CB1Rs) are proposed for treating spasticity and chronic pain. The impact of CB1Rs on mammalian locomotor networks remains, however, incompletely understood. To clarify how CB1Rs may control synaptic activity and locomotor network function, we used the rat spinal cord *in vitro* which is an advantageous model to investigate locomotor circuit mechanisms produced by the local central pattern generator. Neither the CB1 agonist anandamide (AEA) nor the CB1R antagonist AM-251 evoked early (<3 h) changes in mono or polysynaptic reflexes or in locomotor rhythms. Application of AEA (24 h) significantly decreased the ability of dorsal root (DR) afferents to elicit oscillatory cycles, and left synaptic responses unchanged. Similar application of LY 2183240, or JZL 184, inhibitors of endocannabinoid uptake processes, produced analogous results. Application of the antagonist AM-251 (or rimonabant) for >3–24 h largely impaired locomotor network activity induced by DR stimuli or neurochemicals, and depressed disinhibited bursting without changing reflex amplitude or inducing neurotoxicity even if CB1R immunoreactivity was lowered in the central region. Since CB1R activation usually inhibits cyclic adenosine monophosphate (cAMP) synthesis, we investigated how a 24-h application of AEA or AM-251 affected basal or forskolin-stimulated cAMP levels. While AEA decreased them in an AM-251-sensitive manner, AM-251 per se did not change resting or stimulated cAMP. Our data suggest that CB1Rs may control the circuit gateway regulating the inflow of sensory afferent inputs into the locomotor circuits, indicating a potential site of action for restricting peripheral signals disruptive for locomotor activity.

Key words: cannabinoid receptor-1, fictive locomotion, anandamide, AM-251, cAMP, endocannabinoids.

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Abbreviations: 2 AG, 2-arachidonoylglycerol; 5-HT, 5-hydroxytryptamine or serotonin; AEA, N-arachidonoylethanolamine or anandamide; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; cAMP, cyclic adenosine monophosphate; CB1R, cannabinoid receptor-1; DMSO, dimethyl sulfoxide; DR, dorsal root; DRP, dorsal root potential; Frsk, forskolin; GABA, γ-aminobutyric acid; NMDA, N-methyl-D-aspartate; VR, ventral root.

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INTRODUCTION

There is increasing interest in cannabinoid research as this field may yield important therapeutic tools against a range of diseases. A recent report has summarized the North American guidelines for the medical use of cannabinoids, highlighting the principal goals of decreasing chronic pain and spasticity especially in multiple sclerosis patients (Koppel et al., 2014). Conversely, use of endocannabinoid antagonists has been investigated to treat obesity and food craving (Poncelet et al., 2003; Howlett et al., 2004; Pagotto et al., 2005): however, early antagonists have not received approval by drug regulatory agencies because of severe central nervous system effects in man (Jones, 2008; Bifulco and Pisanti, 2009). A large body of evidence supports a critical role of cannabinoid receptors type 1 (CB1Rs; Pertwee et al., 2010) that are physiologically activated by endocannabinoids like anandamide (AEA) and 2-arachidonoylglycerol (2 AG), as pharmacological targets to control aberrant nociceptive transmission in the spinal cord and the maladaptive processing of afferent inputs underlying spasticity (Piomelli, 2005; Manzanares et al., 2006). Nevertheless, the role of CB1Rs in spinal locomotor networks is incompletely understood. *In vivo* observations indicate that activation of CB1Rs usually produces less locomotor activity, although the precise mechanisms remain unknown and the site of action unclear (Lee et al., 2006; Bosier et al., 2010). Previous studies of the lamprey spinal cord *in vitro* have shown that acute CB1R activation by AEA speeds up chemically-stimulated locomotor network function, while the antagonist AM-251 depresses locomotor cycles in a time-dependent fashion (Kettunen et al., 2005). Data on mammalian locomotor networks are currently unavailable. Thus, it seems important to investigate how CB1Rs may modulate mammalian circuits because the pharmacological consequences of CB1R activity largely depends on their coupling to distinct G-proteins, in turn regulating various intracellular effectors that may be species, tissue and cell specific (Hudson et al., 2010).

Most experimental studies have employed short-term application of CB1R agonists or antagonists. Nonetheless, these drugs are usually expected to be administered for a prolonged time, raising the possibility that they might induce adaptive changes in their receptors. To explore the functional consequences of sustained application of such drugs, we used the rat

spinal cord preparation *in vitro* as a model. This is an advantageous system to study locomotor network activity that is generated by a central pattern generator (namely a local neuronal circuitry with intrinsic rhythmicity) located in the lumbar spinal and readily activated by either neurochemicals (N-methyl-D-aspartate (NMDA) and serotonin (5-HT); Grillner, 2003, 2006; Kiehn, 2006, 2011) or trains of electrical stimuli applied to one dorsal root (DR; Marchetti et al., 2001). This pattern, which comprises alternating ventral root (VR) discharges among extensor and flexor motoneuron pools located on both sides of the spinal cord, is termed fictive locomotion because the rhythm is expressed in the absence of hind limbs. One important advantage of the isolated spinal cord is its 24-h survival *in vitro* with intact histological profile (Cifra et al., 2012) and fully preserved locomotor rhythms (Taccola et al., 2008). This property enables studies of delayed consequences of drug action with a timecourse resembling the *in vivo* administration protocols as CB1R ligands are not intended for brief effects only.

The aims of the present study were to clarify the effects of activation or block of CB1Rs on synaptic transmission in the rat spinal cord both acutely or after a 24-h application of CB1R ligands, their role in locomotor networks, any plasticity in the CB1R expression and potential neurotoxicity, and any preferential modulation of CB1R effectors.

EXPERIMENTAL PROCEDURES

In vitro spinal cord preparation

Spinal cords were isolated from neonatal Wistar rats (0–2 days old) in accordance with the guidelines of the National Institutes of Health and the Italian act D.Lgs. 27/1/92 no.116 116 (implementing the European Community directives n. 86/609 and 93/88). All efforts were made to minimize the number of animals used for the experiments and their suffering. Preparations were continuously superfused (7.5 ml min^{-1}) with standard Kreb's solution of the following composition (in mM): 113 NaCl, 4.5 KCl, 1 $MgCl_2 \cdot 7H_2O$, 2 $CaCl_2$, 1 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose, gassed with 95% O_2 and 5% CO_2 (pH 7.4) at room temperature during electrophysiological recording. All the experimental procedures were carried out as previously described (Bracci et al., 1996; Beato and Nistri, 1999; Taccola et al., 2008) with ethical permission granted by the ethics committee of our School.

Experimental protocols for early or delayed effects of CB1R ligands

Reflex and locomotor network activity were first tested in all preparations before any ligand application. On the first day of experimentation, preparations were superfused with cannabinoid ligands (AM-251, AEA), while any changes in electrophysiological responses were continuously monitored. Ligands were then washed out and the preparations were kept in Kreb's solution (24 h) for further electrophysiological testing. In a separate batch of experiments, preparations were treated continuously for 24 h with ligands (AM-251, rimonabant, AEA, LY 218324, 2 AG, or JZL 184) that were washed out before recording on the second day. All experiments were done in parallel with untreated "sham" preparations kept for 24 h in Kreb's solution to be used as control. The same protocol of drug application was used for quantifying basal cyclic adenosine monophosphate (cAMP) levels: these experiments also included an additional experimental group treated with AM-251 for 24 h, washed out and then treated with AEA for 30 min to analyze whether any sustained block of CB1Rs was reversed by short-term application of AEA. Finally, we measured changes in cAMP levels induced by forskolin (Frsk) (1 μM; 30-min application; Steffens et al., 2004) in preparations treated for 24 h with AM-251 or AEA: in this paradigm, CB1R ligands were also co-applied with Frsk.

Drug concentrations used for the present experiments were obtained from previously validated studies with the appropriate references provided for each one of the chemicals listed below. To detect changes in locomotor rhythms by blocking CB1Rs, two antagonist/inverse agonists were employed, namely AM-251 (5 μM; Kettunen et al., 2005; Gonzalez-Islas et al., 2012) or SR 141716A, (rimonabant; 5 μM; Kettunen et al., 2005). CB1Rs were activated by exogenously applying AEA (5–10 μM; Eljaschewitsch et al., 2006) or 2 AG (2–5 μM; Iannotti et al., 2014). We also used the AEA uptake inhibitor; LY 218324 (1–5 μM; Gonzalez-Islas et al., 2012) and the 2 AG uptake inhibitor JZL 184 (1–5 μM; Pan et al., 2009). All these drugs were dissolved in dimethyl sulfoxide (DMSO) except AEA (which was dissolved in ethanol) to reach a 10 mM stock from which final dilutions were made with Kreb's. In all cases, the final concentrations of DMSO or ethanol were also added to the Kreb's solution for control or sham preparations without inducing any detectable change in electrophysiological parameters.

Electrophysiology

Full details of these methods were previously reported (Marchetti et al., 2001; Taccola et al., 2008). In brief, to study reflexes and rhythmicity (flexor and extensor cyclic discharges) of the spinal cord, VRs of the lumbar region segments (L2 and L5) were tightly sucked into monopolar suction electrodes connected to Ag/Ag–Cl micropellets in glass micropipettes. To evoke VR responses, electrical square pulses (0.1-ms duration) were given through a single ipsilateral DR using a bipolar suction electrode. We graded the DR stimulus intensity to evoke either low-threshold monosynaptic responses when the stimulus was just above threshold to induce a detectable response (Fulton and Walton, 1986; Marchetti et al., 2001) or polysynaptic responses when the stimulus was three times higher. Our previous experiments with intracellular recording from motoneurons have validated the functional identification of these responses as either mono or polysynaptic (Ostroumov et al., 2007).

Dorsal-Dorsal root potentials (DR-DRP) were recorded by stimulating (0.1 ms) a single DR and recording from the ipsilateral segmental DR. Reflex responses were the average from 3 to 5 events in each group. To study electrically induced fictive locomotion, a train of 30 pulses (5–7 V) at 2-Hz frequency was given. Alternating rhythmic discharges were also recorded by bath applying NMDA (2–6 μM) and 5-hydroxytryptamine (5-HT; 10 μM) as amply reviewed by Kiehn (2006). To analyze the cycle peak amplitude and periodicity, twenty constitutive oscillations were taken as described in our previous studies (Taccola et al., 2008). The amplitude of each locomotor biphasic cycle was measured from peak to peak, and averaged for at least twenty responses. Disinhibited bursting was elicited by blocking γ-aminobutyric acid (GABA)-A and glycine receptors using bicuculline (20 μM) and strychnine (1 μM), respectively (Bracci et al., 1996). All signals were acquired, digitized and recorded in pClamp version 9.2 (20-kHz acquisition frequency with high- and low-pass filters of 0.1 Hz and 10 kHz, respectively; Molecular Devices, Sunnyvale, CA, USA) using a DP-304 Differential amplifier (Warner Instruments LLC, Hamden, CT, USA) in DC mode.

Responses to exogenously-applied (2 min) transmitter agonists GABA (10 μM), glycine (100 μM), NMDA (10 μM) or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (2.5 μM) were recorded from VRs. Concentrations of these drugs were chosen to produce approximately equi-amplitude depolarizations, with the exception of glycine that was a much weaker agent. After washout and recovery to basal level of VR polarization, AEA (or the CB1 antagonist AM-251) was applied for 30 min and responses re-tested on the same preparation. We also studied whether 24 h *in vitro* might have altered the effects of AEA on these drugs. Thus, after 24 h exposure to AEA (or its antagonist AM-251) and its washout, we tested these transmitter agonists diluted in standard Krebs solution and compared the responses with those from sham spinal cords (24 h) run alongside the treated ones.

In view of the observed late effects induced by CB1 agonist or antagonist application, we also tested whether these phenomena might have actually been due to relatively rapid developmental changes that occurred during 24 h *in vitro* rather than delayed actions due to CB1R modulation. Thus, we studied if spinal cords removed from older rats (P4) could exhibit locomotor-like patterns induced by NMDA (5 μM) and 5HT (10 μM), or DR stimuli. Fictive locomotor patterns are typically studied with spinal cords removed from very young preparations (Cazalets et al., 1992; Kudo and Nishimaru, 1998) because those obtained from older animals are less viable *in vitro* unless hemisected (Long et al., 1988), a procedure that abolishes fictive locomotion. In the present study, stronger DR electrical pulses (5–7 × threshold for monosynaptic reflex vs 2–3 × threshold in P0–P2 spinal cords) were necessary to elicit cumulative depolarization with superimposed oscillations. These older preparations were re-tested after a 3-h application of AEA or AM-251.

Quantification of pyknosis and CB1R immunohistochemistry

Free-floating sections (from the thoraco-lumbar region) of 30-μm thickness were cut (with a sliding microtome) from spinal cords after 4% paraformaldehyde fixation and 30% sucrose cryoprotection. For cell death quantification based on pyknosis counts, images were taken using a Zeiss Axioskop2 microscope (Oberkochen, Germany) and Metavue software (Molecular Devices, Sunnyvale, CA, USA) after DAPI staining. Standard ROIs of $350 \times 350\ \mu m^2$ for dorsal and central, $235 \times 235\ \mu m^2$ for ventral areas were constructed and counted in ImageJ (NIH) in accordance with our former studies (Cifra et al., 2012).

For CB1R immunostaining, sections were incubated in blocking solution (5% normal donkey serum, 5% bovine serum albumin, 0.3% Triton-X 100) for 2 h at room temperature. The primary antibody (1:50 dilution; CB1 (K-15) antibody; Santa Cruz Biotechnology, Inc.) in antibody solution (1% normal donkey serum, 1% bovine serum albumin, 0.1% Triton-X 100) was incubated at 4 °C overnight. Sections were then washed (in phosphate buffer solution) and incubated with the secondary anti-rabbit Alexa Fluor 488 antibody (1:500) in the antibody solution for 2 h. They were finally stained with DAPI and imaged using a Confocal-Nikon microscope with identical capture settings throughout. Five images were acquired at *z* axis intervals of 1 μm at each region. To validate the specificity of the CB1R primary antibody, it was neutralized with its blocking peptide (supplied by the CB1 (K-15) antibody manufacturer) in 1:10 dilution at room temperature as instructed by the supplier. Spinal cord sections were then incubated in the neutralized mixture and processed as mentioned above. No CB1R staining was subsequently observed. The following ROIs were used: Dorsal – $200 \times 300\ \mu m^2$; Central $250 \times 250\ \mu m^2$ and Ventral $200 \times 200\ \mu m^2$ to quantify the intensity of CB1R. Images were processed and analyzed in volocity workstation (Perkin Elmer, London, UK) as previously described by Bianchetti et al. (2013).

cAMP immunoassay

At the end of the experimental protocol of drug application, spinal cords were immediately frozen in liquid nitrogen and processed in accordance with the manufacturers' instructions (Abnova; http://www.abnova.com/products/products_detail.asp?catalog_id=KA0886) on the same day to minimize cAMP degradation. CAMP levels were normalized with respect to the protein concentration assayed with the bicinchoninic acid method.

Drugs

AM-251, AEA, rimonabant, 2 AG, LY 218324, JZL 184 and NMDA were purchased from Tocris (Bristol, UK), while 5-HT and strychnine hydrochloride were from Sigma (Milan, Italy). Bicuculline methiodide was purchased from Abcam (Cambridge, UK).

Statistics

Results are expressed as mean ± SEM, with n = number of preparations. Statistical analysis was performed with SigmaStat (SigmaStat 3.1, Systat Software, Chicago, IL, USA). Data were distinguished as parametric or non-parametric by performing a normality test and then analyzed with the t-test or Mann–Whitney test, respectively, in accordance with the software choice. The accepted significance level was always $P < 0.05$.

RESULTS

AEA-mediated changes in responses evoked by exogenously-applied transmitters

Because of the use of neonatal tissue and its *in vitro* condition, we first explored whether CB1Rs were functional and whether their activation by AEA might change spinal network effects induced by GABA (10 μM), glycine (100 μM), NMDA (10 μM) or AMPA (2.5 μM). Fig. 1A shows, in the same preparation, examples of VR depolarization elicited by superfusing these transmitter agonists (that evoked responses as previously reported; Nistri and Constanti, 1979; Watkins and Evans, 1981), and then repeated 30 min and 24 h after applying AEA (5 μM). On the first day, while no change was observed in the effects by glycine, NMDA or AMPA, a small, yet significant decrease in GABA-mediated depolarization was on average detected (Fig. 1B). This pattern of modulation of GABA-mediated responses was not preserved after a 24-h application of AEA and washout followed by recording agonist-elicited responses in standard Kreb's solution: in fact, depolarizing effects of these agonists were similar to those obtained from sham (24 h) preparations (Fig. 1A, B). It is expected that superfusion of exogenous agonists most likely activated a broad class of receptors widely distributed within spinal networks. Thus, we subsequently investigated whether AEA (or its antagonists) might modulate more focused, i.e. synaptic, actions of these transmitters and locomotor network-dependent activity.

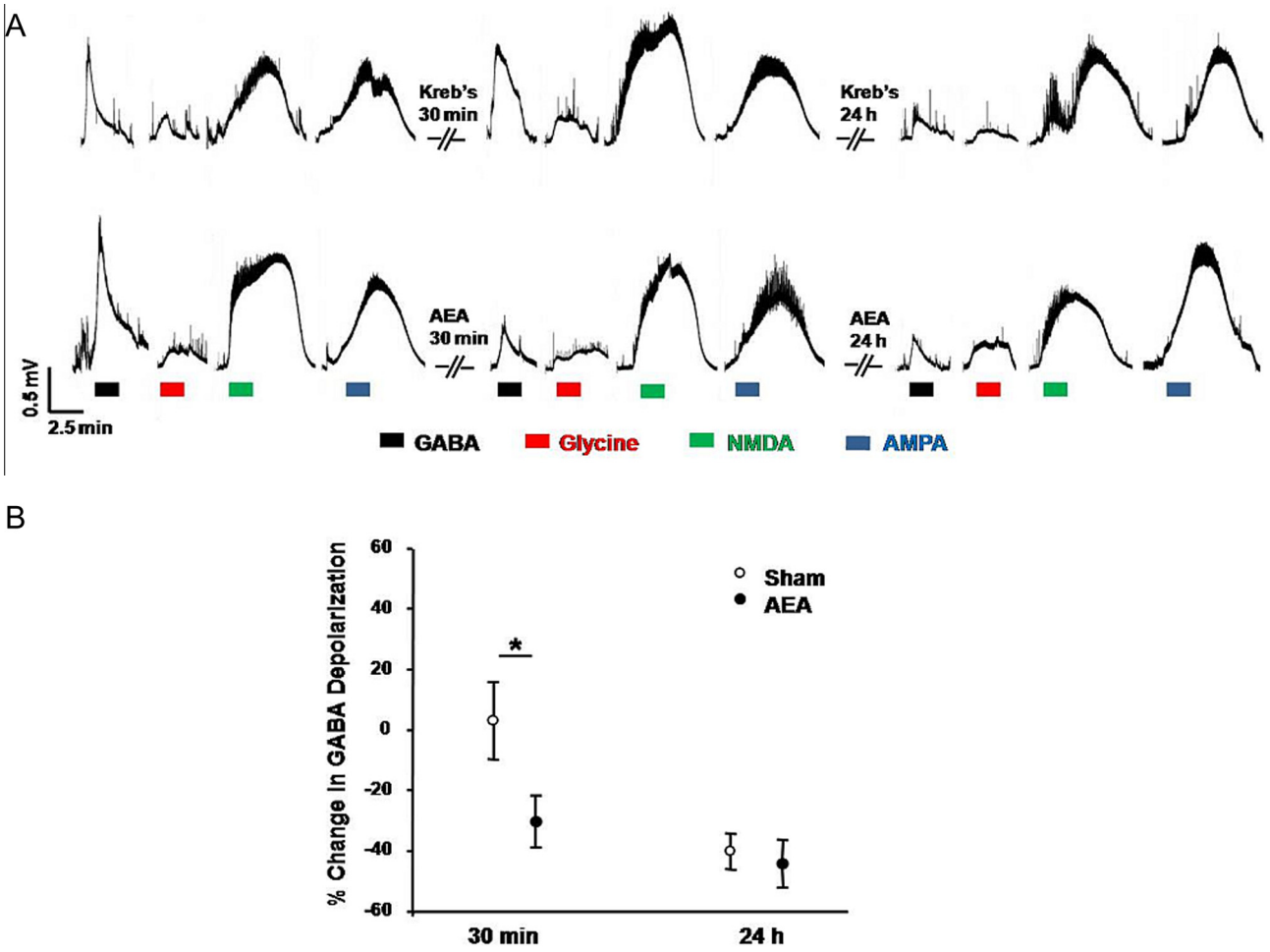

Fig. 1. Effect of AEA on VR depolarization evoked by exogenously-applied transmitter agonists. (A) Top row illustrates examples of responses recorded from a lumbar VR of the same preparation following superfusion with GABA (10 μM), glycine (100 μM), NMDA (10 μM), or AMPA (2.5 μM) in control solution. Bottom row shows that AEA (5 μM) depresses GABA responses after a 30-min application. No significant alteration in the response to the other agonists is observed. (B) Plots show average depression (%) in GABA-mediated depolarization after a 30-min application of AEA (n = 16 vs n = 9 sham; $P < 0.05$), whereas no change is observed after a 24-h application of AEA compared to sham; for the 24 h data % changes refer to the values recorded on the first day.

Changes in spinal reflexes and fictive locomotion evoked by CB1R ligands

We first explored the effects of AEA (5–10 μM) or the CB1R antagonist/inverse agonist AM-251 (5 μM) on mono or polysynaptic transmission elicited by DR stimulation as exemplified in Fig. 2A, B. Neither agent continuously applied for up to 6 h significantly changed monosynaptic reflexes in terms of peak or area values (Fig. 2C, D). Likewise, no significant alteration in the polysynaptic reflex amplitude and area was apparent (Fig. 2E, F). Despite the lack of effect by these ligands on standard tests for synaptic transmission, a clear change was observed when the locomotor network was activated by DR stimulation. This phenomenon is illustrated in Fig. 3A in which a cluster of oscillatory cycles emerged with 2-Hz pulse train applied to a single lumbar DR in control conditions. While AEA did not affect this response, a 3–6-h application of AM-251 significantly depressed such a rhythmic activity (Fig. 3A, B) without changing the cumulative VR depolarization arising from the DR stimulus train (Fig. 3C). Interestingly, no change in cycle amplitude or periodicity appeared when fictive locomotion was evoked by NMDA plus 5-HT (Fig. 3D–F) and recorded for 3–6 h in the presence of AEA or AM-251.

These observations suggested that blocking CB1Rs inhibited the integration of repeated afferent inputs into the locomotor networks, yet it did not affect the locomotor central pattern generator when this was stimulated with neurochemicals. One possibility was that AM-251 largely modulated GABA-mediated synaptic transmission especially at dorsal level. This issue could be examined in the spinal cord by recording the DR-DRP, namely the primary afferent depolarization functionally expressed as presynaptic inhibition for which GABA receptor activation is one important contributor (Levy, 1977; Rudomin, 2009). Nonetheless, DR-DRP amplitude and area (control amplitude = 0.49 ± 0.1 mV; area = 827.89 ± 279.03 mV × ms; n = 3) were not significantly different after applying AM-251 for 3 h (amplitude = 0.38 ± 0.1 mV; area = 726.11 ± 292.78 mV × ms; n = 3). In keeping with these observations, there was no change induced by AM-251 on GABA- (10 μM) mediated VR depolarizations (0.84 ± 0.13 mV vs 0.71 ± 0.10 mV in control solution; n = 7).

Fictive locomotion is just one of the rhythmic patterns originated in the isolated rodent spinal cord. To check for the pattern selectivity of CB1R ligand-mediated changes, we investigated the intrinsic rhythmicity that emerges when synaptic inhibition is blocked by strychnine and bicuculline. Thus, slow and large bursts (disinhibited bursting) appear through the recurrent excitation mediated by glutamatergic synapses and modulated by intrinsic neuronal conductances (Bracci et al., 1996). Fig. 4A shows examples of disinhibited bursting in control conditions (3 h in Kreb's solution), or in the continuous (3 h) presence of AEA or AM-251. No statistical difference in the number of bursts, burst amplitude or duration was found (Fig. 4B–D), suggesting that the oscillatory cycles elicited by DR stimulation were the primary functional target for CB1R block.

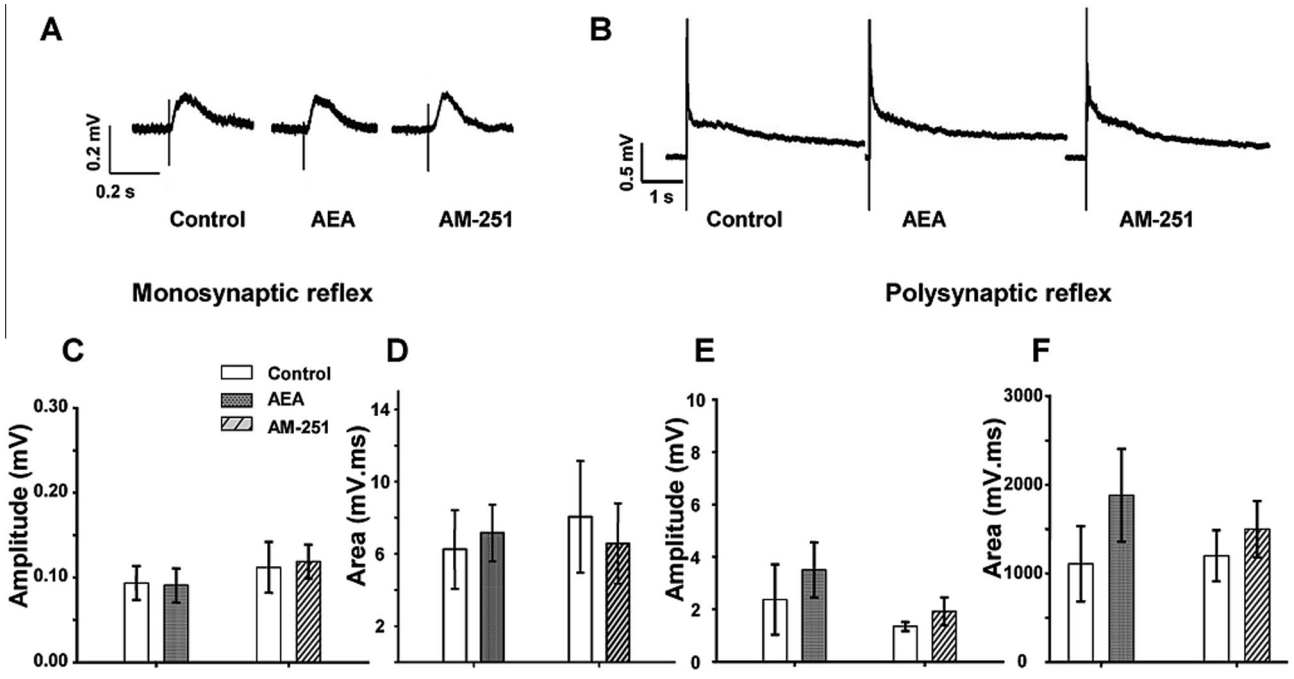

Fig. 2. Effects of AEA and AM-251 on reflex activity after a 3–6-h application. (A, B) Examples of average monosynaptic (A) or polysynaptic (B) reflex responses recorded from lumbar 5 VR in control, AEA or AM-251 solution. (C, D) Histograms showing the peak amplitude of mono (C) and polysynaptic (E) responses after AEA or AM-251 treatment compared with time-matched control (n = 14), AEA (n = 5) or AM-251 (n = 14). (D, F) Histograms representing the area of mono and polysynaptic reflex responses following AEA or AM-251 application (same data as shown in C, E).

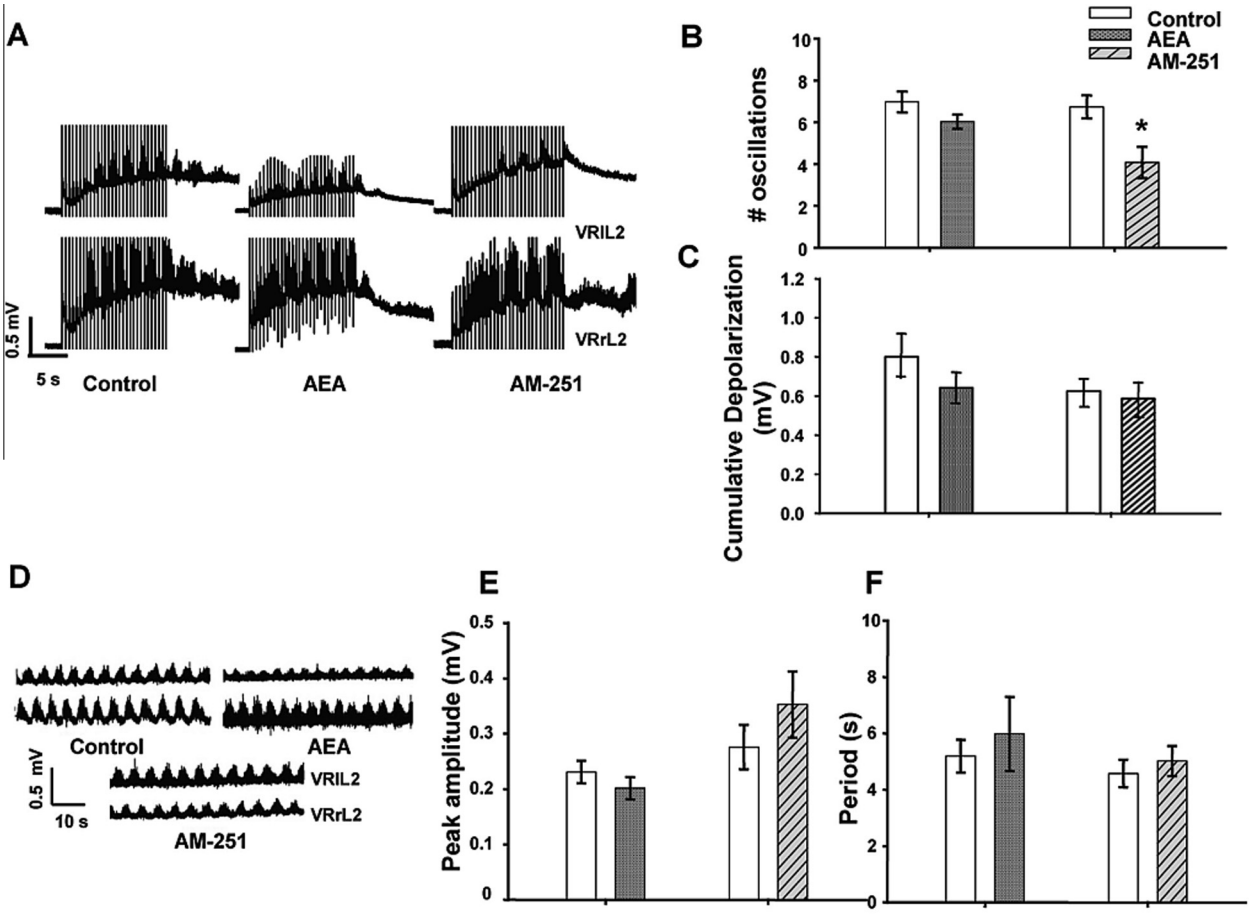

Fig. 3. Changes in network responses after AEA or AM-251 treatment. (A) Electrically evoked fictive locomotion in control (left), AEA (middle) or AM-251 (right) solution. Note depression of rhythmic discharges by AM-251 treatment (3–6 h). (B) Histograms of the number of oscillations induced by DR stimulation. Note that there is a significant decline in the number of oscillations after AM-251 3–6-h application (n = 14). (C) Bar graphs showing no significant change in cumulative depolarization after AEA or AM-251 application. (D) Examples of fictive locomotion induced by NMDA and 5-HT in control (top left), AEA (top right) or AM-251 (bottom), 3–6-h application. (E) Histograms showing no change in peak amplitude of chemically evoked oscillations after AEA or AM-251 treatment compared with control (same preparations shown in B). (F) Histograms showing the periodicity of the rhythm generated by NMDA and 5-HT after AEA or AM-251 application in comparison with time-matched controls. Note that there is no change in the parameters of chemically evoked fictive locomotion. $^{*}P < 0.05$.

Longer (24 h) term changes in reflex activity evoked by CB1R ligands

We wondered if the delayed network depression observed in the presence of AM-251 might be intensified for longer application times (24 h). For this purpose, experiments were performed on the second day from the spinal cord dissection and used ''sham'' preparations (namely maintained *in vitro* for 24 h in Kreb's solution) for comparison. We also explored the long-term consequences of a 24-h application of other CB1R ligands (see review by Pertwee et al., 2010) like AEA, the CB1R antagonist rimonabant, the AEA uptake inhibitor LY 2183240, the CB1R agonist 2 AG, or the 2 AG uptake inhibitor JZL 184. In all these protocols (see Experimental procedures), the ligands were washed out just prior to the recording session on the second day of each experiment, in order to enable detection of delayed consequences on network function.

On average sham monosynaptic responses were 0.14 ± 0.02 mV in amplitude (n = 15). There was no difference in monosynaptic reflexes after overnight exposure to AEA (0.10 ± 0.01 mV, n = 17), or AM-251 (0.18 ± 0.03 mV, n = 19). Likewise, neither 2 AG (2–5 μM; 0.21 ± 0.11 mV; n = 8), nor LY 2183240 (1–5 μM; 0.06 ± 0.01 mV; n = 6), nor JZL 184 (5 μM; 0.11 ± 0.02 mV; n = 7) changed the monosynaptic reflex amplitude.

Fig. 5A shows representative records of the depression of polysynaptic transmission evoked by a 24-h application of AM-251. While the average peak amplitude was unchanged (0.87 ± 0.13 vs 0.84 ± 0.28 mV for treated, n = 19, and sham, n = 15, respectively), the area of the response became significantly smaller as indicated in Fig. 5B, C. Thus, the average reflex time course plot demonstrated faster decay to the baseline (Fig. 5C). In analogy with data observed on the first day of treatment, the DR-DRP parameters were not changed by a 24-h administration of AM-251 (amplitude: 0.25 ± 0.02 vs 0.20 ± 0.03 mV; area: 379.03 ± 52.28 vs 741.04 ± 195.88 mV × ms for

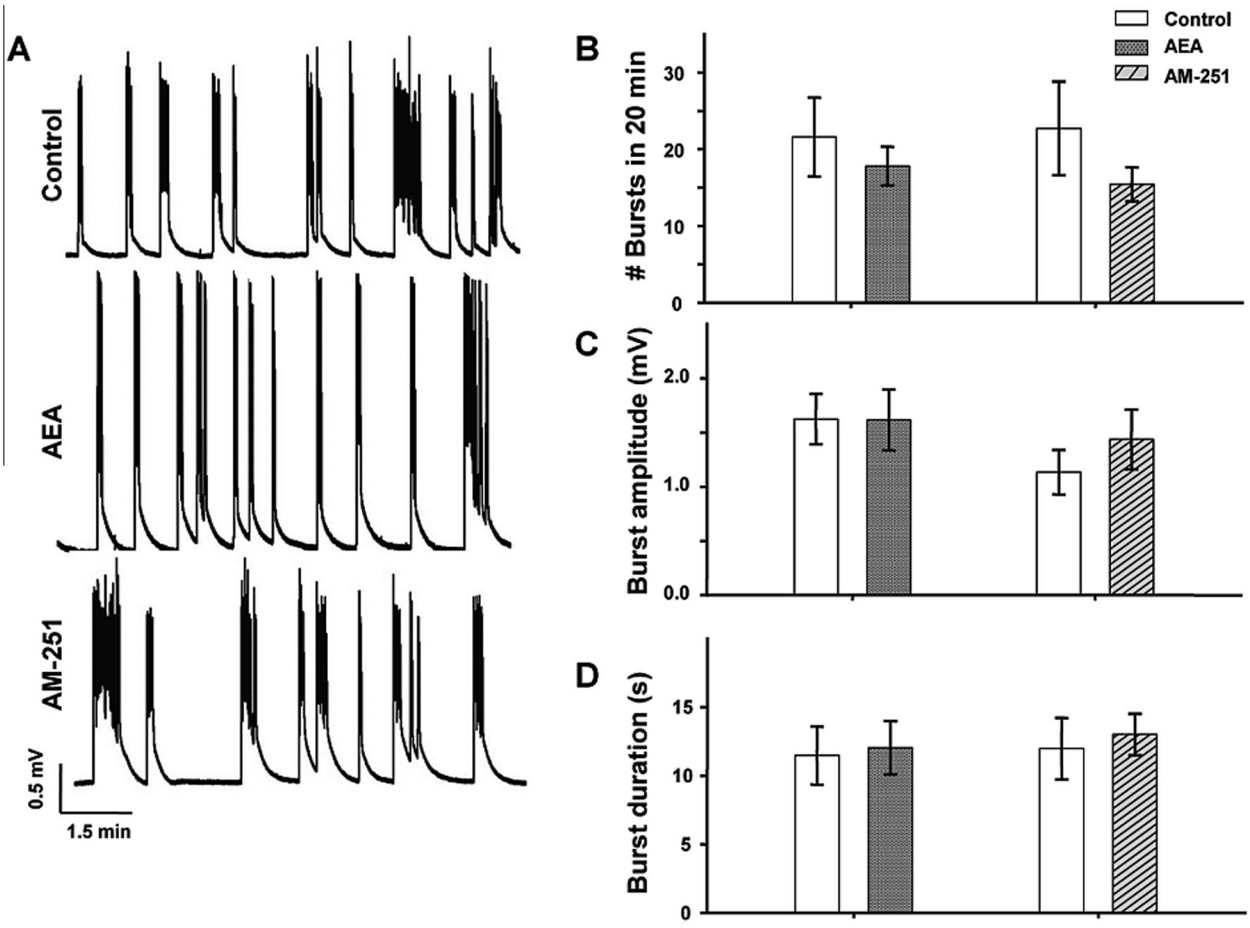

Fig. 4. Disinhibited bursts after 3–6 h of CB1R ligand application. (A) Sample disinhibited burst recordings obtained from control (top), AEA-treated (middle) or AM-251-treated (bottom) preparations. Bursts were evoked by strychnine and bicuculline application. (B–D) Histograms showing no effects by AEA (n = 5) or AM-251 (n = 7) on number of bursts in 20 min (B), burst amplitude (C), and duration (D).

sham and AM-251, respectively; n = 5). Table 1 summarizes the effect of the other CB1R ligands on polysynaptic reflexes.

Network rhythmicity after a 24-h exposure to CB1R ligands

Fig. 6A shows that AEA treatment (24 h) significantly impaired the ability to generate oscillations induced by a DR stimulus train. This effect was associated with slightly lower amplitude of cumulative depolarization (0.60 ± 0.05 vs 0.89 ± 0.09 sham preparations; n = 17, P < 0.05).This phenomenon was even more dramatically observed after 24 h AM-251 application as no (or rare) oscillations were present despite strong cumulative depolarization and irregular discharges associated with the DR train. Fig. 6B quantifies these observations for AEA or AM-251 treatments. Table 1 shows that rimonabant, LY 2183240 or JZL 184 also decreased the number of rhythmic oscillations, while 2 AG was ineffective.

A different picture emerged from experiments with chemically-evoked fictive locomotion. In fact, as shown in Fig. 6C, while a 24-h treatment with AEA had no blocking effect (data are summarized in Fig. 6D, E) in analogy with LY 2183240, 2 AG or JZL 184 (see Table 1), preparations treated with AM-251 for 24 h could be split into two groups. The majority of them (11/19 spinal cords) could not generate any fictive locomotor cycles (Fig. 6C) despite systematic stepwise changes in the NMDA concentration (2–6 μM). In the remaining eight spinal cords, fictive locomotion was evoked, however the rhythm was very much slower than the sham one with diminished cycle amplitude (Fig. 6D, E).To validate this result, we also explored the effect of a chemically different CB1R antagonist, rimonabant (5 μM; 24 h) that significantly prolonged the cycle period (7.7 ± 2.2 s; n = 6, three further preparations lacked fictive locomotion; Table 1). The slowly developing depression of fictive locomotion by AM-251 was confirmed by examining the outcome after just 3–6 h of treatment with AM-251 on the first experimental day followed by a 24-h washout with Kreb's solution. In this case, on a database of 15 spinal cords, we observed a lower number of oscillations (3.8 ± 0.3; n = 14, p < 0.001) induced by standard DR trains, while the chemically-elicited fictive locomotion showed a significantly smaller cycle amplitude (0.23 ± 0.03 mV; n = 14, p < 0.05) and slower period (6.4 ± 0.40 s; n = 14, p < 0.05). These results indicated that any

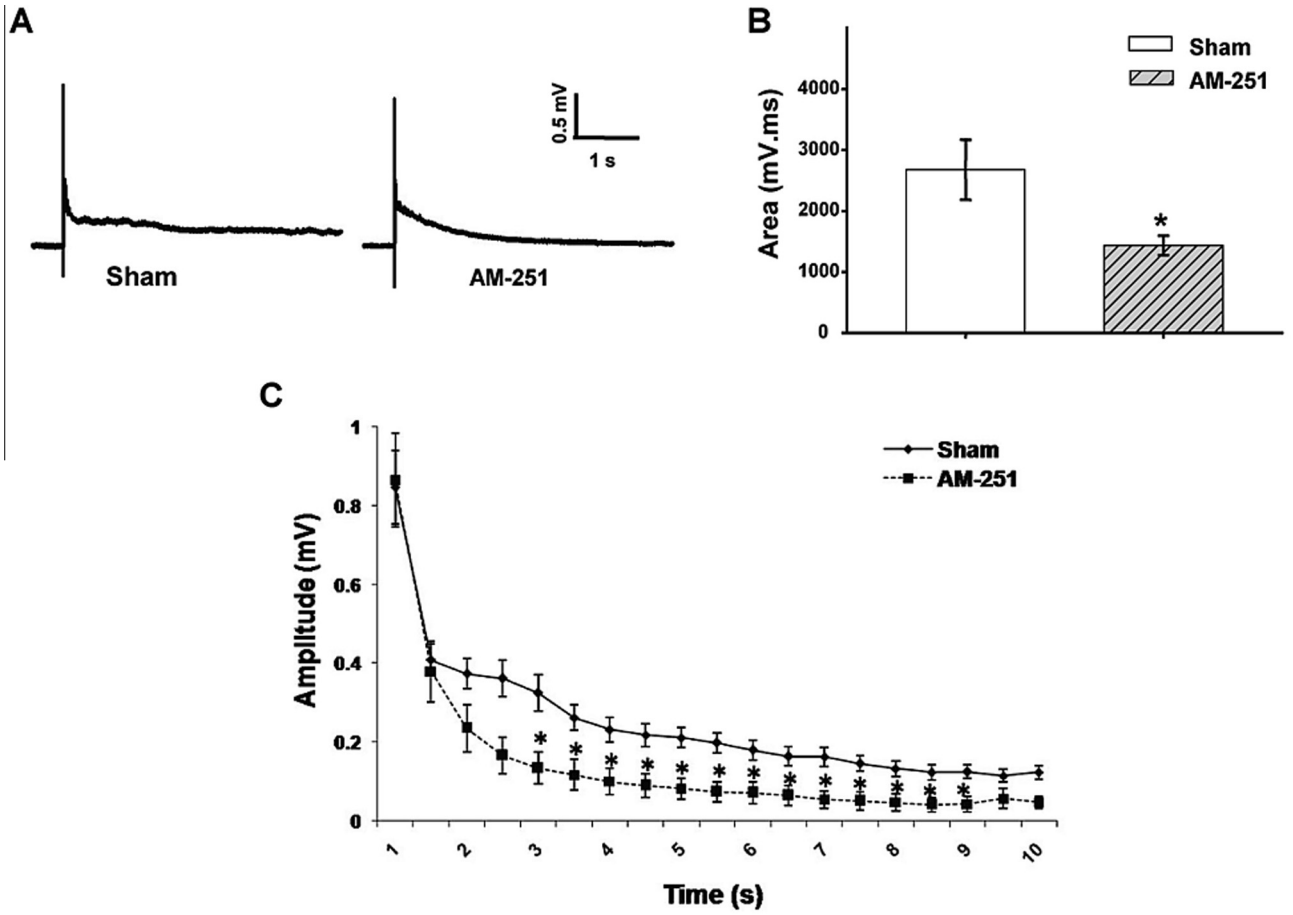

Fig. 5. Changes in polysynaptic reflex response after AM-251 24-h treatment. (A) Sample recordings of control (left) and AM-251 24-h-treated (right) preparations. (B) Bar graph indicating the significant reduction in polysynaptic reflex response area after AM-251 24-h application, $n = 19$. (C) Plot of decay time course of the polysynaptic reflex showing significant change in AM-251-treated preparations compared with sham (same spinal cords as in B). $^{*}P < 0.05$.

change induced by AM-251 was actually quite slow, as it grew over a matter of a few hours and could not be rapidly arrested by washout. On disinhibited bursting (Fig. 7A, B), a 24-h application of AM-251 produced a comparatively lower number of bursts vs sham preparations and no further change in burst properties. Conversely, AEA application for 24 h had no effect (see Fig. 7B–D).

Downregulation of CB1R expression by AM-251

We first examined whether a 24-h application of AM-251 had any neurotoxic effect. We looked for pyknosis in the three main ROIs as used in previous reports (Cifra et al., 2012). Thus, we saw no evidence for pyknosis in dorsal, central or ventral ROIs as illustrated in Fig. 8B, C that confirmed full histological viability of the preparations after 24 h *in vitro*. We next looked for immunofluorescence expression of CB1Rs in dorsal, central and ventral ROIs as shown in Fig. 9A. The non-nuclear localization of the CB1R staining made it difficult to count the number of positive cells. Hence, in analogy with our previous reports (Bianchetti et al., 2013), we recorded the average fluorescence intensity (AU) in dorsal, central and ventral regions (Fig. 9A, B). A significant fall in immunofluorescence intensity was observed for the central area after application of AM-251 for 24 h, suggesting that main downregulation of CB1R expression was topographically-localized to this region.

CB1R signaling following sustained application of ligands

One principal effector of CB1R activation in the central nervous system is the depression of cAMP synthesis, whereas CB1R block may be associated with a rise in cAMP levels depending on the degree of constitutive activity of CB1Rs (Howlett et al., 2004). We used this phenomenon as a readout for the ability of AEA or AM-251 to activate or inhibit cAMP formation either under basal conditions or following Frsk (1 μM)-evoked activation of adenylyl cyclase (Steffens et al., 2004). For this purpose, we employed an ELISA-based assay with spinal cord samples collected after 24 h *in vitro* with (or without) subsequent application of Frsk for 30 min. Fig. 10A shows that, under basal conditions, the cAMP levels at 24 h were similar for sham or AM-251-treated spinal cords. A significant reduction was, however, observed after a 24-h application of AEA. Fig. 10A also shows that, on AM-251-treated preparations, AEA (30 min) did not depress basal cAMP.

Table 1. Effect of cannabinoid ligands after a 24-h treatment on reflex and rhythmic discharges

Parameters		Sham (n = 15)	Rimonabant (24 h; n = 6)	LY 2183240 (24 h; n = 6)	2 AG (24 h; n = 8)	JZL 184 (24 h; n = 7)
Reflex	Monosynaptic amplitude (mV)	0.14 ± 0.02	0.15 ± 0.03	0.06 ± 0.01	0.21 ± 0.11	0.11 ± 0.02
	Monosynaptic area (mV ms)	15.13 ± 3.25	12.35 ± 2.31	11.92 ± 2.09	13.33 ± 1.42	11.55 ± 1.07
	Polysynaptic amplitude (mV)	0.84 ± 0.28	0.79 ± 0.04	0.61 ± 0.13	0.69 ± 0.06	0.79 ± 0.10
	Polysynaptic area (mV.ms)	2675.15 ± 491.36	1354.74 ± 219.42*	771.00 ± 456.25	1814.52 ± 368.58	1346.37 ± 394.47
Dorsal root train	# oscillations	6.83 ± 0.63	1.44 ± 1.06**	1.83 ± 1.13*	5.78 ± 0.64	2.81 ± 0.38*
	Cumulative depolarization (mV)	0.80 ± 0.08	1.13 ± 0.17	0.320 ± 0.12	0.61 ± 0.18	0.67 ± 0.07
	Area (mV.ms)	18385.22 ± 2105.22	24091.89 ± 4706.64	5832.01 ± 2367.88	14719.73 ± 6633.74	16654.38 ± 2415.18
NMDA/5-HT	Amplitude (mV)	0.26 ± 0.04	0.22 ± 0.09	0.13 ± 0.04	0.17 ± 0.03	0.18 ± 0.04
	Period (s)	4.75 ± 0.34	7.74 ± 1.29* (3 cords no oscillation)	4.90 ± 0.68 (1 cord no good oscillation)	5.05 ± 0.35	4.36 ± 0.11
Disinhibited bursts	# bursts (in 20 min)	25.13 ± 3.90	14.25 ± 1.11*	25.67 ± 10.04	15.50 ± 2.79	38.67 ± 5.84*
	Burst amplitude (mV)	0.92 ± 0.14	1.14 ± 0.26	0.86 ± 0.09	0.89 ± 0.13	0.65 ± 0.14
	Burst duration (s)	18.08 ± 4.95	18.68 ± 4.20	9.277 ± 1.75	23.94 ± 5.27	9.42 ± 1.19

* $P < 0.05$.
** $P < 0.005$.

Fig. 10B shows that Frsk-stimulated cAMP to similar levels in sham or AM-251-treated (24 h) preparations. The outcome of AEA treatment (24 h) was different as the action of Frsk was significantly decreased (Fig. 10B). Furthermore, co-application (30 min) of AEA together with Frsk to preparations previously treated for 24 h with AM-251 24 evoked a cAMP rise as much as in sham preparations (Fig. 10B).

Effects of AEA or AM-251 on network activity in older spinal cords

The delayed effects observed after a 24-h application of CB1R ligands might have been actually due to ongoing developmental maturation of this tissue even in the *in vitro* condition. To assess this possibility, we recorded responses taken acutely from P4 spinal cords *in vitro*. As shown in Fig. 11A, fictive locomotion induced by NMDA and 5HT was observed with periodicity and alternation similar to the values found at P0–P2. Table 2 summarizes the main characteristics of network effects at this postnatal age. When AM-251 or AEA was applied for 3 h, no significant alteration in any of these parameters was observed with the notable exception of the number of oscillations associated with DR stimulation during AM-251 application (Fig. 11B and Table 2).

DISCUSSION

The principal finding of the present report is the novel observation that application of the CB1R antagonist AM-251 (or the pharmacological analogue rimonabant) triggered off a delayed (24 h) strong depression of locomotor network activity in the rat spinal cord *in vitro* despite modest changes in fast synaptic transmission. Such a modulatory action showed a gradient of neurodepression that was first targeted to the locomotor-like activity recruited by afferent impulses. Conversely, CB1R agonists evoked just moderate depression of locomotor networks. These data have implications for the prolonged use of CB1 R drugs *in vivo* and are the first electrophysiological description of how mammalian locomotor circuits react to their sustained administration.

CB1Rs in spinal networks

A large body of evidence shows that CB1Rs potently modulate nociceptive signal processing in the dorsal horn (Kato et al., 2012; Piomelli et al., 2014) by regulating synaptic inhibition mediated by GABA and glutamatergic synaptic excitation (Castillo et al., 2012; Katona and Freund, 2012). These effects are typically, though not exclusively, exerted at presynaptic level via control of neurotransmitter release and are usually observed within a short time period. In this framework, CB1Rs are known to inhibit the synthesis of cAMP, an important intracellular regulator of ligand and voltage-gated channels (Howlett et al., 2004). On the other hand, CB2 receptors are poorly expressed by spinal neurons and appear to be mainly associated to activated microglia after experimental chronic inflammation (Burston et al., 2013).

Despite extensive studies carried out on the lamprey spinal cord (El Manira and Kyriakatos, 2010) indicating the modulatory role of CB1Rs on locomotor networks (Kettunen et al., 2005), the involvement of CB1Rs in mammalian locomotion remains unclear. While CB1R activation globally reduces *in vivo* locomotion in mammals (Lee et al., 2006; Bosier et al., 2010; Chaouloff et al., 2011; McLaughlin et al., 2013), the mechanisms underlying this phenomenon are not readily resolved with motor behavior experiments. It is, however, clear that, even after a single injection of a CB1R agonist, decrease in locomotion occurs rapidly (Di Marzo et al., 2000) and can persist for many hours with concomitant changes in brain metabolic activity (Whitlow et al., 2002). This issue seems important because of the increasing focus on the use of cannabinoid agonists to control chronic pain and spasticity as demonstrated by recent clinical guidelines (Koppel et al., 2014). Conversely, there is large interest in developing novel antagonists to suppress food craving or cannabis dependence (Borgelt et al., 2013) as the more traditional CB1R blockers possess major neuropsychiatric side effects in man (Jones, 2008; Bifulco and Pisanti, 2009). None of these therapeutic targets are achieved with short-term pharmacological treatments. Thus, the present study sought to investigate how rat locomotor networks may react to sustained application of cannabinoid agonists and antagonists.

Early effects of cannabinoid drugs

Rather unexpectedly, we found that the CB1R agonist AEA or the antagonist AM-251 had no significant effect on synaptic transmission and network function despite the fact that these receptors are widely expressed in the rat spinal cord (Herkenham et al., 1991; Farquhar-Smith et al., 2000). It is interesting to note that CB1Rs are developmentally regulated in so far that neonatal and young preparations express them at higher level with full functional activity (Kato et al., 2012; Galve-Roperh et al., 2013). Furthermore, we observed similar results even when recording from P4 preparations, a finding that made unlikely that developmental changes occurring during the long *in vitro* experiment were confounding our observations at P0–P2. Hence, it is not simple to attribute lack of acute changes in synaptic transmission merely to immaturity of CB1Rs in the neonatal rat spinal cord: in fact, their immunohistochemical expression was readily observed in the present study. Even the very large disinhibited bursts were not changed by AEA or AM-251 application, suggesting that CB1Rs were not directly contributing to the control of such events contrary to our early expectation that they should perhaps be capable of retrogradely affecting synaptic release (Castillo et al., 2012; Katona and Freund, 2012). These data led us to actually query if CB1Rs had any functional activity in the neonatal spinal cord. Thus, we investigated whether VR depolarizations induced by transmitter agonists were affected by AEA and observed that GABA responses were decreased by approximately 30%. Such VR responses presumably included a broad contribution by extrasynaptic and synaptic receptors distributed within the wide spinal network and could not pinpoint the site of GABA response modulation. Thus, while these data could not per se clarify the mechanism of action of CB1Rs in this preparation, they did show that such receptors were functionally active (as confirmed by studying changes in cAMP concentrations) and that their acute impact on glutamatergic receptors was minimal. The depression of GABA responses by AEA accords with previous reports of CB1R-mediated inhibition of GABAergic transmission in the rat dorsal horn (Jennings et al., 2001; Pernía-Andrade et al., 2009).

We, therefore, examined the possibility that CB1Rs had a subtler action involved in the maintenance and modulation of important network activities besides any rapid control over synaptic transmission. Indeed, a potential role of these receptors was hinted by the fact that sustained ($\geqslant$3 h) application of AM-251 significantly decreased the number of oscillations elicited by a train of DR pulses even though the amplitude of mono and polysynaptic reflexes was unchanged. In addition, lack of changes in DR-DRPs confirmed that DR impulse conduction and primary afferent depolarization were not the origin of this depression. When we tried to wash out this effect and looked for any recovery one day later, we actually observed further deterioration of locomotor network function, suggesting that the former application of AM-251 had set off a slow depression targeted to locomotor circuits whose delayed consequences were apparent 24 h later. There was, however, no neurotoxic effect. It is noteworthy that clearance of AM-251 from a single administration *in vivo* requires just a few hours, indicating that this drug is not an irreversible CB1R antagonist (Liu et al., 2008). The present data do not conflict with a former study showing reversible depression of excitatory synaptic currents elicited by rather strong electrical pulses in superficial dorsal horn neurons of the adult mouse spinal cord (Kato et al., 2012): in fact, our report is based on responses elicited by weaker DR stimuli through activation of a complex and extended circuitry that presumably could compensate any discrete deficit in glutamatergic transmission detected by Kato et al. (2012) at single-cell level in the superficial dorsal horn laminae.

Delayed effects of cannabinoid drugs

It is noteworthy that the present protocols to examine delayed drug actions comprised full washout of the previously applied agents. Hence, any observed change was likely due to formerly developed phenomena. When AM-251 was applied for 24 h, the locomotor depression was very intense. Thus, oscillations induced by DR stimuli were almost completely suppressed together with a decrease in the area of single polysynaptic responses (implying impaired synaptic integration at network level), although the amplitude of the VR cumulative depolarization was not significantly altered. In line with this finding, expression of chemically-triggered fictive locomotion became impossible in the majority of preparations or very impaired in periodicity and cycle amplitude in the rest. It was interesting that, when very slow fictive locomotor cycles remained, they preserved alternation between flexor and extensor motor pools, suggestive of a deficit in the operation of the generator

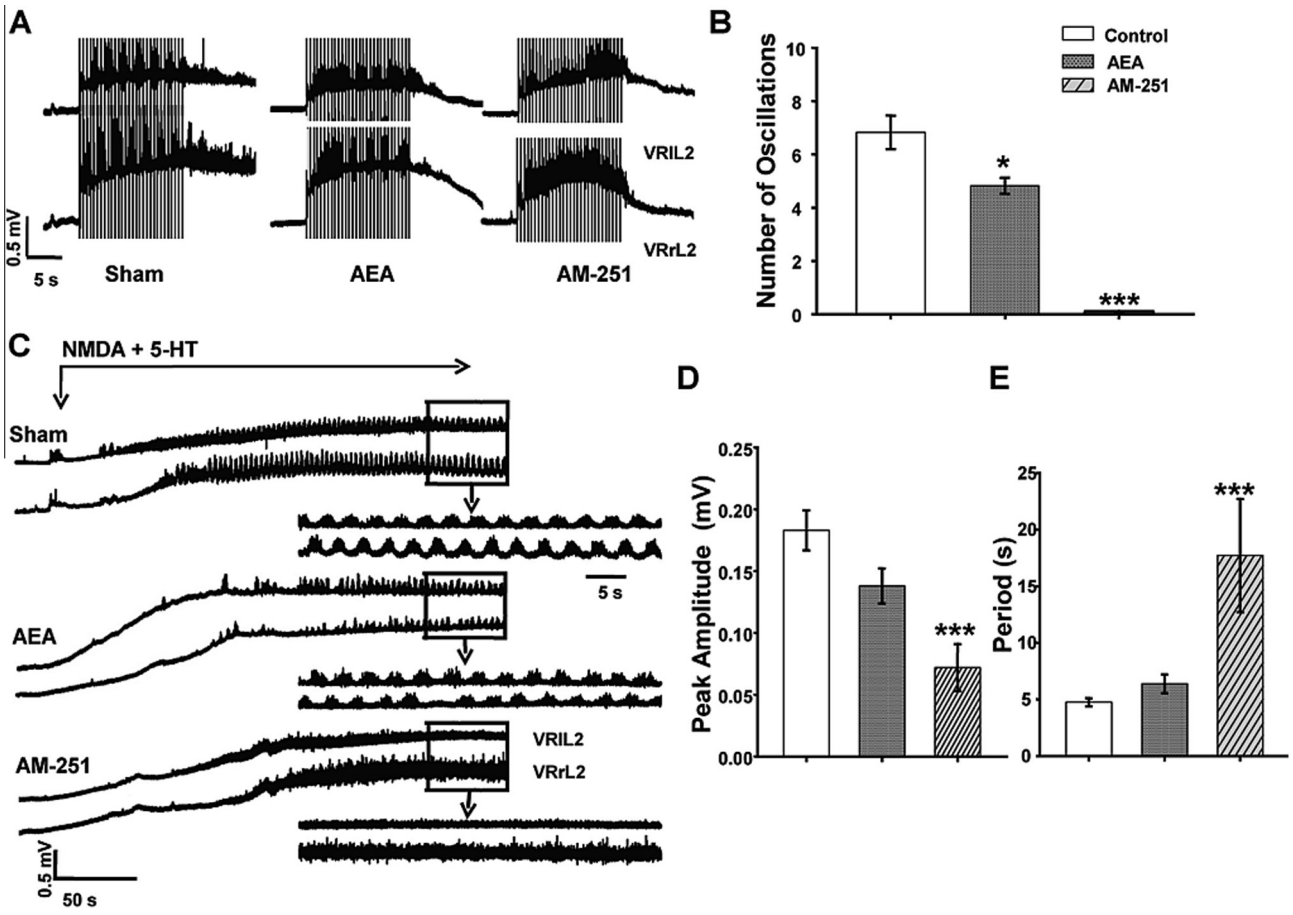

Fig. 6. Fictive locomotion changes after AEA or AM-251 24-h treatment. (A) Examples of electrically evoked fictive locomotion in control (left), AEA-treated (middle), or AM-251-treated (right) preparations. Note depression of oscillatory activity which is very intense after AM-251 treatment. (B) Histograms showing the number of oscillations evoked by electrical stimulation is significantly decreased in AEA- or AM-251-treated spinal cords; $n = 17, 19$, respectively. (C) Examples of fictive locomotion induced by NMDA and 5-HT in control (top), AEA-treated (middle), or AM-251-treated (bottom) preparations. The original records depict the onset of fictive locomotion that is also illustrated on a faster time scale taking the traces from the records in the box. (D, E) Histograms plotting the peak amplitude of locomotor cycles (D) or cycle period (E) for sham ($n = 15$), AEA-($n = 16$) or AM-251-($n = 8$) treated spinal cords. The AM-251 group comprises those preparations (8/19) in which chemically induced oscillations remained after AM-251 prolonged treatment. In the latter case, a significant fall in cycle amplitude and lengthening of period are observed. $^{*}P < 0.05$, $^{***}P < 0.001$.

clock rather than the pattern formation circuitry (McCrea and Rybak, 2008). These observations were validated by analogous results obtained with a 24-h application of another CB1R antagonist, namely rimonabant, indicating that the network depression was not a peculiar effect by AM-251, but it was probably a consequence of CB1R block. Interestingly, a slow depression of locomotor network function was also observed to gradually develop over the first few hours of CB1R antagonist application to the lamprey spinal cord (Kettunen et al., 2005) even if the underlying mechanism remains to be fully elucidated.

Close inspection of the effects of AM-251 revealed that there was a certain gradient of network sensitivity to this blocker-evoked depression. In fact, oscillatory cycles triggered by repeated afferent impulses appeared to be the most sensitive to AM-251, whereas monosynaptic reflexes or DR-DRPs were always unscathed. The periodicity of disinhibited bursting was also decreased, yet never eliminated, indicating that chronic block of CB1Rs decreased network excitability even when $GABA_A$ and glycine receptors were pharmacologically blocked. Although GABAergic mechanisms responsible for DR-DRPs were unchanged, this finding may simply reflect a rather limited role of CB1Rs in this specific pathway, while their function remains prevalent on laminae I-II neurons predominantly involved in nociceptive processing (Kato et al., 2012).

The delayed effects of CB1R agonism were qualitatively similar to those arising from chronic block, although quantitatively much less intense. Thus, only the number of DR train-evoked oscillations was diminished by a 24-h AEA, LY 2183240, or JZL 184 application. Interestingly, studies of rat locomotion *in vivo* have actually reported that systemic injection of either agonists or antagonists slowly depresses locomotion (Järbe et al., 2006). This apparent paradox might be explained if antagonists blocked CB1Rs and agonists partially desensitize them (Howlett et al., 2004; Martin et al., 2004) with delayed downstream effects on locomotor networks. Another possibility is that chronic application of agonist or antagonist led to differential coupling to G-proteins with distinct impact on neuronal

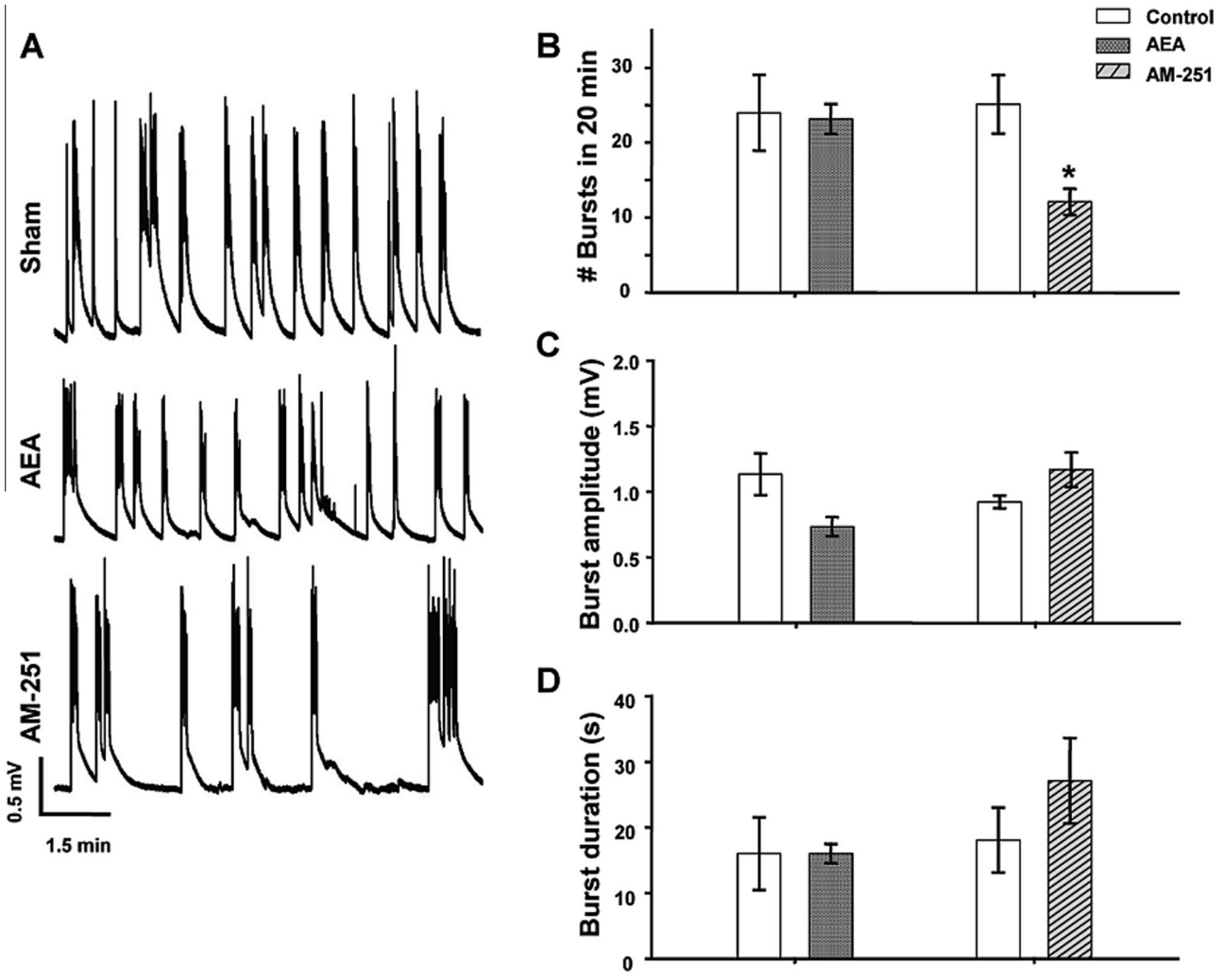

Fig. 7. Disinhibited bursts after 24 h of AEA or AM-251 application. (A) Examples of disinhibited burst recordings obtained from sham- (top), AEA-treated (middle) or AM-251-treated (bottom) preparations. (B–D) Histograms showing the effects by AEA or AM-251 on number of bursts in 20 min (B), burst amplitude (C), and duration (D). Note the significant reduction in number of bursts after AM-251 24-h treatment. $n = 9$, $^{*}P < 0.05$.

activity (Childers and Deadwyler, 1996), or to CB1R heteromerization with other membrane proteins possessing certain properties (Hudson et al., 2010; Pertwee et al., 2010). To further explore the canonical biochemical effectors of CB1R ligands, experiments on cAMP levels were performed.

Assaying cAMP as readout of CB1R activity

The use of a specific signal transduction assay was critical for the identification of CB1Rs whose activation was demonstrated to decrease cAMP (Howlett et al., 2004). When the cAMP synthesis by synaptosomal preparations is stimulated by the adenylyl cyclase activator Frsk, the maximal inhibition induced by AEA via native CB1Rs is observed at 1–10 μM concentration and amounts to about 25% fall in cAMP levels (Steffens et al., 2005). It has, however, been repeatedly pointed out that, while the pharmacology of cannabinoid-inhibited adenylyl cyclase remains an essential property for signal transduction studies, it is difficult to directly relate it to changes in integrated functions of neurobiological systems and behavior (Childers and Deadwyler, 1996; Howlett et al., 2004). Thus, the present study sought to employ the cAMP assay simply as a tool to find out if, after sustained application of CB1R agonist/antagonist, these receptors could still transduce such a biochemical signal. Basal cAMP was significantly lower after a 24-h AEA application, suggesting that CB1Rs remained at least partly operational following the agonist persistent administration and that any desensitization (Howlett et al., 2004; Martin et al., 2004) was incomplete. Caution is, however, required to relate this observation to any actual degree of CB1R desensitization, or downregulation because of the complexity of intermediate pathways (Katona and Freund, 2012; Piomelli, 2014). After applying AM-251 for 24 h, there was no significant change in basal cAMP levels, indicating that cAMP synthesis was under minimal constitutive control by CB1Rs. The Frsk-stimulated large rise in cAMP was unaffected by

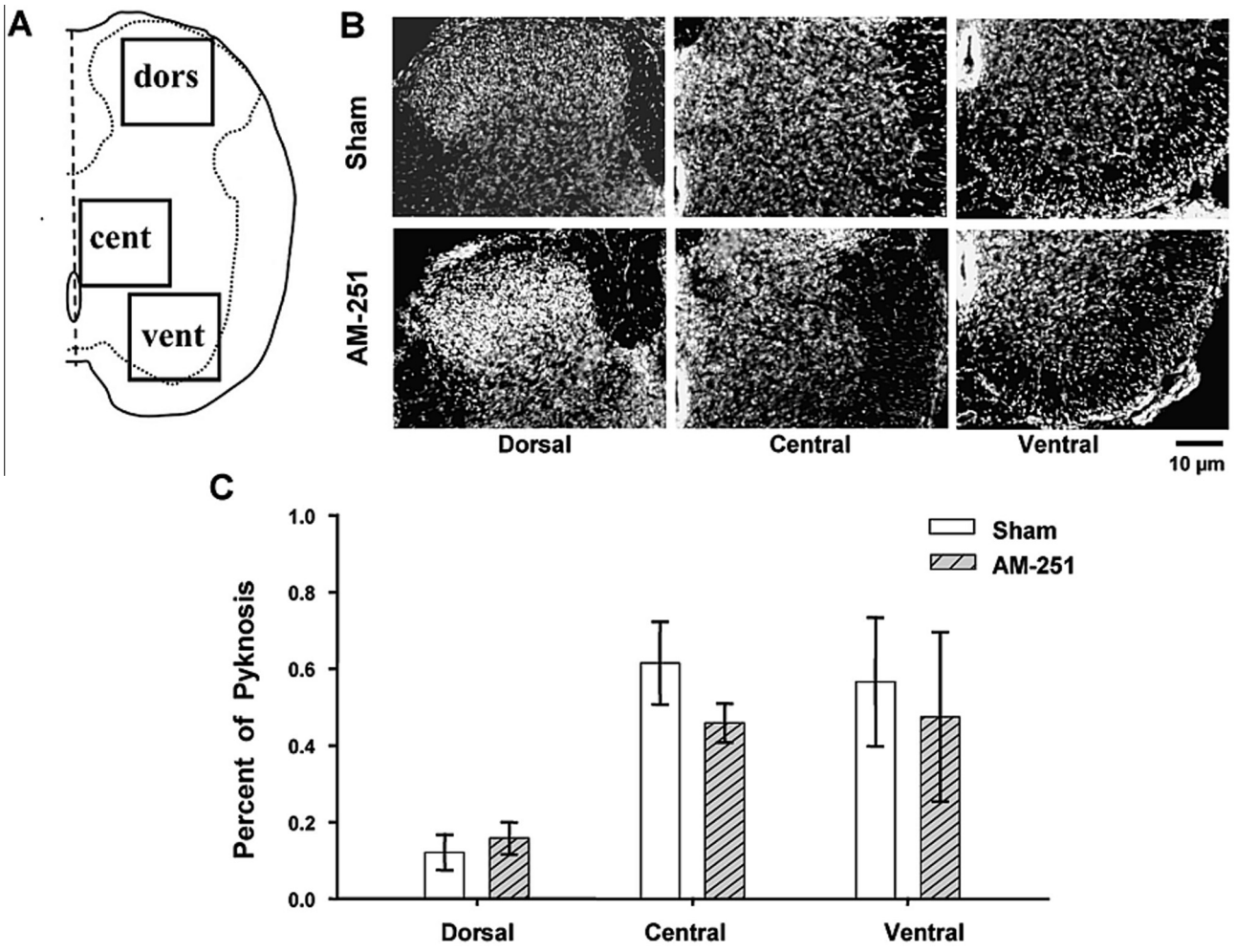

Fig. 8. Histology of sham and 24-h AM-251-treated spinal cord preparations. (A) Schematic representation of the ROIs used for analysis. Dors, dorsal horn; Cent, central region; Vent, ventral region. (B) Examples of sham- (top) and AM-251-treated (bottom) spinal cord cross sections stained with DAPI in dorsal (left), central (middle) and ventral (right) regions. Scale bar = 10 μm. (C) Histograms showing that there is no change in percent of pyknosis after AM-251 24-h treatment in comparison with sham in all the three regions. $n = 9$.

chronically-applied AM-251, yet depressed by AEA in an AM-251-sensitive fashion. Thus, the present results may suggest that CB1Rs, notwithstanding the agonist-sustained application, remained at least partly functional in terms of regulation of cAMP synthesis.

A number of investigations have clearly shown that the actual concentration of cAMP has little impact on locomotor activity because inhibitors of adenylyl cyclase (Barrière et al., 2004), of cAMP-dependent protein kinases (Parker et al., 1998), or of the cAMP-metabolizing enzyme phosphodiesterase-4 (Heidemann et al., 2014), have no effect on spinal rhythmicity *in vivo* or *in vitro*. Furthermore, the CB1R antagonism of the present observations makes it unlikely that the depressant effects by exogenous AEA or endocannabinoids were caused by AEA-mediated activation of TRPV1 spinal receptors expressed by a subgroup of nociceptive afferents and capable of modulating mouse locomotor networks in biphasic fashion (Mandadi et al., 2009). Furthermore, while the TRPV1 agonist capsaicin rapidly facilitates fictive locomotion and then depresses it within minutes (Mandadi et al., 2009), we could never observe a similar effect with AEA (or related ligands) application.

A HYPOTHESIS ON THE ROLE OF CB1RS IN THE RAT SPINAL CORD *IN VITRO*

The focus of the present study was on the action of CB1Rs on locomotor networks of the rat spinal cord. The most parsimonious interpretation of our data is that CB1Rs were functional as demonstrated with their inhibitory action on GABA VR depolarization and cAMP synthesis. These experimental approaches are, however, unsuitable to understand where these receptors precisely acted because spinal homogenates or summated network activity are, of course, not the best tools to answer such a question. CB1 immunohistochemistry indicated their sparse distribution in line with the minimal changes in synaptic or network activity observed after short-term CB1 agonist/antagonist application. In fact, AM-251 did not change basal cAMP levels suggesting that CB1Rs

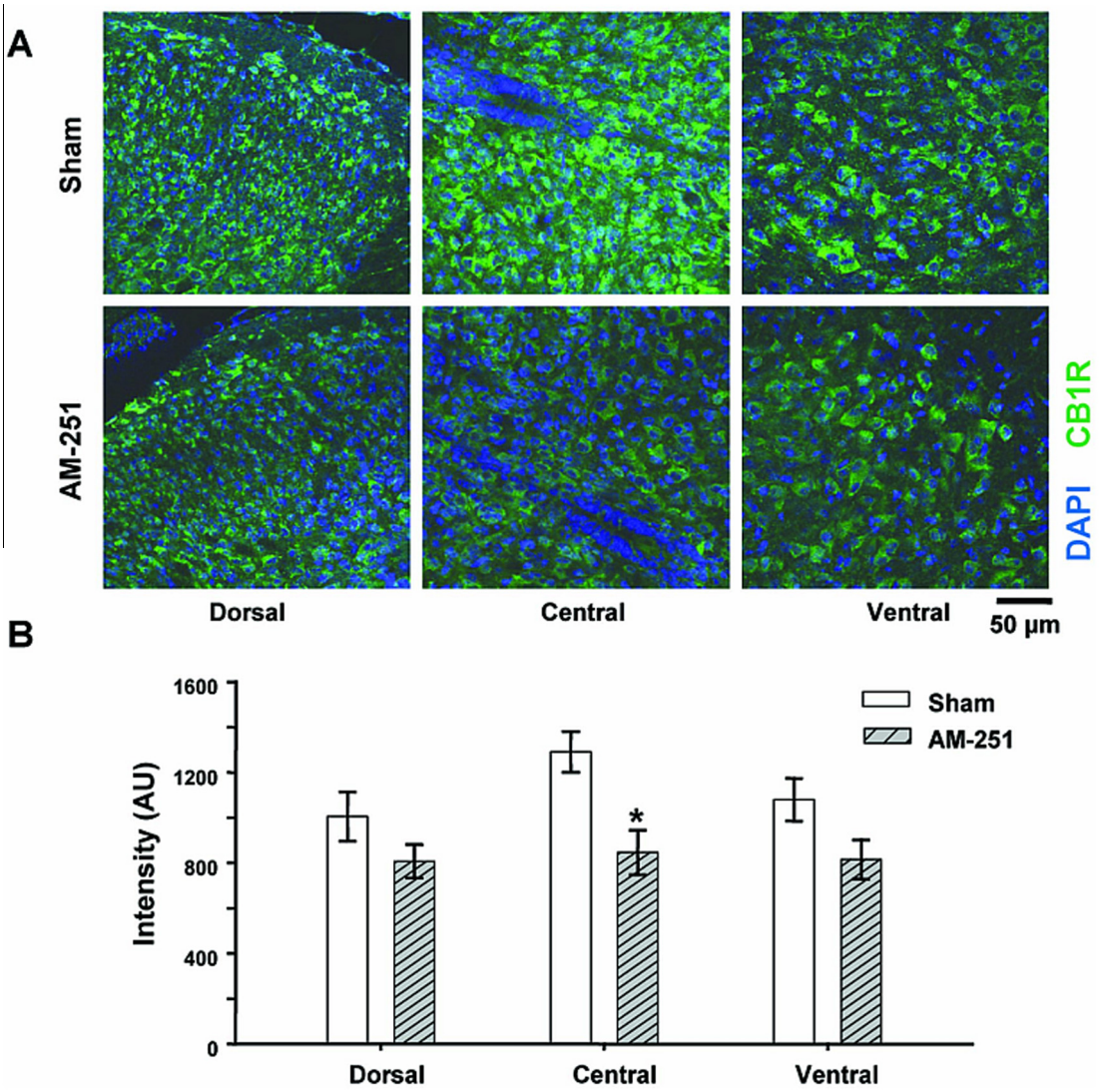

Fig. 9. Changes in CB1R intensity after AM-251 24-h treatment. (A) Examples of immunostaining for CB1Rs in dorsal (left), central (middle) and ventral (right) regions of sham (top) and AM-251 24-h-treated (bottom) preparations. Scale bar = 50 µm. (D) Bar diagram showing the difference in CB1R immunoreactivity after AM-251 24-h treatment in all the three regions. Note the significant reduction in CB1R intensity in central region of AM-251-treated preparations. $n = 12$, $^{*}P < 0.05$.

possessed low constitutive activity and their action was best demonstrated only when cAMP synthesis was stimulated by Frsk. The role of CB1Rs clearly emerged when they were pharmacologically manipulated for many hours. In particular, after a 24- hblock, a significant downregulation appeared at the level of the spinal central region that is critical for locomotor activity generation (Grillner, 2003, 2006; Kiehn, 2006, 2011). This phenomenon was functionally associated to severe depression of fictive locomotion. Thus, it is hypothesized that CB1Rs stabilized the operational activity of the locomotor central pattern generator, an effect reminiscent of the slow regulatory role of CB1Rs in tuning neuronal networks (Kim and Alger, 2010).

Previous studies have shown that, in the brain and spinal cord, CB1Rs are particularly important during the prenatal stage to ensure correct axon guidance to establish effective synapses (Berghuis et al., 2007). Furthermore, persistent block of CB1Rs with AM-251 drastically disrupts neuronal maps in the somatosensory cortex (Li et al., 2009), outlining a distinct function of CB1Rs in circuit stabilization. We propose that pharmacological inhibition of CB1Rs destabilized a critical subset of synapses that are essential contributors to the locomotor pattern generation. Future studies will be necessary to identify the molecular pathways responsible for the delayed loss of locomotor network function after AM-251. This is likely to be a complex task because of the diffuse multisegmental projections of primary afferents to the spinal cord (Kremer and Lev-Tov, 1997; Etlin et al., 2010) and the widespread distribution of the locomotor central pattern generator that demands at least three intact lumbar segments to express the locomotor cycles (Kiehn and Kjaerulff, 1998). Former evidence has indicated that CB1Rs strongly control, in a time-dependent fashion, locomotor activity *in vivo* by acting on multiple

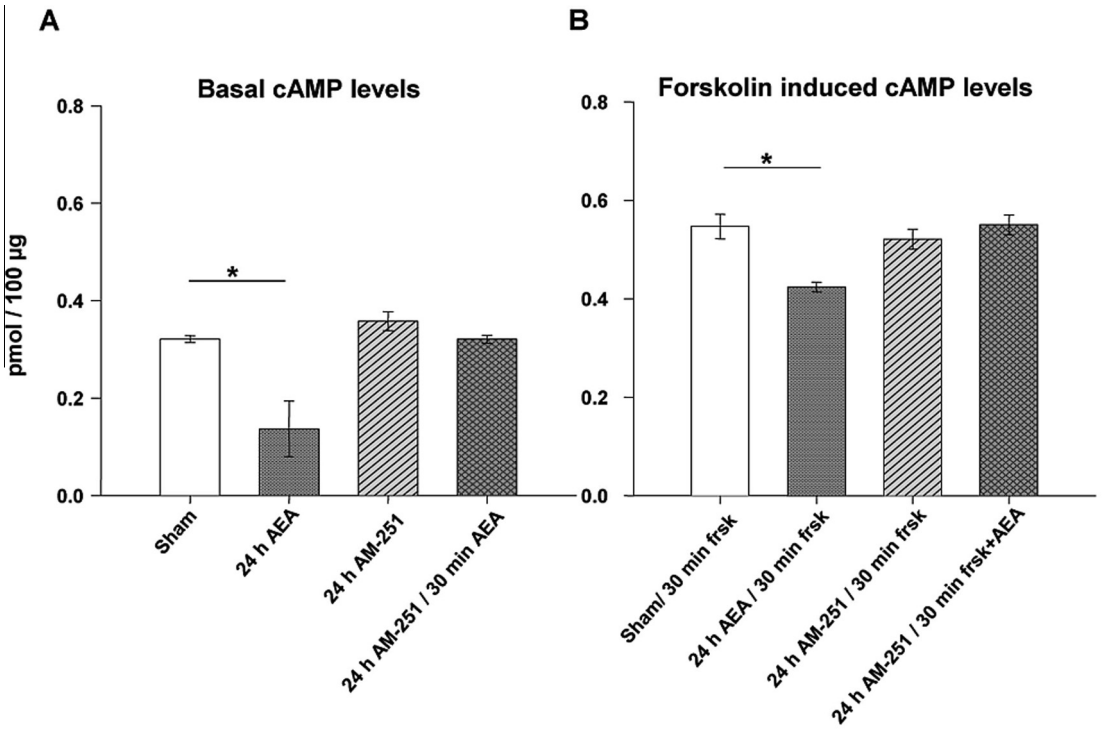

Fig. 10. Effects of AEA or AM-251 on basal and forskolin-induced cAMP levels. (A) Histograms showing the changes in basal cAMP levels after AEA 24 h, AM-251 24 h or AM-251 24 h/AEA 30 min. Note the significant reduction in basal cAMP levels after AEA 24-h treatment compared to sham. (B) Bar graphs showing the effects of AEA 24 h, AM-251 24 h or AM-251 24 h/AEA 30 min on forskolin- (1 μM; 30 min) induced cAMP levels. Note the significant decline of forskolin-induced cAMP levels after AEA 24-h treatment. Data are from duplicate experiments using 18 spinal cords. $^{*}P < 0.05$.

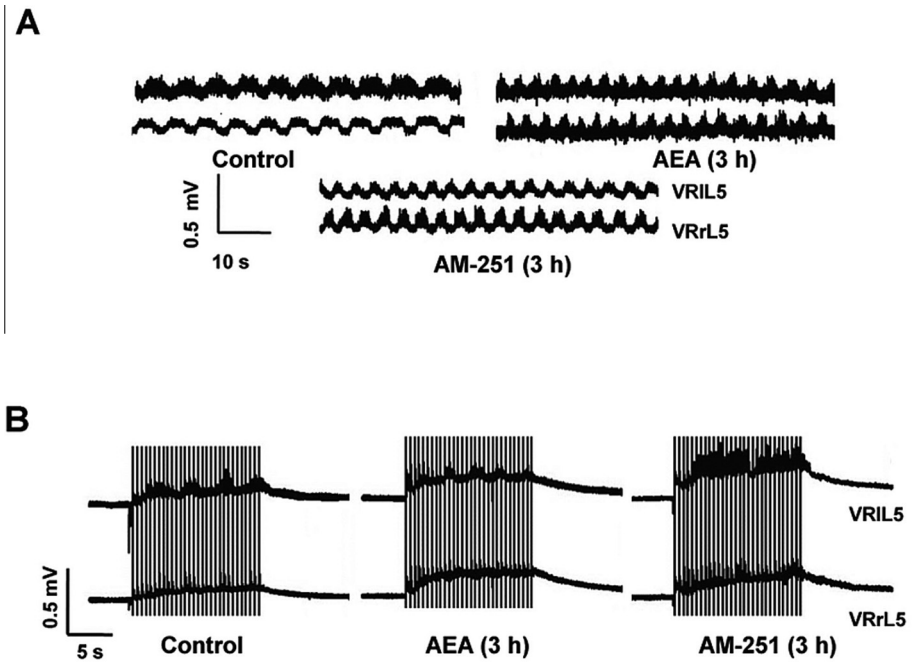

Fig. 11. Fictive locomotion changes in P4 spinal cord preparations after AEA or AM-251 3-h treatment. (A) Examples of fictive locomotion induced by NMDA and 5-HT in control (top left), AEA-treated (top right) or AM-251-treated (bottom) preparations. (B) Representative records of DR-evoked oscillations in control (left), AEA (middle) and AM-251 (right) preparations. Note the slight depression of oscillations in the AM-251-treated preparation.

Table 2. Effect of cannabinoid ligands after a 3-h treatment on rhythmic discharges of P4 spinal cord preparations

Parameters		Control (3 h) (n = 6)	AM-251 5 μM (3 h) (n = 7)	AEA 5 μM (3 h) (n = 4)
Response to dorsal root train	# oscillations	4.61 ± 0.67	**2.62 ± 0.31***	4.00 ± 0.51
	Cumulative depolarization (mV)	0.39 ± 0.05	0.41 ± 0.05	0.37 ± 0.04
	Area (mV.ms)	8318.50 ± 1091.35	7894.36 ± 948.66	7830.39 ± 594.13
NMDA/5-HT fictive locomotion	Amplitude (mV)	0.15 ± 0.03	0.16 ± 0.02	0.11 ± 0.01
	Period (s)	4.37 ± 0.70	4.47 ± 0.60	4.17 ± 0.42

* $P < 0.05$.

dopaminergic systems in the basal ganglia (Ginovart et al., 2012): since in the isolated spinal cord preparation this site of action was eliminated, the present work demonstrated a site of action of CB1Rs on the locomotor central pattern generator rather than on the motor behavior, outlining a target for CB1R modulation on movement generation in addition to its role in the origin and selection of motor commands (Grillner et al., 1998).

CONCLUSIONS

The current results on the partial depression of oscillatory cycles by CB1R agonism do not conflict with the guidelines for using such an approach to treat spasticity or chronic pain (Koppel et al., 2014). These syndromes are likely to arise at the spinal level because a discrete lesion had induced aberrant motor activity with hyperresponsiveness to afferent inputs (Dietz, 2010; D'Amico et al., 2014). Our observations that CB1R agonism could depress DR-evoked oscillations without affecting the intrinsic activity of the locomotor central pattern generator or standard synaptic transmission are in line with a selective decrease in peripherally triggered hyper-reflexia and hyperalgesia especially when supraspinal descending control is damaged in neurological disease. Conversely, the motor network depression induced by persistent CB1R antagonism indicates this phenomenon as a further potential side-effect of chronic treatment with CB1R antagonists like rimonabant that caused significant adverse effects in a number of patients who then withdrew from the clinical trial (Després et al., 2005).

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A STUDY OF CANNABINOID-1 RECEPTORS DURING THE EARLY PHASE OF EXCITOTOXIC DAMAGE TO RAT SPINAL LOCOMOTOR NETWORKS *IN VITRO*

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Abstract—Endocannabinoids acting on cannabinoid-1 receptors (CB1Rs) are proposed to protect brain and spinal neurons from excitotoxic damage. The ability to recover from spinal cord injury (SCI), in which excitotoxicity is a major player, is usually investigated at late times after modulation of CB1Rs whose role in the early phases of SCI remains unclear. Using the rat spinal cord *in vitro* as a model for studying SCI initial pathophysiology, we investigated if agonists or antagonists of CB1Rs might affect SCI induced by the excitotoxic agent kainate (KA) within 24 h from a transient (1 h) application of this glutamate agonist. The CB1 agonist anandamide (AEA or pharmacological block of its degradation) did not limit excitotoxic depolarization of spinal networks: cyclic adenosine monophosphate (cAMP) assay demonstrated that CB1Rs remained functional 24 h later and similarly expressed among dead or survived cells. Locomotor-like network activity recorded from ventral roots could not recover with such treatments and was associated with persistent depression of synaptic transmission. Motoneurons, that are particularly vulnerable to KA, were not protected by AEA. Application of 2-arachidonoylglycerol also did not attenuate the electrophysiological and histological damage. The intensification of damage by the CB1 antagonist AM251 suggested that endocannabinoids were operative after excitotoxic stimulation, yet insufficient to contrast it efficiently. The present data indicate that the early phases of excitotoxic SCI could not be arrested by pharmacologically exploiting the endocannabinoid system, consistent with the notion that AEA and its derivatives are more useful to treat late SCI phases.

Key words: CB1, spinal cord injury, endocannabinoids, kainate, fictive locomotion, neuroprotection.

INTRODUCTION

Spinal cord injury (SCI) is a major health challenge that currently occurs with an estimated annual incidence of over 50 cases per million population with uncertain recovery despite the fact that the primary lesion is often incomplete (Singh et al., 2014; Jain et al., 2015). The SCI process has a complex time course starting from the initial insult and followed by a cascade of prolonged, secondary injury events. The secondary injury process includes early increase in the extracellular level of the excitatory transmitter glutamate, i.e. the principal activator of excitotoxicity that irreversibly damages spinal neurons (Park et al., 2004; Oyinbo, 2011; Borgens and Liu-Snyder, 2012). This death process is brought about by over activity of glutamate receptors with subsequent rise in intracellular Ca^{2+} that triggers formation of reactive oxygen species, and inflammatory mediators which all contribute to excitotoxicity, mitochondrial damage, demyelination and neuronal and glial loss (Dumont et al., 2001; Rowland et al., 2008; Fakhoury, 2015). It is, therefore, important to identify the most suitable targets to arrest the progression of the secondary lesion and provide neuroprotection.

Since many years cannabis and its derivatives have been used as an (un)conventional tool to treat pain and spasticity that can appear after chronic SCI (Teasell et al., 2010; Arevalo-Martin et al., 2016; Boadas-Vaello et al., 2016). This approach is, however, not supported by some animal studies indicating that inhibiting endocannabinoids might ameliorate chronic pain (Toniolo et al., 2014; Ueda et al., 2014). Furthermore, little is known about any early role of cannabinoids and their receptors (that in the central nervous system predominantly belong to the cannabinoid-1 receptor (CB1R) type; Pertwee, 2005) in controlling the lesion process during the first few hours after acute spinal injury. Exploring this issue is prompted by the fact that retrograde signaling by endocannabinoids via downregulation of glutamate release physiologically contributes to restrain excessive excitation (Schlicker and Kathmann, 2001; Brown et al., 2003; Monory et al., 2015) and by endocannabinoid-modulation of postsynaptic N-methyl-D-aspartate (NMDA) receptor trafficking (Sánchez-Blázquez et al., 2014).

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Abbreviations: 2-AG, 2-arachidonoylglycerol; 5-HT, 5-hydroxytryptamine or serotonin; AEA, N-arachidonoylethanolamine or anandamide; cAMP, cyclic adenosine monophosphate; CB1R, cannabinoid receptor-1; DAPI, 4,6-diamidino-2-phenylindole; DR, dorsal root; FL, fictive locomotion; Frsk, forskolin; GABA, γ-aminobutyric acid; KA, kainate; NMDA, N-methyl-D-aspartate; ROI, regions of interest; SCI, spinal cord injury; SEM, standard error mean; VR, ventral root.

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The endocannabinoid function is exerted via activation of CB1Rs widely expressed in the spinal cord of adult or neonatal rats (Farquhar-Smith et al., 2000; Veeraraghavan and Nistri, 2015). Using an *in vivo* rat SCI model, Garcia-Ovejero et al. (2009) have investigated changes in the major endogenous cannabinoid agonists anandamide (AEA) and 2-arachidonoylglycerol (2-AG) and their receptor expression. Thus, levels of AEA (and 2-AG) are increased a few hours after injury with gradual return to basal level, while global CB1R expression remains unchanged for the first few days. It remains, however, to be explored how changes in endocannabinoid levels in this time interval might be related to locomotor network activity, their neuroprotection, and any region-specific damage.

To pursue this issue, we used an *in vitro* rat spinal cord model of SCI in which rhythmic patterns (fictive locomotion) produced from the locomotor pattern generator intrinsic to the spinal cord (Kiehn, 2006, 2016; Grillner et al., 1998) can be electrophysiologically recorded over the first 24 h from injury and related to the extent of tissue damage evoked by the glutamate analog kainate (KA; Taccola et al., 2008; Kuzhandaivel et al., 2011). One important feature of this paradigm is that transient application of KA (50 μM) reliably produces a histologically-moderate lesion that destroys 12.5–23% neurons in the central and dorsal gray matters plus a more severe (50%) loss of motoneurons (Mazzone et al., 2010). The consequence of such lesion is to bring the locomotor network cells below the threshold for "minimal membership", namely the size of the neuronal circuitry capable of expressing alternating locomotor patterns (Kuzhandaivel et al., 2011). Although it is difficult to establish the actual neuronal loss after human SCI, the large majority of SCI cases are incomplete indicating significant neuronal and glial preservation (Sekhon and Fehlings, 2001; Hillen et al., 2013) that, however, does not enable recovery of locomotion as only $<5\%$ of these patients will walk again (Dobkin and Havton, 2004). Thus, moderate neuronal losses are translated into major deficit as observed in our model. In addition, our experimental protocol replicates the clinical condition whereby intensive care support is usually provided at the earliest possible time to arrest lesion progression (Hillen et al., 2013).

In this context, the present study pursued several aims: (1) To understand if enhancing the endogenous levels of AEA might inhibit excitotoxicity by applying LY2183240, a well-known pharmacological blocker of the endocannabinoid inactivation mechanisms (cell uptake and degradation by fatty acid amide hydrolase; Alexander and Cravatt, 2006). (2) To study if the CB1Rs remain functional to inhibit cAMP synthesis in the rat spinal cord (Howlett et al., 2004; Veeraraghavan and Nistri, 2015) after the excitotoxic insult and how their cellular expression might be altered. (3) To find out if pharmacological activation (or block) of CB1Rs might improve the outcome of KA-evoked neurotoxicity. This possibility was pursued by applying exogenous AEA as the prototypic CB1 agonist, its inverse agonist AM251 (Piomelli, 2005), or 2-AG that possess stronger agonist activity on CB2 than CB1 receptors (Di Marzo and De Petrocellis, 2012).

EXPERIMENTAL PROCEDURES

Spinal cord *in vitro* preparations

Isolated spinal cords were removed from neonatal (0–2- day-old) Wistar rats under urethane anesthesia. The experimental protocols were approved by the ethics committee of the Scuola Internazionale Superiore di Studi Avanzati and are in accordance with the current European Union guidelines. A total of 119 animals were used for the entire study. Details of animals in each group are as follows: Sham = 21; KA = 21; LY = 4; LY/KA + LY = 9; KA/LY = 10; AEA = 4; AEA/KA + AEA = 10; KA/AEA = 14; AM/KA + AM = 3; KA/AM = 12; 2-AG/KA + 2-AG = 3; KA/2-AG = 8. All efforts were made during experiments to minimize the number of animals and their suffering, and to observe the three Rs objective. Isolated preparations were continuously superfused at the rate of 7.5 ml min^{-1} with standard Kreb's solution of the following composition (in mM): 113 NaCl, 4.5 KCl, 1 $MgCl_2 \cdot 7H_2O$, 2 $CaCl_2$, 1 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose, gassed with 95% O_2 and 5% CO_2 (pH 7.4) at room temperature (Bracci et al., 1996; Beato and Nistri, 1999; Taccola et al., 2008).

Experimental spinal injury model

The present study used a model of SCI *in vitro* based on excitotoxic stimulation elicited by the potent glutamate agonist KA (Taccola et al., 2008; Mazzone et al., 2010). The rationale for this approach stems from the important role of excitotoxicity in generating neuronal damage during SCI (Park et al., 2004). Thus, the goal is trying to identify neuroprotective mechanisms directly related to the nervous tissue without the complexity of vascular changes or immune reactions. Furthermore, advantages of the present model are the absence of general anesthesia during the lesion in analogy to human clinical lesions and the transient nature of the excitotoxic stimulation that lasts for 1 h and is followed by sustained washout with oxygenated Krebs solution: this procedure intends to mimic the intensive care approach that aims at stabilizing patients as soon as possible with correction of intervening metabolic dysfunctions and any respiratory depression. It is noteworthy that, despite the washout of KA, the large decline of glutamate release (Mazzone and Nistri, 2011) and the dissipation of network depolarization (Taccola et al., 2008), the secondary damage progresses and peaks within the first few hours (Kuzhandaivel et al., 2011) because neuronal losses are stabilized by 24 h *in vitro* as indicated by long-term slice cultures (Mazzone et al., 2013). During this early time frame, the present model enables repeated testing of locomotor network activity, a parameter not often investigated as clinical reports simply indicate "spinal shock" as a condition of temporary paralysis of locomotion (Dietz, 2010).

The present model has also some intrinsic limitations because it relies on neonatal spinal cords that may have responses somewhat different from adult ones, although it should be borne in mind that pediatric SCI are a significant percentage of all human SCI (Vogel et al., 2012). Further limitations of this model are its

time-restricted survival *in vitro* (usually two days) that precludes investigating delayed, important components of SCI like neuroinflammation and scar-forming processes that influence the lesion outcome. While temporary oxygen/glucose deprivation mainly affects spinal glia (Taccola et al., 2008), the present excitotoxic model preferentially damages neurons, especially motoneurons that are rather vulnerable cells (Mazzone et al., 2010). Our hypothesis is that, if significant neuroprotection can be observed during the early hours after the lesion, the pathological late consequences can probably be strongly decreased.

Protocols for experimental SCI and cannabinoid ligand treatment

After testing normal locomotor patterns, excitotoxicity was induced by bath applying KA (50 μM) for 1 h, followed by washout in standard Kreb's solution for 24 h. This protocol was sufficient to evoke a standard lesion that was suprathreshold to abolish locomotor function with moderate histological damage (Mazzone et al., 2010). Immediately after washout of KA, a group of preparations were continuously treated with cannabinoid ligands (LY2183240 3 μM or AEA 5 μM or 2-AG 2 μM or AM251 5 μM) for 24 h after which functional and histological observations were done. The drug concentrations were chosen in accordance with previous studies (Kettunen et al., 2005; Eljaschewitsch et al., 2006; Gonzalez-Islas et al., 2012; Iannotti et al., 2014). The canonical effector of CB1 receptor activity is inhibition of forskolin-stimulated adenylyl cyclase that synthesizes cyclic adenosine monophosphate (cAMP; Steffens et al., 2004). The aforementioned drug application protocols were used to explore the CB1 receptor activity at the end of the experiment. In a few tests, we also investigated the outcome of cannabinoid ligands (pre-applied for 30 min and then applied together with KA) on KA-induced depolarization recorded from VRs. For the sake of comparison, all experiments were run in parallel with sham preparations.

Electrophysiological recordings

DC-coupled recordings were performed in the lumbar region (ventral L2 and L5) of isolated spinal preparations as described in our previous studies (Marchetti et al., 2001; Taccola et al., 2008) using a DP-304 Differential amplifier (Warner Instruments LLC, Hamden, CT, USA). Reflex and locomotor-like discharges (fictive locomotion) were elicited by stimulating a dorsal root (DR; usually L5) through a bipolar suction electrode and recorded from ipsilateral and contralateral ventral roots (VRs) at segmental level L2 and L5. In each preparation the smallest near threshold response elicited by 0.1 ms DR stimulation at low stimulus intensity was recorded from the ipsilateral VR and was interpreted as monosynaptic (Fulton and Walton, 1986; Marchetti et al., 2001; Ostroumov et al., 2007). Polysynaptic reflexes were evoked by stimulating the same DR with intensity of three times the threshold. To study fictive locomotion (FL) consisting of alternated rhythmic VR discharges (Marchetti et al., 2001; Taccola et al., 2008), a train of 30 DR pulses (2–3 times the threshold) was delivered while recording responses characterized by cumulative depolarization with superimposed alternated oscillations from at least 3 VRs. FL was also evoked by bath applying NMDA (2–6 μM) and 5-hydroxytryptamine (5-HT; 10 μM). Fictive locomotor parameters were the amplitude of oscillations and their periodicity. Disinhibited bursting elicited by blocking γ-aminobutyric acid (GABA)-A and glycine receptors by bicuculline (20 μM) and strychnine (1 μM), respectively (Bracci et al., 1996), was also monitored to assess the intrinsic network excitability. Signals were acquired using pClamp version 9.2 at 20-kHz acquisition frequency (Molecular Devices, Sunnyvale, CA, USA).

cAMP immunoassay

Full details of this assay based on inhibiton of cAMP synthesis have recently been published (Veeraraghavan and Nistri, 2015). Because basal cAMP levels are usually low in the spinal cord and, upon CB1R activation, may go even below the detection limit, we measured cAMP levels stimulated by forskolin (Frsk) (1 μM for 30 min) in accordance with standard methods (Howlett et al., 2004; Steffens et al., 2004). All measurements were performed on the second day *in vitro*. Briefly, to study changes in cAMP levels downstream of CB1 receptor activity, at the end of the experimental protocols preparations were immediately frozen in liquid nitrogen to block cAMP degradation and processed in accordance with the manufacturers' instructions (Abnova; http://www.abnova.com/products/products_detail.asp?catalog_id=KA0886).

Fluorescence immunostaining procedure

After electrophysiological recordings, spinal cords were fixed in 4% paraformaldehyde, cryopreserved in 30% sucrose and then sectioned to obtain 30-μm-thick sections. The free-floating immuno-fluorescence procedure used fully validated antibodies and was previously described by Taccola et al. (2008), Mazzone et al. (2010) and Veeraraghavan and Nistri (2015). The tissue staining with primary antibodies, namely, NeuN (neuron-specific nuclear marker, 1:50 dilution, Chemicon, Millipore MAB377), CB1R (1:50 dilution, CB1 (K-15) antibody, Santa Cruz Biotechnology, Inc.), or SMI-32 (anti-neurofilament H non-phosphorylated mouse mAb used to stain spinal motoneurons; Mazzone et al., 2010; 1:200 dilution, Covance, SMI-32 R) was visualized using the secondary fluorescent Alexa Fluor 488 or 594 antibodies (1:500 dilution, Invitrogen, Carlsbad, CA, USA). The CB1R antibody was previously validated by us through the use of the specific blocking peptide (Veeraraghavan and Nistri, 2015). Sections were stained with a solution (1 μg/ml) of 4,6-diamidino-2-phenylindole (DAPI) for 20 min to label cell nuclei and mounted on Superfrost Plus slides (Menzel-Glaser, Braunschweig, Germany). The immunostaining signal was analyzed by either Zeiss Axioskop2 (Oberkochen, Germany) or confocal-Nikon microscope. Each treatment group contained at least three preparations and three lumbar slices were analyzed for every spinal cord.

Quantification of pyknosis, NeuN positive cells, motoneurons and CB1R staining

These procedures were recently described (Cifra et al., 2012). In brief, to analyze the extent of neuronal damage, the number of NeuN-positive cells was counted using the ImageJ Cell counter (NIH, USA), while DAPI-positive nuclei were counted with'eCELLence' software (Glance Vision, Trieste, Italy). The following standard regions of interest (ROI) were selected: $350 \times 350\ \mu m^2$ for dorsal and central, $235 \times 235\ \mu m^2$ for ventral areas (Cifra et al., 2012). The average percent values of nuclei with condensed chromatin (taken as index of pyknosis) were normalized with respect to the total number of nuclei and compared between different ROI and treatments. Additionally, the number of SMI-32-positive cells located in the ventral ROI and with somatic diameter $>25\ \mu m$ were considered as motoneurons (Cifra et al., 2012) and counted using ImageJ Cell counter. CB1R intensity (AU) was quantified in a "volocity" workstation (Perkin Elmer, London, UK) as previously described by Veeraraghavan and Nistri (2015) using a manual ROI constructed every time according to the size of the cell in different regions. From each experimental preparation three slices were taken and from each slice three cells were quantified and averaged.

Drugs

AEA, AM251, LY2183240, 2-AG, KA and NMDA were obtained from Tocris (Bristol, UK), while bicuculline methiodide was purchased from Abcam (Cambridge, UK). 5-HT (5-hydroxytryptamine or serotonin) and strychnine hydrochloride were purchased from Sigma (Milan, Italy).

Data analysis

All parametric data are expressed as mean ± SEM, while non parametric data are shown as median with whole distribution. Statistical analysis was carried out with SigmaStat 3.5 (Systat Software, Chicago, IL, USA) and plotted in Sigmaplot 10. Depending on the parametric or non-parametric distribution of the data indicated by the software, the analysis was performed using either the Student t-test or the one-way analysis of variance followed by the Tukey test or the Kruskal–Wallis One-Way Analysis of Variance on Ranks accompanied with the Dunn's method.

RESULTS

Effect of cannabinoid ligands on KA induced depolarization

Our previous studies show that 50 μM KA (1 h) evokes a very large VR depolarization followed by irreversible excitotoxic damage to the *in vitro* neonatal spinal preparation (Mazzone et al., 2010; Nasrabady et al., 2011). Furthermore, we have previously demonstrated that acute application of the CB1R agonist AEA does not change network depolarization evoked by AMPA or NMDA (Veeraraghavan and Nistri, 2015). In the present study we first examined if CB1R pharmacological modulation might change VR depolarization evoked by sustained (1 h) KA superfusion: for this purpose we pre-applied ligands for 30 min (to allow for drug equilibration within the tissue) that were then continuously superfused together with KA for 1 h. Fig. 1A shows examples of VR depolarization induced by KA (see arrows) that was not prevented by LY2183240 (3 μM), AEA (5 μM), 2-AG (2 μM), or AM251 (5 μM). The bar graph of Fig. 1B indicates that, on average, the mean KA depolarization plateau was not changed by anyone of such treatments ($n = 9$, $n = 10$, $n = 3$, or $n = 3$, respectively vs. KA; $n = 17$).

Since CB1R activity did not appear to regulate the KA depolarization from which spinal networks are known to recover regardless the onset of later damage (Taccola et al., 2008), we queried whether CB1R activity might actually contrast the slowly developing evolution of secondary damage over the subsequent 24 h.

CB1R activity after KA treatment

In order to find out if CB1Rs could modulate the progression of excitotoxic lesions, it was first necessary to find out if the spinal cord would still express functional CB1R one day after KA application. Thus, we examined whether, 24 h after KA application, CB1R activity was present by measuring changes in cAMP levels, because the chief target of CB1R is inhibition of adenylyl cyclase (Howlett et al., 2004). We used forskolin (1 μM; 30 min) to largely stimulate cAMP synthesis (Steffens et al., 2004) in the rat spinal cord (Veeraraghavan and Nistri, 2015) and verified how CB1R ligands could affect it. Fig. 2A shows basal cAMP concentration in samples treated with KA did not significantly deviate (within 10% variation) from sham ones. A significant ($P < 0.05$) reduction in cAMP levels was, however, observed after LY 2183240 applied alone or after KA washout (KA/LY). Likewise, AEA, applied either alone or after KA (KA/AEA), significantly decreased cAMP (Fig. 2A; $n = 4$ in each treatment). These data suggest that residual CB1R activity (expressed as inhibition of cAMP synthesis) after KA application could be enhanced by either blocking endocannabinoid degradation or by AEA: this observation led us to examine the expression pattern and electrophysiological correlates of these phenomena.

Effect of KA and cannabinoid ligand treatment on CB1R expression

Many studies have shown that there is a change in cannabinoid receptor intensity after neuropathic pain or any excitotoxic insult to brain and spinal regions (Mitrirattanakul et al., 2006; Garcia-Ovejero et al., 2009; Miller and Devi, 2011). To find out if excitotoxicity could alter CB1R expression in the present spinal cord model, we studied the CB1R immunohistochemical changes after KA, KA/LY, KA/AEA or KA/AM treatments. Fig 2B shows examples of CB1R expression after KA and CB1R

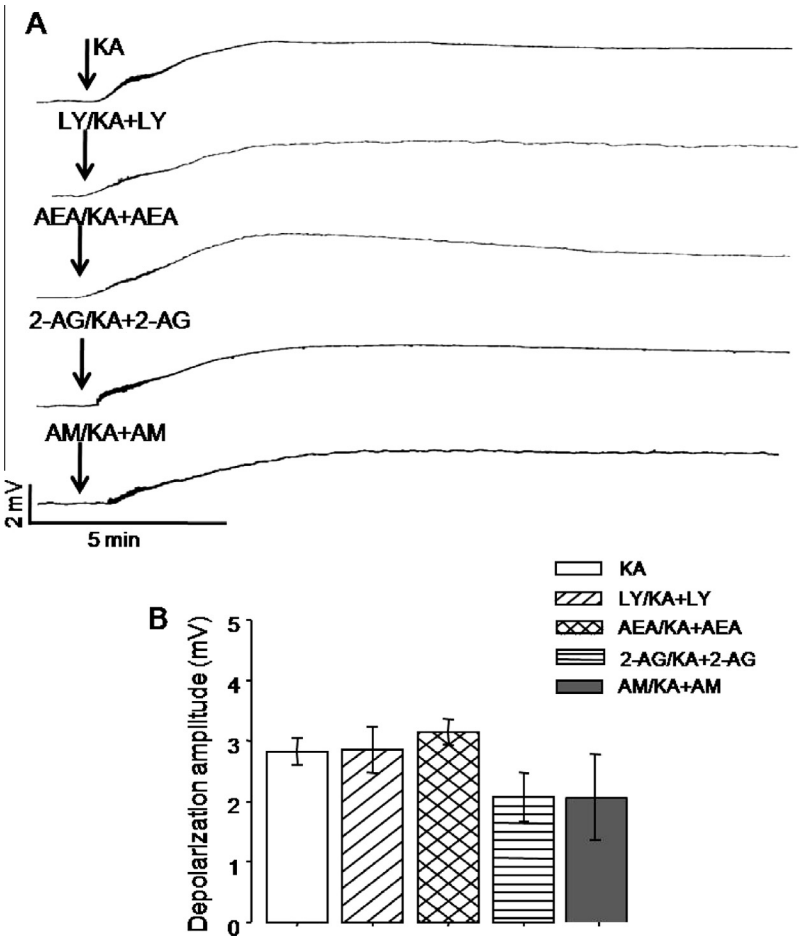

Fig. 1. Effect of cannabinoid ligands on KA-induced VR depolarization. (A) Examples of KA (50 μM)-evoked depolarization following pre-application of cannabinoid ligands as indicated by vertical arrows. (B) Histograms show that the amplitude of the KA-evoked response is unchanged by LY2183240 (3 μM; $n = 9$), AEA (5 μM; $n = 10$), 2-AG (2 μM; $n = 3$), or AM251 (5 μM; $n = 3$) compared to KA only treated preparations ($n = 17$; data here are shown as mean ± SEM).

ligands' application in cross sections of the spinal cord, indicating scattered damage that also comprised motoneurons. To quantify the changes in CB1R expression followed by these protocols, individual (surviving/pyknotic) cells were analyzed (as discussed in the methods section). In the ventral region that is critical for locomotor activity (Kiehn, 2016), there was no marked difference in CB1R immunosignal compared to sham spinal cords regardless whether we examined non-pyknotic or pyknotic cells for any tested treatment (Fig 3A–C). Conversely, in the dorsal region, the cells survived after KA or KA/AM treatment showed lower intensity of CB1R expression ($n = 11$ or 12 spinal cords, respectively; Fig 3A, B). In the survived cells of the central region, CB1R intensity was greatly reduced by KA/AM treatment ($n = 12$) in comparison with KA only treated preparations (Fig 3B). KA/LY spinal cords showed an increase in the CB1 receptor expression around the pyknotic cells in the dorsal region ($n = 6$; Fig 3A, C). These data suggest that, within the first 24 h following excitotoxic stimulation, there was a region-related change in the CB1R expression.

CB1R modulation on synaptic reflexes after KA mediated excitotoxicity

Our recent report has examined the effects of sustained application of AEA, LY2183240, 2-AG or AM251 on synaptic transmission of rat spinal networks (Veeraraghavan and Nistri, 2015). In the present study we confirmed that monosynaptic reflexes recorded from VRs 24 h after KA application (1 h) were significantly depressed in their amplitude and area (Fig. 4A–C; Mazzone et al., 2010). When LY2183240 or AM251 were applied after KA wash (KA/LY $n = 6$, or KA/AM $n = 11$, respectively), monosynaptic responses were further decreased in amplitude and area in comparison to KA-only treated preparations ($n = 17$; Fig 4A–C). The polysynaptic reflex depression induced by KA remained unchanged in amplitude by LY2183240 or AM251 application (Fig 4D, E), while the decrease in the response area was intensified (Fig 4F). A modest improvement in the monosynaptic reflex area with no apparent change in peak amplitude was observed in spinal cords treated with AEA after KA washout (KA/AEA), while depression of polysynaptic responses persisted ($n = 10$; Fig 4A–F).

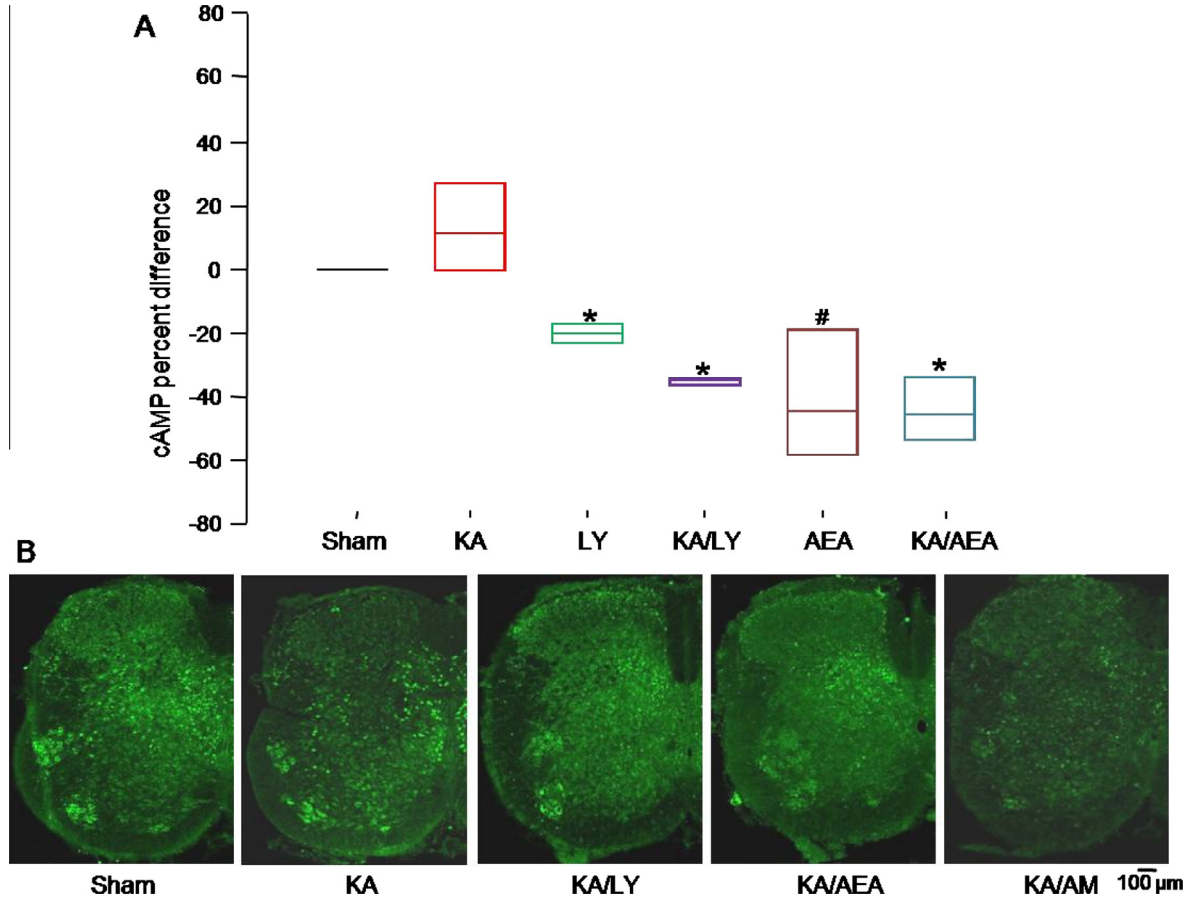

Fig. 2. Effect of KA and cannabinoid ligands on CB1R activity and its expression. (A) Box plots illustrating the distribution of changes in induced cAMP levels (expressed as % of sham samples). Data are shown as median with 25th and 75th quartile distribution. $^{*}P < 0.05$, $^{\#}P < 0.001$ (Kruskal–Wallis one-way analysis of variance on ranks). (B) Examples of CB1R immunostaining of sham, KA or KA/cannabinoid ligands treated preparations at low magnification (for experimental protocols see Methods). Scale bar = 100 μm.

In analogy, reflex parameters were not improved by 24-h administration of 2-AG after KA. Monosynaptic amplitude and area were 0.03 ± 0.01 mV and 3.61 ± 0.50 mV.ms, respectively (n = 8), thus largely depressed with respect to sham data (see Fig. 4). Likewise, polysynaptic amplitude and area values were 0.37 ± 0.08 mV and 366.93 ± 61.31 mV.ms, respectively (n = 8), results that showed no improvement vs. sham data (cf. Fig. 4).

Effect of CB1R modulation on network rhythmicity after KA treatment

DR train-evoked VR oscillations typical of FL were abolished by KA and not restored by LY2183240, AEA, AM251 or 2-AG application (Fig 5A, B). The depression in cumulative depolarization (and its area) induced by KA treatment was not changed by AEA (KA/AEA, n = 10 vs. KA, n = 17, respectively; Fig 5A–D), whereas LY2183240 (KA/LY, n = 6), AM251 (KA/AM, n = 11) or 2-AG (KA/2AG, n = 8) further increased it (Fig. 5A, C). In analogy with these data, chemically (NMDA plus 5-HT)-induced FL was suppressed by KA treatment and was not rescued by anyone of the CB1R ligands used. Sporadic, irregular VR bursts were observed in a few preparations treated with KA (8/17), KA/LY (2/6) and KA/AEA (6/10) with rather low amplitude and variable periodicity (Fig 6A–C): this observation confirms that rhythmic networks were not completely destroyed by KA, yet no FL recovery could be produced by CB1R modulation. None of the KA/AM treated preparations showed any burst like activity in NMDA/5HT application (n = 11, Fig 6A). Furthermore, only two out of eight preparations treated with 2-AG after KA displayed low amplitude, sporadic oscillations (Fig. 6A–C).

The intrinsic rhythmicity of spinal networks emerging from pharmacological inhibition of GABA and glycinergic transmission is a reliable index of the basic excitability of these simplified circuits (Bracci et al., 1996). KA-mediated excitotoxicity strongly scaled down the burst frequency, amplitude and intraburst occurrence without changing single burst duration (Fig 7A). These effects were mainly unchanged by LY2183240 or AEA application after KA washout. Only burst duration was prolonged by AM251 together with even lower burst frequency (Fig. 7B, D).

Histological changes induced by KA and cannabinoid ligand treatment

Lack of neuroprotection by LY2183240 or AEA despite persistence of functional CB1R activity could simply

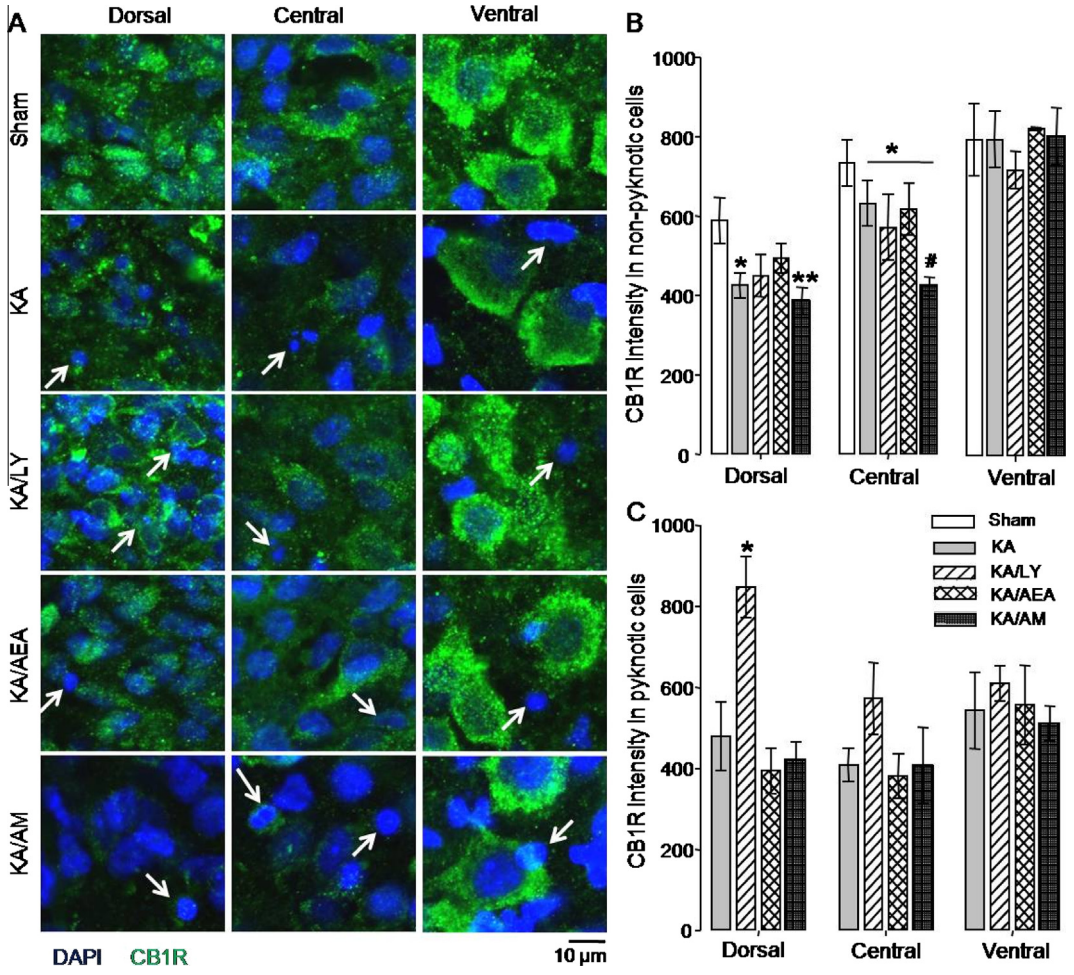

Fig. 3. Analysis of single-cell CB1R expression after KA or KA/cannabinoid ligands. (A) Examples of CB1R immunoreactivity in dorsal, central and ventral regions of spinal cords treated with KA or KA followed by cannabinoid ligands (for experimental protocols see Methods). Cell nuclei are stained with DAPI. Scale bar = 10 μm. (B) Bar graphs showing changes of CB1R immunostaining (in AU values; mean ± SEM) in non-pyknotic cells after KA (n = 11), KA/LY (n = 6), KA/AEA (n = 8) or KA/AM (n = 12) treatments compared to sham (n = 9) for the three indicated ROIs. (C) Histograms showing the mean (± SEM) of CB1R intensity in pyknotic cells after KA, KA/LY, KA/AEA or KA/AM treatments. Number of spinal cords as in (B). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.001$ (pyknotic cells are indicated in arrows; one-way analysis of variance with Tukey test for pairwise multiple comparison procedures).

indicate that CB1Rs were not neuroprotective in this model because their expression was inadequate in regions strategic for locomotor function. This possibility prompted us to look more closely at the regional topography of the excitotoxic damage following application of CB1R ligands (Fig. 8A).

Fig. 8B shows the distribution of pyknosis within dorsal, central and ventral ROI after KA application with or without subsequent administration of CB1R ligands. As expected, the dorsal ROI was the most intensely lesioned one because it contains the largest number of neurons (Cifra et al., 2012). LY2183240, AEA or 2-AG did not significantly alter the pyknosis data in any ROI compared to KA. However, when AM251 was applied after KA, a threefold increase ($P < 0.001$) in the percent of pyknotic cells was observed in the central ROI (Fig. 8B), suggestive of a certain role of CB1R intrinsic tone in modulating excitotoxicity.

Characterization of neuronal damage evoked by KA and its changes after cannabinoid ligand treatment

Fig 9C summarizes the changes in the number of NeuN-positive cells followed by KA and CB1R ligand treatments in the dorsal and central region. The dorsal region had a higher loss of neurons after KA treatment (around 20%). It is known from previous studies that KA receptors are predominantly present in the dorsal horn of the rat spinal cord (Tölle et al., 1993) and that excitotoxicity is primarily directed to nerve cells (Kuzhandaivel et al., 2011). None of the cannabinoid ligand treatments changed the number of dorsal horn neurons compared to KA treatment (Fig. 9A, C). KA/LY- or KA/2-AG-treated cords showed no change in the number of neurons compared to KA in the central region. Conversely, after KA/AEA application only a minimal neuronal loss was observed in the central region compared to KA-only treated preparations ($P < 0.05$; Fig. 9B, C). AM251 treatment significantly

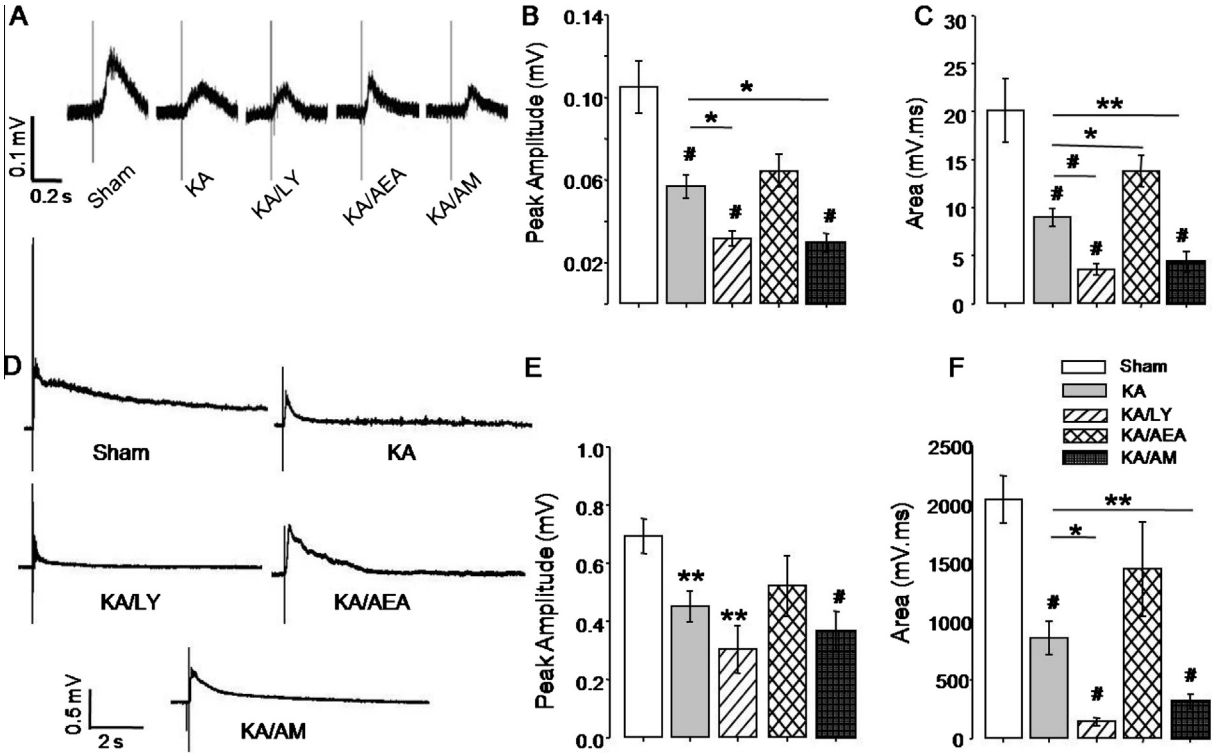

Fig. 4. Effect of KA and cannabinoid ligands on DR-stimulation-evoked synaptic transmission after 24 h *in vitro*. Representative traces of monosynaptic (A) and polysynaptic (D) reflex responses in sham, KA or KA/cannabinoid ligand treated preparations (for experimental protocols see Methods). (B, C) Histograms showing the peak amplitude (B) and area (C) of monosynaptic responses after KA (n = 17), KA/LY (n = 6), KA/AEA (n = 10) or KA/AM (n = 11) treated cords vs. sham (n = 17). (E, F) Bar graphs showing the changes in polysynaptic reflex amplitude (E) and area (F) after various treatments as above (same preparations as in B, C). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.001$ (All data are expressed in mean ± SEM; one-way analysis of variance with Tukey test for pairwise multiple comparison procedures).

($P < 0.05$) reduced the NeuN-positive cells in the central region in accordance with pyknosis data shown in Fig. 8B. Large SMI-32-positive neurons were counted in the ventral region to determine changes in motoneuronal survival after treatments with the same protocols shown above (Fig. 10A, B). The number of motoneurons was halved after KA treatment compared to sham and it was left unchanged with any of the CB1R ligand application.

DISCUSSION

The present study demonstrated two substantive outcomes after an excitotoxic insult to the rat spinal cord: (1) Even 24 h later and despite the associated neuronal loss, CB1R activity was well preserved, (2) CB1R activity was probably an intrinsic defense mechanism, yet its role was insufficient to bring about structural and functional protection of locomotor networks even when enhanced by AEA, LY2183240 or 2-AG.

Early effect of cannabinoid ligands on KA evoked damage

The endocannabinoid system has been shown as a promising therapeutic target for a spectrum of spinal neurological diseases including multiple sclerosis, spasticity, neuropathic pain, and SCI (Pacher et al., 2006; Koppel et al., 2014). Endocannabinoids are retrogradely released by strongly excited neurons to depress further transmitter release in a negative feedback process (Piomelli, 2014). In principle, this mechanism might be expected to contrast the spinal lesion caused by KA excitotoxicity associated with a large release of endogenous glutamate (Mazzone and Nistri, 2011). On the other hand, in hippocampal slices endogenous cannabinoids are rapidly mobilized to the neuronal membrane after KA receptor activation resulting in a decline of GABA release (Lourenço et al., 2011) which could perhaps intensify any excitation-related damage. In fact, our former data show early depression of GABA-mediated responses by AEA in the rat spinal cord (Veeraraghavan and Nistri, 2015). Within this context, the impact of CB1R activation on spinal network responsiveness to KA could not be predicted *a priori*. Hence, we studied whether early block or activation of CB1Rs (for 30 min) should affect KA-induced depolarization, and whether sustained manipulation of CB1R activity at later times might determine the outcome after the excitotoxic paradigm.

The present report indicates that AEA was not a functional antagonist of KA because it could not contrast the KA-induced network depolarization. Analogous results were obtained after applying 2-AG.

Delayed CB1R modulation in a SCI model

Using an *in vivo* rat model of contusion SCI, Arevalo-Martin et al. (2010, 2012) have shown that injection of

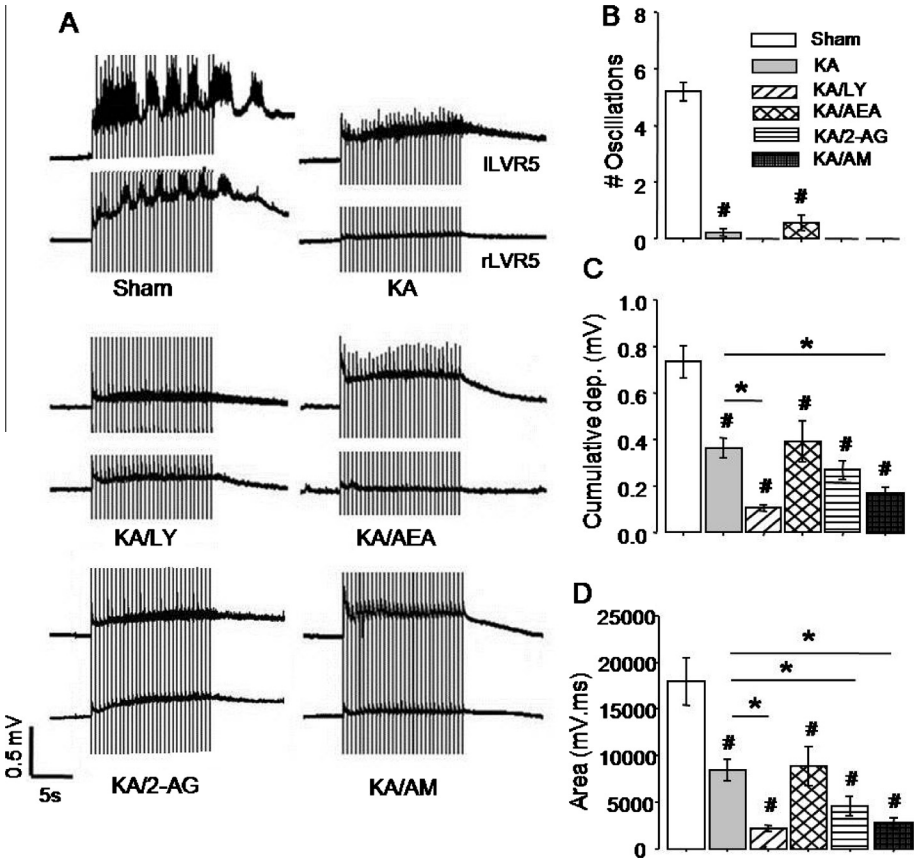

Fig. 5. Changes in DR train-evoked VR responses after 24 h *in vitro*. (A) Examples of fictive locomotion evoked by DR train in sham (top left), KA (top right), KA/LY (middle left), KA/AEA (middle right), KA/2-AG (bottom left) or KA/AM (bottom right). For experimental protocols see Methods. (B) Histograms showing the very intense depression of oscillations in the same spinal cords shown in Fig. 4. (C) Bar graphs indicating the decline in the amplitude of cumulative depolarization after KA and KA/cannabinoid ligand treatments. (D) Plots showing the fall in area of the FL after the same treatments as in B, C. KA/LY, KA/2-AG ($n = 8$) and KA/AM treated cords showed large depression in area. $^{*}P < 0.05$, $^{\#}P < 0.001$ (mean ± SEM; one-way analysis of variance with Tukey test for pairwise multiple comparison procedures).

the endocannabinoid agonist 2-AG 30 min after the mechanical lesion exerts beneficial effects (persisting for a few weeks) on the white matter lesion. Although the extent of functional recovery was not reported in those experiments, the data indicate that administration of a mixed agonist like 2-AG, that can activate CB1 and CB2 receptors (Di Marzo and De Petrocellis, 2012), may improve recovery when the target is primarily the spinal white matter. Because our excitotoxic model targets predominantly neurons rather than glia (see review by Kuzhandaivel et al. (2011), we did not observe neuroprotection by 2-AG. Tissue degradation of 2-AG into potentially toxic metabolites (Sang et al., 2007) might have contributed to the intensification of the KA evoked functional deficit.

The specific role of CB1Rs, that are mainly expressed by the spinal gray matter (Sideris et al., 2012), has been investigated by Hong et al. (2015) who also used an *in vivo* model of mouse SCI. In their study, daily administration of a selective CB1R agonist improved spinal tissue preservation and functional recovery after one or more weeks with respect to the normal degree of spontaneous recovery. These studies support a positive role of endocannabinoids during the late injury stages probably by modulating the neuroinflammatory process associated with the complex SCI pathophysiology (Piomelli, 2005). It remained, however, to be clarified the impact of endocannabinoids on neuronal numbers and the activity of the locomotor CPG shortly after the primary lesion. This issue was explored in the present report that inquired whether delayed CB1R activation was still possible after an excitotoxic insult and could provide functional and histological neuroprotection during the first 24 h. This approach also stemmed from *in vivo* SCI reports showing an early phase (4–24 h) of increased endocannabinoid levels (Garcia-Ovejero et al., 2009; Arevalo-Martin et al., 2012).

An excitotoxic insult may cause not only cell death, but also a compensatory mechanism in surviving cells whose resistance may depend on their receptor expression ability (Friedman et al., 1994). In this context we studied the CB1R function in terms of cAMP inhibition (Howlett et al., 2004) that was clearly observed with application of AEA or LY2183240 one day after KA treatment. This result was validated by the CB1R expression profile in both pyknotic and non-pyknotic cells. Although the intensity of immunofluorescence signal even at single-cell level is not an absolute index of receptor expression

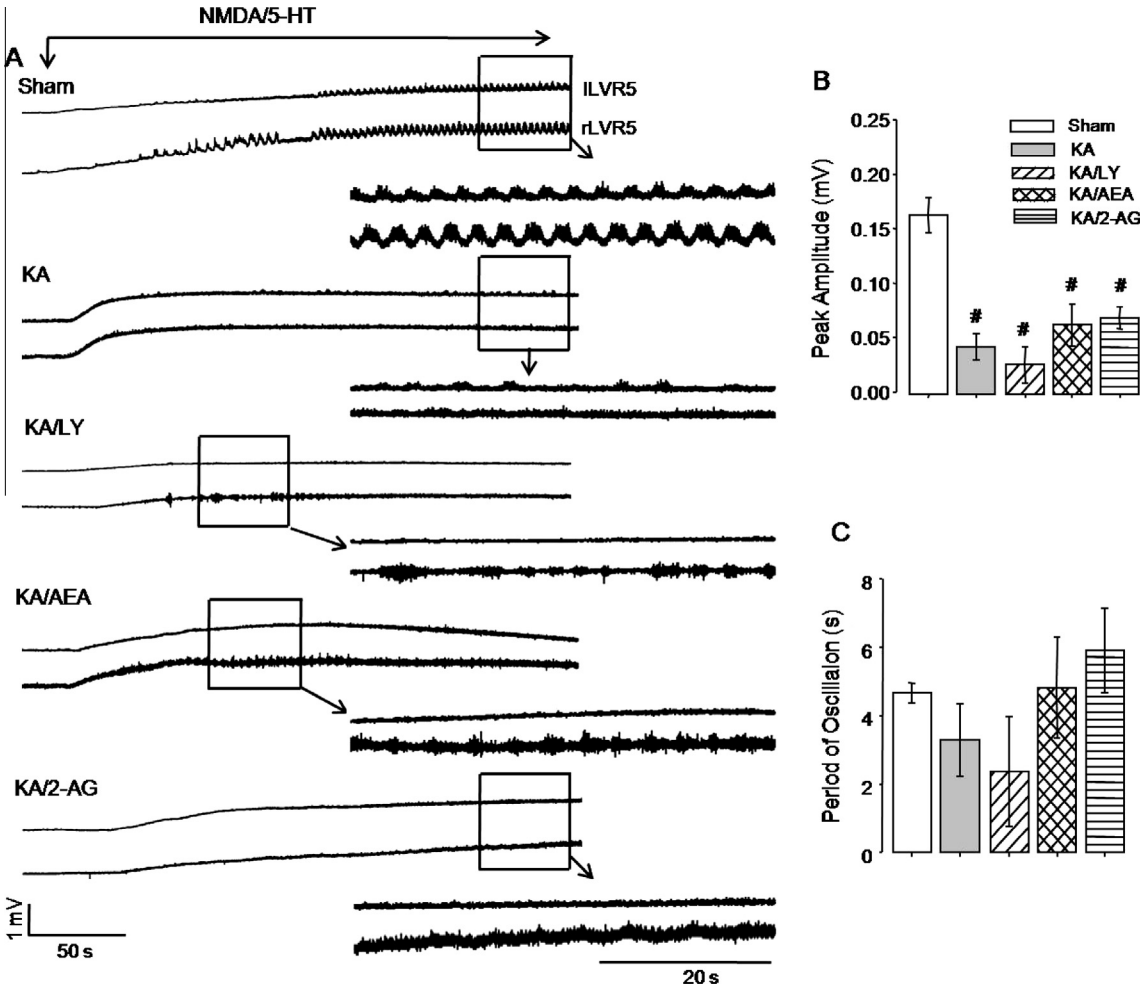

Fig. 6. Expression of fictive locomotion evoked by NMDA plus 5-HT after 24 h *in vitro*. (A) Examples of onset and maintenance of fictive locomotor patterns in sham, KA or KA/cannabinoid ligands treated spinal cords (for experimental protocols see Methods). Note the VR alternating rhythmic activity evoked by chemicals is disrupted by KA alone or followed by cannabinoid ligands treatment when compared to sham. Boxed sections show at faster time speed the typical patterns of fictive locomotion. (B) Bar graphs showing the significant depression of peak amplitude of few bursts seen after KA, KA/LY, KA/2-AG or KA/AEA treatments compared to sham. (C) Histograms indicating the periodicity of the sparse oscillatory cycles detectable after KA and cannabinoids treatment. $^{\#}P < 0.001$ (mean ± SEM; one-way analysis of variance with Tukey test for pairwise multiple comparison procedures). Full details are in Figs. 4 and 5 legends.

strength, the present data did not indicate a large deficit in CB1Rs.

Impact of CB1Rs on cell survival in a model of SCI

Enhancing CB1R activity with AEA or LY2183240 had differential consequences on cell survival depending on the spinal ROI examined. Thus, in the dorsal ROI that contains the largest number of neurons (Cifra et al., 2012), the CB1R expression was the lowest and their activation had no or slight consequence for neuronal survival. The central ROI expressed stronger CB1R immunoreactivity, significant (though not complete) neuroprotection by AEA and larger damage when CB1Rs were pharmacologically inhibited. The ventral ROI inclusive of motoneurons showed large CB1R expression, poor neuroprotection and particularly severe loss of motoneurons that could not be altered by endocannabinoids. These results suggest that there was no straightforward relation between topography of CB1R expression and neuronal resilience to insult. Immaturity of the CB1R system was unlikely to account for this neuroprotective failure because endocannabinoids are neuroprotective against excitotoxicity by KA applied to neonatal rat brain (Fernández-López et al., 2010). Thus, the functional consequences of the present data were explored with electrophysiological recording 24 h after applying KA.

Electrophysiological characteristics of locomotor networks after excitotoxic stimulation

Following application of KA, the rather modest improvement in spinal reflexes evoked by AEA was not translated into recovery of FL elicited by either DR stimulus trains or NMDA plus 5-HT. Although previous experiments have shown that AEA can partially depress VR oscillations evoked by DR stimuli (Veeraraghavan and Nistri, 2015), in the present study the modest depressant action by AEA did not appear to be additive to the strong depression by KA, perhaps suggesting that the

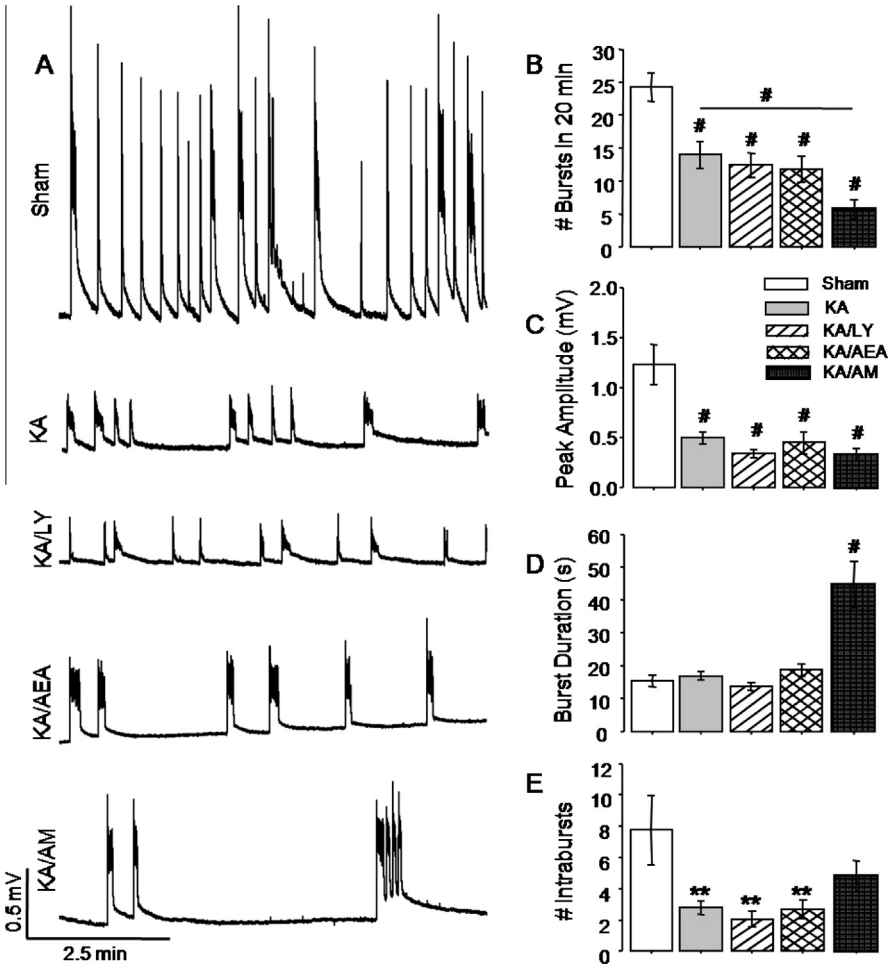

Fig. 7. Intrinsic rhythmicity of spinal cords following KA and cannabinoid ligands' treatment. (A) Examples of disinhibited bursts induced by inhibiting GABA and glycinergic transmission with bicuculline and strychnine (for experimental protocols see Methods) after 24 h *in vitro*. (B) Bar graphs showing significant decrease in number of bursts (in 20 min observation time) after KA (n = 13), KA/LY (n = 5), KA/AEA (n = 9) or KA/AM (n = 10) compared to sham (n = 15). (C) Histograms indicating the significant reduction in peak amplitude after various treatments compared to sham. (D) Bar graphs indicating changes in single burst duration after KA and cannabinoid ligand treatments compared to sham. (E) Histograms showing significant reduction in the number of fast oscillations within each burst after KA, KA/LY or KA/AEA treatments. $^{**}P < 0.01$, $^{\#}P < 0.001$ (mean ± SEM; one-way analysis of variance with Tukey test for pairwise multiple comparison procedures).

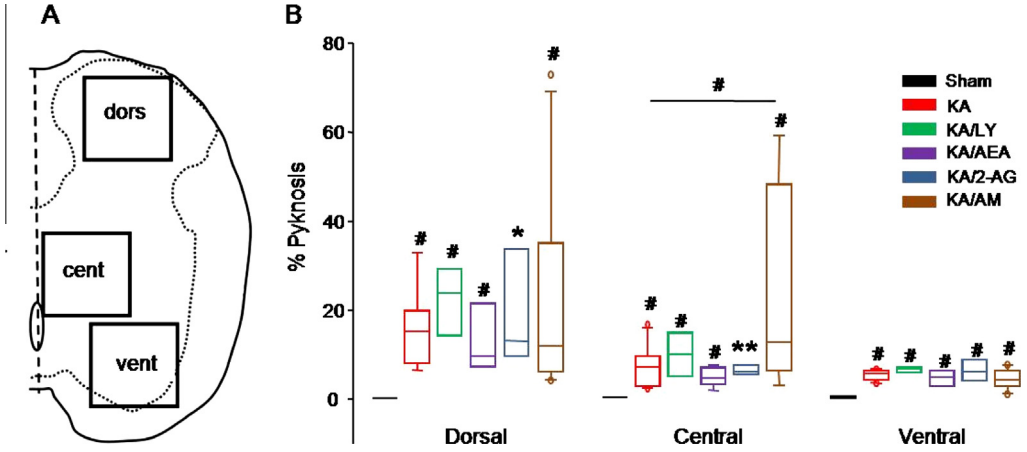

Fig. 8. Histological changes induced by KA or KA/cannabinoid ligand treatments after 24 h *in vitro*. (A) Schematic representation of the dorsal, central and ventral ROIs taken for analysis. (B) Box plots showing the percent of pyknotic nuclei after KA (n = 11), KA/LY (n = 6), KA/AEA (n = 10), KA/2-AG (n = 5) or KA/AM (n = 12) treatments compared to sham (n = 8) in each region. A rather strong increment is observed as significant in the central ROI after KA/AM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.001$ (Median and quartile distribution of all the data sets are shown; Kruskal–Wallis one-way analysis of variance on ranks).

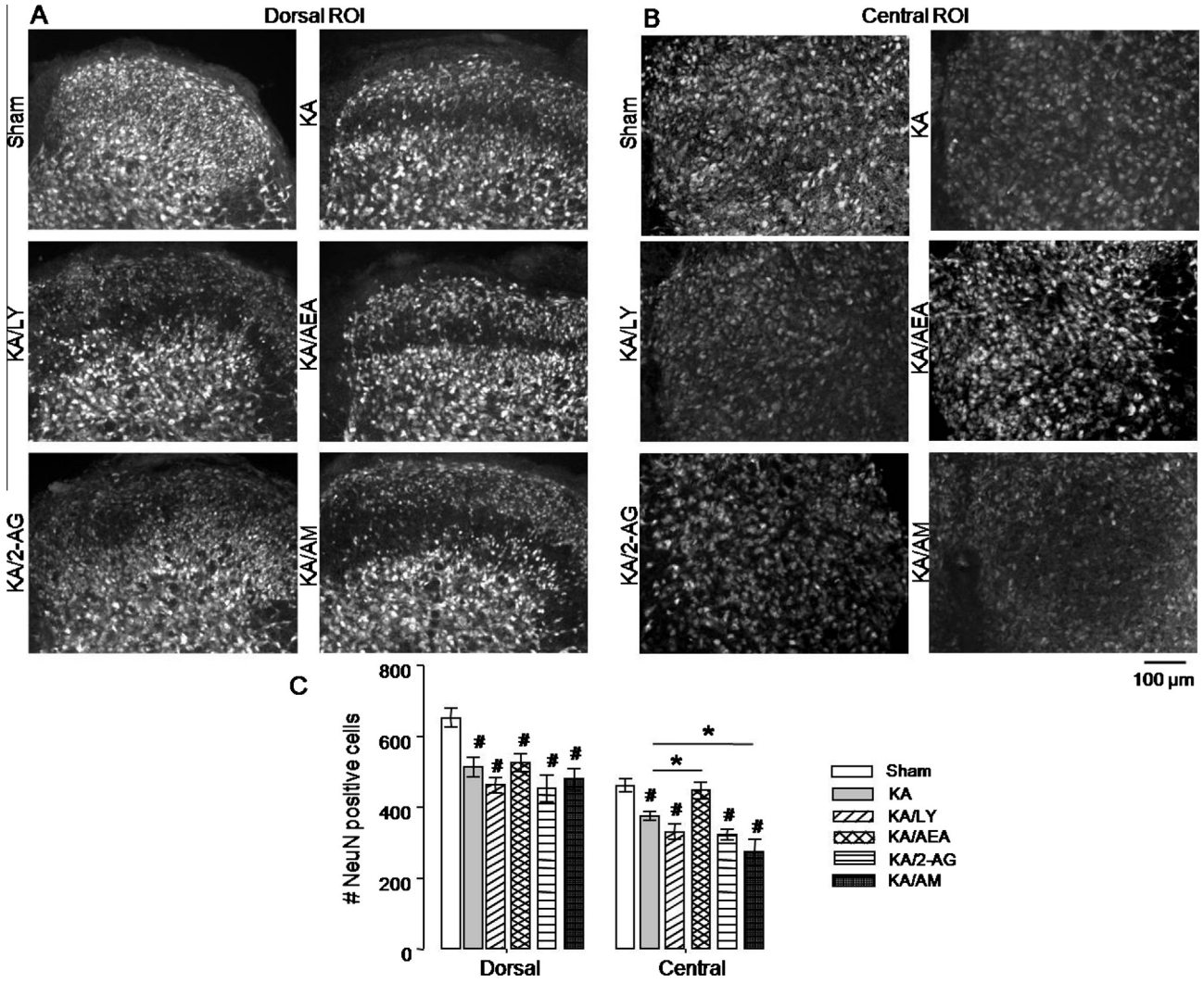

Fig. 9. Changes in neuronal numbers (identified with NeuN positivity) induced by KA or KA/cannabinoid ligand treatments after 24 h *in vitro*. (A, B) show examples of neuronal distribution in dorsal or central ROIs, respectively. Data pertain to treatment with KA (n = 11), KA/LY (n = 3), KA/AEA (n = 9), KA/2-AG (n = 5) or KA/AM (n = 12) and are compared to sham (n = 9). (C) Summarizes neuronal numbers after the treatments indicated above. $^{*}P < 0.05$, $^{\#}P < 0.001$ (mean ± SEM; one-way analysis of variance with Tukey test for pairwise multiple comparison procedures).

neuronal pathways depressed by AEA had been destroyed by KA. While the mechanism responsible for this occlusion remains unclear, the main implication is that AEA could not ameliorate this neurotoxic effect. In certain protocols like the use of LY2183240 with the aim of boosting the extracellular concentration of AEA, further deterioration of electrophysiological responses became apparent. This result was consistent with the depressed network activity elicited by LY2183240 applied for 24 h (Veeraraghavan and Nistri, 2015) and might be due to alterations in the endogenous level of glutamate (Gonzalez-Islas et al., 2012). FL elicited by DR trains was more severely depressed than the one induced by NMDA plus 5HT. In the latter, few sporadic cycles appeared probably indicating that the severity of the excitotoxic damage was not very large, yet capable of suppressing locomotor generation. Thus, bath-applied neurochemicals that are expected to excite a broader network that electrical pulses to a single DR can do, could apparently stimulate fragmentary elements of the locomotor program without reviving a full network output. The 2-day limit of *in vitro* survival of our preparation did not allow studying whether these primitive oscillatory components could be reorganized into locomotor patterns at later times.

The central region of the spinal cord is believed to contain the principal neural elements critical for generating locomotion (Kiehn, 2006). One might therefore expect that even relatively small changes in neuronal numbers can profoundly impact on the operation of these networks (Kuzhandaivel et al., 2011). Although AEA applied after KA almost restored neuronal numbers in this ROI, FL did not return. It is also noteworthy that the intrinsic rhythmicity observed as disinhibited bursting was also irreversibly depressed. We propose that one major contributor to this functional deficit was the strong damage to motoneurons unmitigated by either AEA or LY2183240. Because motoneurons are thought to be not only the output elements of the locomotor networks but also to actively contribute to rhythm generation

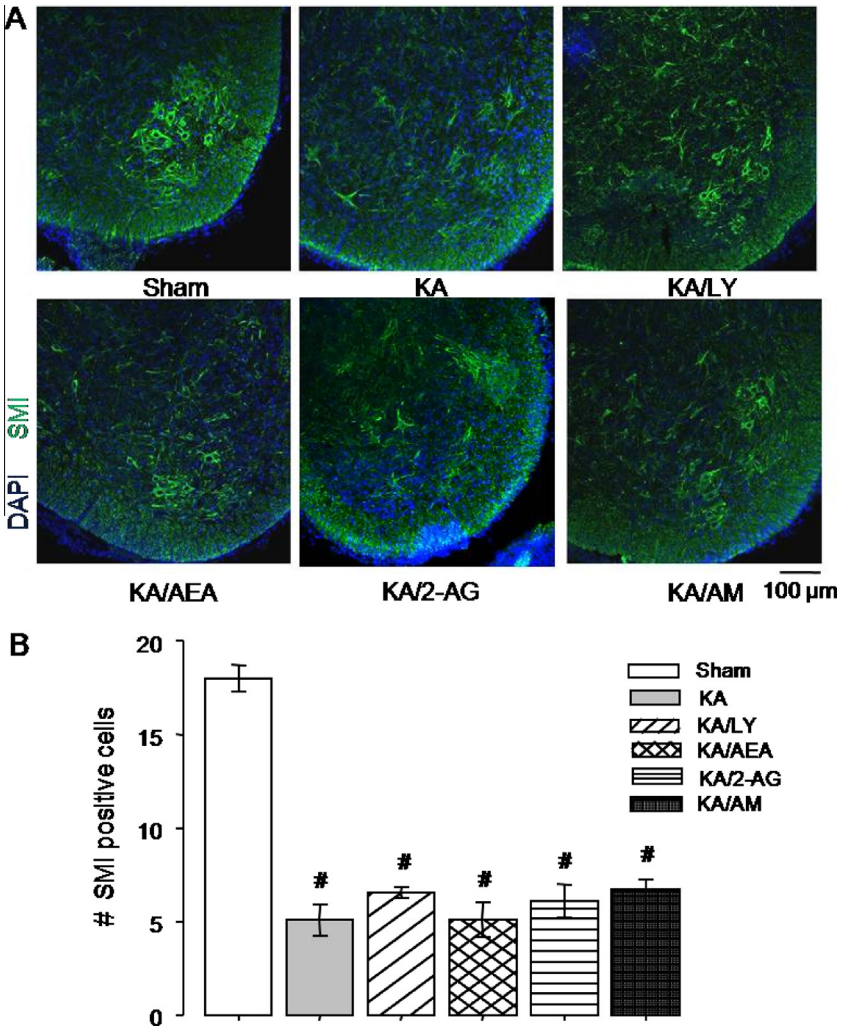

Fig. 10. Effect of KA and cannabinoid ligand treatment on motoneurons after 24 h *in vitro*. (A) Examples of large ventral horn neurons stained with SMI-32 and identified as motoneurons following the experimental protocols indicated in Methods. (B) Histograms quantify the number of motoneurons in the ventral ROI following various treatments. Sham ($n = 4$), KA ($n = 7$), KA/LY ($n = 3$), KA/AEA ($n = 3$), KA/2-AG ($n = 5$) and KA/AM ($n = 7$) spinal cords. Cannabinoid ligands do not change the large fall in motoneuron number evoked by KA. $^{\#}P < 0.001$ (mean ± SEM; one-way analysis of variance with Tukey test for pairwise multiple comparison procedures).

(Tresch and Kiehn, 2000), our proposal is that loss of motoneurons was a critical phenomenon underlying lack of locomotor network activity. Thus, attempts to repair the damaged networks become a desirable goal (Bradbury and McMahon, 2006).

The mixed CB1R and CB2R agonist 2-AG applied after the excitotoxic stimulation also proved to be ineffective to neuroprotect locomotor network activity and neuronal numbers in the rat spinal cord *in vitro*, suggesting that even encompassing CB2R activation did not confer additional amelioration. We propose that activation of endocannabinoid systems might be beneficial with respect to the late phases evolving from the primary lesion when neuroinflammatory processes become important to determine the lesion outcome (Park et al., 2004; Oyinbo, 2011).

Our current data do not exclude some mitigation of KA neurotoxicity by local endocannabinoid release because the functional and morphological damage was strongly enhanced by applying the CB1R antagonist AM251. Interestingly, damage intensification was detected when the antagonist was applied after KA washout, suggesting that endocannabinoid release perhaps continued after the first stage of acute excitotoxicity, although it was inadequate to arrest subsequent damage.

CONCLUSIONS

It seems likely that the CB1R activity developing in the aftermath of the KA insult was partially neuroprotective. Since CB1Rs were probably nearly saturated by endogenous ligands in this pathological condition, any further increase in CB1R activity with an exogenous agonist would be ineffective. On the other hand, inhibiting CB1Rs with AM251 could result in the loss of a naturally occurring protective mechanism as demonstrated by the present experiments.

This scenario indicates that, despite reports that in the rat brain CB1R agonists reduce short-term mitochondrial dysfunction and oxidative stress after excitotoxicity

(Rangel-López et al., 2015) and minimized matrix metalloproteinase (MMP) activity with consequently minimal cell loss after SCI (Hong et al., 2015), spinal networks rely only partially on their endocannabinoid system to contain early excitotoxic damage. Even the observed small neuronal losses in the central region, that is important for rhythm generation, may bring the locomotor network below the threshold value for network membership adequate to support such a coordinated and complex function (Kuzhandaivel et al., 2011). This situation is likely to be aggravated by the motoneuron loss.

The beneficial effects of CB1R activity may be better expressed at later times when a complex series of cellular events including activation of microglia and gliosis are called upon to transform the acute lesion into a chronic condition (Arévalo-Martín et al., 2003; Pacher et al., 2006). During such a phase, spasticity and chronic pain may also benefit from cannabinoids (Boadas-Vaello et al., 2016) as supported by recently issued clinical guidelines (Koppel et al., 2014) and by experimental studies indicating that hypoactivity of the spinal cannabinoid system results in NMDA-dependent hyperalgesia (Richardson et al., 1998).

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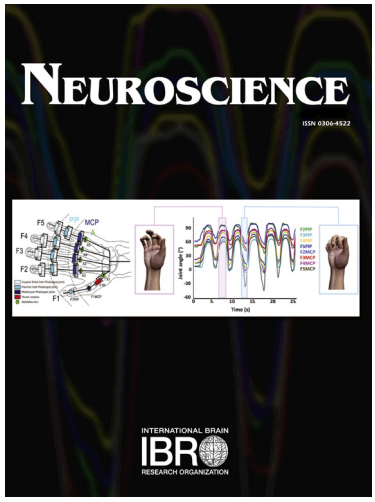

ASIC channel inhibition enhances excitotoxic neuronal death in an in vitro model of spinal cord injury

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ASIC channel inhibition enhances excitotoxic neuronal death in an in vitro model of spinal cord injury

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Highlights

- ASIC channels are nonselective pores activated by mild acidification and modulating neuronal function

- Expression of ASIC channels by mouse spinal cord was increased after a borderline excitotoxic stimulation
- ASIC pharmacological blockers or the ASIC1a knockout phenotype intensified excitotoxicity
- Electrophysiological network oscillations were suppressed by mild excitotoxic stimuli followed with ASIC block
- ASIC activity was an unexpected controller of neurotoxicity to restrain spinal network damage

Abstract

In the spinal cord high extracellular glutamate evokes excitotoxic damage with neuronal loss and severe locomotor impairment. During the cell dysfunction process, extracellular pH becomes acid and may activate acid-sensing ion channels (ASICs) thought to be important contributors to neurodegenerative pathologies. Our previous studies have shown that transient application of the glutamate analogue kainate (KA) evokes delayed excitotoxic death of spinal neurons, while white matter is mainly spared. The present goal was to enquire if ASIC channels modulated KA damage in relation to locomotor network function and cell death. Mouse spinal cord slices were treated with KA (0.01 or 0.1 mM) for 1 h, and then washed out for 24 h prior to analysis. RT-PCR results showed that KA (at 0.01 mM concentration that is near-threshold for damage) increased mRNA expression of ASIC1a, ASIC1b, ASIC2 and ASIC3, an effect reversed by the ASIC inhibitor 4',6-diamidino-2-phenylindole (DAPI). A KA neurotoxic dose (0.1 mM) reduced ASIC1a and ASIC2 expression. Cell viability assays demonstrated KA-induced large damage in spinal slices from mice with ASIC1a gene ablation. Likewise, immunohistochemistry indicated significant neuronal loss when KA was followed by the ASIC inhibitors DAPI or amiloride. Electrophysiological recording from ventral roots of isolated spinal cords showed that alternating

oscillatory cycles were slowed down by 0.01 mM KA, and intensely inhibited by subsequently applied DAPI or amiloride. Our data suggest that early rise in ASIC expression and function counteracted deleterious effects on spinal networks by raising the excitotoxicity threshold, a result with potential implications for improving neuroprotection.

Keywords: pH; acid sensing ion channels (ASICs); neuroprotection; kainic acid; spinal cord injury; fictive locomotion.

Abbreviations

ANOVA, analysis of variance; ASICs, Acid-sensing ion channels; AU, arbitrary unit; DAPI, 4',6-diamidino-2-phenylindole; DIV, days in vitro; DME/HIGH, Dulbecco's modified Eagle's medium high glucose; DR, dorsal root; FCS, fetal calf serum; KA, kainate; NGF, nerve growth factor; NeuN, neuronal specific nuclear protein; PBS, phosphate-buffered saline; PI, propidium iodide; S100β, astroglial calcium-binding protein S100 β; SCI, spinal cord injury; SEM, standard error of the mean; VR, ventral root.

Introduction

Spinal cord injury (SCI) is a devastating event marked by a series of cellular and molecular mechanisms that promote or contrast neuronal death and loss of locomotor activity in a process slowly developing after the primary lesion (Borgens and Liu-Snyder, 2012; Anwar et al., 2016). During this dynamic disturbance it is likely that neuronal acid homeostasis needs tight control to avoid aggravating the pathophysiological outcome (Ruffin et al., 2014). Acid pH fluctuations at synaptic (and perisynaptic) level may originate, at least in part, from the high proton content of synaptic vesicles (Sinning and Hübner, 2013) during exaggerated synaptic transmission as well as lesion-evoked neuronal disruption. Acidosis has been recently demonstrated to be a Janus-like process that may lead, on the one hand, to hyperexcitability due to dysregulation of cortical GABAergic neurons and alterations in glutamate re-uptake by astrocytes (Huang et al., 2015a), or to inhibition of glutamate receptors to curtail excessive excitation (Giffard et al., 1990; Tang et al., 1990). Furthermore, several types of voltage or ligand gated channels are modulated by pH with complex impact on neuronal excitability (Holzer, 2009), for instance activation of certain background K^+ conductances by acid pH can stabilize the resting membrane potential and depress excitability (Ma et al., 2012).

In addition to classical modulation of synaptic transmission by pH, more recent targets of protons have been identified as a subpopulation of membrane channels termed ASICs (Gründer and Pusch, 2015; Boscardin et al., 2016). ASIC channels are proton-gated, non-selective cationic channels belonging to the degenerin epithelial sodium channel superfamily (Osmakov et al., 2014). ASIC channels are activated when pH falls below 7 and generate a transient inward current that rapidly fades to a shallow, persistent current potentially suitable to induce longterm effects on neurons (Alexander et al., 2011; Osmakov et al., 2014). Recent studies have proposed that activation of ASICs, particularly the ASIC1a subtype that mediates Na^+, K^+ and Ca^{2+} transmembrane flow (Yang and Palmer, 2014), plays a critical role in neuronal injury associated with neurological disorders such as brain ischemia (Krishtal, 2003), multiple sclerosis (Arun et al., 2013), and spinal cord lesion (Hu et al., 2011). Furthermore, ASIC1a channels are important mediators of aberrant plasticity of AMPA receptor-mediated synapses in the hippocampus following transient anoxia (Quintana et al., 2015). The elucidation of the acidosis mechanism involvement in acute SCI remains, however, to be clarified. Our previous studies were focused on exploiting a lesion model based on transient excitotoxic stress as the trigger mechanism for delayed damage of the spinal cord (Taccola et al., 2008; Mazzone et al., 2010; Mazzone and Nistri, 2011a). This approach stems from the notion that excitotoxicity (due to temporary, large release of glutamate) is critical for lesion amplification and severity (Oyinbo, 2011; Borgens and Liu-Snyder, 2012), and that, in a clinical setting, the associated metabolic impairment is promptly corrected with intensive care treatment (Park et al., 2004; Dietz, 2010). This model, based on the neonatal rodent isolated spinal cord and organotypic spinal slices, offers an advantageous device to test preclinical approaches to neuroprotection because excitotoxicity (evoked by the glutamate analogue kainate, KA) destroys neurons with only modest white matter damage (Taccola et al., 2008; Mazzone et al., 2010; Mazzone and Nistri, 2011a). The damage is produced without intervening changes in blood pressure or general metabolism, thus providing a platform for testing proof of principle of direct neuroprotection at central level. The aim of the present study was to understand whether ASIC channel activity might have a beneficial or negative effect on the outcome of excitotoxicity studied 24 h after the primary insult and investigated with histological and electrophysiological methods. In particular, we wished to

explore if modulation by ASIC channels might transform a near-threshold neurotoxic action of KA into a more damaging one in terms of histology and function.

Experiments were carried out with mouse spinal cord preparations, using wild type or ASIC1a knockout mice (Price et al., 2001; Wemmie et al., 2002), and were also performed with ASIC channel inhibitors like DAPI (Chen et al., 2010) and amiloride (Alexander et al., 2011; Leng et al., 2016) even if such drugs may exert additional effects on the nervous system.

Materials and Methods

Choice of spinal preparation

The present study used mouse spinal cord tissue for three preparations in vitro: organotypic slices, acute spinal cord slices and the isolated spinal cord. Because of the ease of accessibility and positioning of the electrochemical biosensor, the organotypic slices were used for measuring glutamate release and pH changes plus correlated tests of neuronal survival and gene expression (Mazzone and Nistri, 2011b). Acute spinal slices were employed for evaluating the local distribution of neurotoxicity assessed with fluorescence changes due to intracellular accumulation of propidium iodide (PI) in topographically-intact sub-regions and laminae to which PI could be readily distributed via bath-application. For monitoring delayed changes in neuronal survival and network activity, we used the isolated spinal cord preparation that requires several intact segments to express coordinated alternating rhythmic discharges (Kjaerulff and Kiehn, 1996) investigated electrophysiologically (Taccola et al., 2008). This approach enabled us to examine the issue of neurotoxicity from various research angles converging onto the same spinal networks (Mazzone et al., 2010; Cifra et al., 2012).

Thoracolumbar spinal cord preparations were isolated from neonatal C57BL/6J mouse (3 days old). The experiments were conducted with the approval from the Scuola Internazionale Superiore di Studi Avanzati (Trieste) Ethical Committee, in accordance with the National Institutes of Health (NIH) guidelines and the Italian act D.Lgs. 27/1/92 n. 116 (implementing the European Community directives n. 86/609 and 93/88). The experiments performed in Argentina were carried out according to National guidelines and approved by local Ethical Committees, Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio (CICUAL, FCEN-

UBA, act C.D. Nº 3141/11, protocol Nº 42). A total of 103 animals were used for the immunohistochemistry and functional analysis. Details of animal numbers in each group are as follows: sham = 19; KA = 22; KA/DAPI = 13; KA/amiloride = 27; DAPI = 9; amiloride = 13. All efforts were aimed at minimizing animal use and suffering. Spinal cords were continuously superfused in a recording chamber with Krebs' solution of the following composition (in mM): NaCl, 113; KCl, 4.5; $MgCl_2.7H_2O$, 1; $CaCl_2$, 2; NaH_2PO_4, 1; $NaHCO_3$, 25; glucose, 11; gassed with 95% O2 5% CO_2; pH 7.4 at room temperature. All details about laboratory procedures have been previously published and the experimental setup has been fully reported (Taccola et al., 2008). Drugs were dissolved in Krebs solution and bath applied at the concentrations indicated in the text.

Spinal cord organotypic slice cultures were prepared from pregnant C57BL/6J mouse, at 13 days of gestation, in accordance with our standard procedure as previously reported (Mazzone et al., 2010; Cifra et al., 2012). This study comprised 6 series of different cultures, each one yielding, on average, 40 slices for immunohistochemistry, gene expression and electrochemical release. Briefly, culture slices were grown in a medium containing 82 % Dulbecco's Modified Eagle medium (DME/HIGH; osmolarity 300 mOsm, pH 7.35, "complete medium"), 8 % sterile water for tissue culture, 10 % foetal bovine serum (FBS Invitrogen, Carlsbad, CA, USA), 5 ng/ml nerve growth factor (NGF; Alomone Laboratories; Jerusalem, Israel), and maintained in culture for 22 days in vitro (DIV) in accordance with standard procedures (Gahwiler, 1981; Mazzone et al., 2010). DME/HIGH, penicillin and streptomycin were purchased from Euroclone (Paignton,UK), chicken plasma was from Rockland Immunochemicals (Gilbertsville, PA, USA), thrombin was from Merck KGaA (Darmstadt, Germany). KA was purchased from Ascent Scientific (Weston-Supermare, UK). Unless otherwise indicated, other reagents were purchased from Sigma-Aldrich (Milan, Italy and Buenos Aires, Argentina).

Protocol for spinal cord excitotoxic injury

Excitototoxicity was induced by applying KA for 1 h (10 µM or 100 µM) dissolved in Krebs' solution for isolated in vitro spinal preparations, or complete culture medium for organotypic cultures, and then washed out to induce a minimal to moderate injury (Mazzone et al., 2010). 4', 6-Diamidino-2-phenylindole (DAPI, Sigma-Aldrich, 3.5 µM) (Chen et al., 2010) or amiloride

(Sigma-Aldrich, 100 µM) (Chen et al., 2010) was administered for 24 h after KA washout. Following KA washout, cultures were used for mRNA RT-PCR analysis, electrophysiological recordings and/or immunohistochemistry studies. Each experiment contained untreated preparations (sham) that were maintained for the same time and subjected to the same experimental procedures.

Electrochemical Release

Electrochemical biosensors (Sarissa Biomedical Ltd, Coventry, UK) were used in accordance with our previous reports (Mazzone and Nistri, 2011b; Mazzone et al., 2013). On-line records were integrated with a potentiostat (Pinnacle Technology Inc., Lawrence, KS, USA) and analysed off-line with PAL software (V1.6.7; Pinnacle Technology Inc.). Thus, we employed glutamate and null biosensors placed at each side of the ventral fissure (Fig. 1 A). The current generated by released glutamate was monitored with the glutamate-sensing electrode, while the null electrode was used for recording the background current known to be directly proportional to the extracellular pH change (Llaudet et al., 2005; Robinson et al., 2008). Hence, as previously demonstrated (Llaudet et al., 2005), in control solution the null electrode current linearly grew in relation to test acidification of the medium within the 5.8-7.4 pH range (see linear plot in Fig. 1 B with data fitted by the equation $y = -0.06626x + 0.486$, $R^2 = 0.9933$, $n=6$, $p \leq 0.05$, t-test from 7.4 pH). Thus, a calibration curve for the null sensor was obtained by using different basal solutions adjusted within the 5.8-7.4 pH range. The level of acidification detected by the null sensor was estimated as a mean current change expressed in nA. Because the experimental data were obtained from organotypic cultures whose structure normally comprises two cell layers only and, thus, they are comparatively small with respect to the extracellular volume, it seems unlikely that the calculated values reflected changes in the extracellular space. At the beginning of each experiment the specificity of the glutamate sensor was validated by using a glutamate calibration curve (0.5-50 µM) and a single point calibration (with 25 µM) was repeated at the end of each experimental day. The change of glutamate and null electrode signals were recorded simultaneously by using a basal solution (containing in mM: NaCl, 152; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; glucose, 10, pH 7.4; 300–320 mOsm; Sigma-Aldrich, Milan, Italy).

Real-time PCR expression levels

The expression levels of ASIC1a, ASIC1b, ASIC2 and ASIC3 were evaluated in spinal cord organotypic slice cultures by real-time PCR using specific primers (Table 1). The design of primers and/or their specificity check was performed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The total RNA was isolated using Tri Reagent solution according to the manufacture's protocol (Invitrogen). RNA samples were quantified in a spectrophotometer at 260 nm. Retrotranscription using 1 µg of total RNA was performed with an iScript cDNA Synthesis Kit (BIORAD Cat. No 170-8891) according to the manufacture's suggestions. The reaction was run in a thermocycler at 25°C for 5 min, 42°C for 45 min, 85°C for 5 min. The reaction was performed in the iQ Real Time PCR Thermal Cycler (BIO RAD) using the fluorescent dye SYBR Green (SYBR Green Supermix kit IQTM; BIORAD). The analysis of the results was performed using iCycle iQ Real Time PCR Detection System (BIORAD) program. mRNA samples were calibrated to obtain similar amplification of the 18S and β-actin as housekeeping mRNA. Cycling parameters were determined, and calculations for relative mRNA transcript levels were performed using the comparative CT method (ΔΔCT; Pfaffl, 2001)) between cycle thresholds of different reactions, normalized to the housekeeping gene. Melting curve analysis was performed to assess product specificity.

Immunostaining and cell counting

Immunohistochemistry experiments were performed in accordance with previous reports (Mazzone et al., 2010; Cifra et al., 2012). Spinal cord preparations were fixed in 4 % paraformaldehyde for 60 min at room temperature and stored in phosphate buffer saline (PBS) until use or in 30 % sucrose PBS for cryoprotection (24 h at 4°C). Cryoprotective slices were cryostat-sectioned and collected sequentially on histology slides. Organotypic slices were blocked with 3 % fetal calf serum (FCS), 3 % bovine serum albumin (BSA), 0.3 % Triton in PBS (blocking solution) for 1 h at room temperature, followed by overnight incubation at 4°C in a blocking solution containing the previously-validated primary antibodies using the following dilutions: NeuN (1:250; Millipore, Billerica, MA, USA) and S100β (1:1000; Dako, Glostrup, Denmark). For neonatal spinal cord preparations, sections were pre-incubated in blocking solution containing with 5 % FCS, 5 % BSA, 0.3 % Triton in PBS for 1 h at room temperature.

Slices were incubated overnight at 4°C in a solution containing 1 % FCS, 1 % BSA, 0.1 % Triton in PBS, with primary antibodies using the following dilutions: NeuN (1:200; Millipore) and S100β (1:500; Dako). Primary antibodies were visualized using a secondary fluorescent antibody (Alexa Fluor 488 or 594 at 1:500 dilution, Invitrogen, Carlsbad, CA, USA) after mounting the sections with Vectastashield medium (Vector Laboratories, Burlingame, CA, USA).

For each slice culture, the number of NeuN and S100β positive cells was obtained by counting images with a Zeiss Axioskop2 microscope (Zeiss Axioskop2, Carl Zeiss MicroImaging, Thornwood, NY, USA) or FV300 confocal microscope (Olympus Optical, Tokyo, Japan), as previously reported (Cifra et al., 2012). The counting was performed with "eCELLence" software (Glance Vision Tech, Trieste, Italy) or by counting manually with using Image J software (version 1.49v, Wayne Rasband, National Institutes of Health, Bethesda, MD). NeuN positive cells were counted in three regions of interest (ROIs, namely: dorsal, central and ventral). When biomarker staining was diffuse, like in the case of S100β, signals were collected as mean fluorescence intensity, expressed in arbitrary units (AU) with densitometry analysis using a Zeiss Axioskop2 microscope with MetaVue imaging software (Molecular Devices, Sunnyvale, CA) in fields of 500x500 μm area. For neonatal spinal cord preparations NeuN and S100β positive cells were counted in four different ROIs: dorsal grey matter, ventral grey matter, and central grey matter (110 x110 μm), using FV300 confocal microscope by counting stacks of 4 images (60x magnification, pinhole was airy 1 corresponding to 102 μm) in an area of 52,900 μm^2. The total number of neurons, NeuN positive cells, and the mean fluorescence intensity for S100β astrocytes, were normalized by the ROI size (μm^2) to obtain cell density.

Propidium iodide staining

Thoracolumbar spinal cord preparations isolated from neonatal C57BL/6J mouse (3 days old), were embedded in 1.5% Agarose (Sigma)/Krebs' solution at 35°C and allowed to solidify on ice for 5 min (Mitra and Brownstone, 2012). For these experiments, a total of 10 mice were used, 5 of them belonged to the C57BL/6J strain and 5 ASIC1a$^{-/-}$ genotype: 67 slices were obtained in total. Slice numbers used for each experimental group are as follows: C57BL/6J control = 9; KA = 13; ASIC1a$^{-/-}$ control = 9; KA = 15. Slices (300 μm) were obtained by using a vibrating tissue slicer (Integraslice 7550PSDS, Campden Instruments Limited, UK). Slices were then

incubated in PEG solution (30% v/v, PEG pure in distillated water) for 60 s, and washed twice in Krebs' solution. Slices were kept in Krebs' solution (at 35°C) gassed with 95% O_2 5% CO_2 for 1 h. After this incubation time, slices were maintained at RT for spinal cord excitotoxic injury protocol as previously described. Slices were incubated with propidium iodide staining solution (PI, P3566 - Molecular Probes, Invitrogen, Buenos Aires, Argentina, 0.2 µL/1 mL in Krebs' solution) at room temperature for 5 min in the dark. Slices were observed under a BX51WI upright microscope with a 10X objective lens (LUMPlane FI, Olympus) and an EMCCD camera (AndoriXon, Oxford Instruments), together with cell-M System Coordinator/ cell-R Real Time controller software. PI quantification was done with Image J software. Fig. 1 C, D shows an example of enhanced PI signal when the same spinal cord slice was treated (for 5 min) with 100 µM HCl that induced strong dye accumulation. For the present study we also used a batch of ASIC1a$^{-/-}$ mice (Wemmie et al., 2002) kindly donated by Dr. J.A. Wemmie, University of Iowa, USA. PCR tests confirmed that these mice were knockout for the ASIC1a$^{-/-}$ gene (Fig. 1 E) in accordance with previous data (Price et al., 2001; Wemmie et al., 2002).

Electrophysiological recordings

Using tight-fitting Ag/Ag-Cl monopolar suction electrodes, DC-coupled records were obtained from lumbar (L2 and L5) ventral roots (VRs) that contain the motor axons of flexor-extensor motor pools of hind limbs (Taccola et al., 2008). Electrical square pulses (0.1 ms) were delivered to a single lumbar dorsal root (DR) through a bipolar suction electrode and the responses from VRs were simultaneously recorded at 10 KHz with a DP-304 Differential amplifier (Warner Instruments LLC, Hamden, CT, USA). Response threshold was determined for each preparation by applying graded electrical stimuli and detecting a minimal response in ipsi-lateral and ipsi-segmental VRs. Two to three times threshold stimulus intensity at frequency of 2 Hz for 15 s was given to induce DR evoked fictive locomotion (Marchetti et al., 2001). Chemically-induced fictive locomotion was obtained by bath-applying N-methyl-D-aspartate (NMDA) (1.5-6 µM) plus 5-hydroxytryptamine (Kiehn and Kjaerulff, 1998) (5-HT; 10 µM). Signals were acquired and processed with pClamp software (version 9.2; Molecular Devices, Sunnyvale, CA, USA). Measurements concerning locomotor rhythm were in accordance with Taccola et al. (Taccola et al., 2008).

Statistics

Results were expressed as means ± SEM unless otherwise indicated; n refers to the number of sections (acute spinal cord slices), organotypic cultures, or isolated spinal cords. Statistical calculations were done using SigmaStat 3.11 (Systat Software, Chicago, IL, USA). To distinguish between parametric or non-parametric data, the normality test equal variance criteria were routinely used in accordance with the software directed choice. For multiple comparisons the ANOVA test for parametric data followed by the Tukey-Kramer post-hoc test or Fisher's least significant difference test were used. Non-parametric values were analyzed with the Kruskal-Wallis test followed by Dunn's Method for multiple comparisons. When two groups were compared, the Student's t-test for parametric data or the Mann-Whitney Rank Sum Test for non-parametric data was applied. Two groups of data were considered statistically different if $p \leq 0.05$.

Results

Excitotoxic cell death is modulated by H^+ release

In our previous study concerned with an vitro model of SCI, we have shown that KA induces delayed excitotoxic cell death (Taccola et al., 2008) and evokes release of endogenous glutamate (Mazzone and Nistri, 2011b) with insult-related proliferation of endogenous spinal neuroprogenitors over a relatively brief time course (Mazzone et al., 2013). The early factors controlling this process and determining the functional outcome remain incompletely understood. Our previous experiments have also indicated that the release of endogenous glutamate can be quantified with a real-time electrochemical assay as a useful, simple index of spinal network activity in culture (Mazzone and Nistri, 2011b). A useful adjunct of this method is that the null electrode can be successfully employed for monitoring pH changes alongside glutamate concentrations (Llaudet et al., 2005; see Methods), thus enabling concurrent estimation of extracellular glutamate and pH. Fig. 2A, B shows average time course and extent of glutamate release induced by 10 (left) or 100 (right) μM KA from a mouse organotypic slice culture. As indicated by the different vertical scales in Fig. 2 A, B, the peak amplitude of the glutamate sensor response was significantly higher with 100 than 10 μM KA (4.39±1.08 vs

0.061±0.004 nA, respectively, n=6, * p≤ 0.05, t-test): the higher gain of the latter response is accompanied by larger basal noise. For either KA concentration the signal peaked within 10 min from the start of KA application and then gradually waned to reach baseline by 30 min in analogy with former data with rat spinal slice cultures (Mazzone and Nistri, 2011b). Fig. 2 C, D illustrates the concomitant changes in current recorded by the glutamate-insensitive null electrode and representing extracellular acidification. Although the response size of the null electrode was similar to the glutamate one when 10 μM KA was applied (Fig. 2 C), in the presence of 100 μM KA the null electrode response peak was ten times smaller than the glutamate current signal (Fig. 2 D). On the basis of the calibration plot (see Methods and Fig. 1 B), it was estimated that the average pH attained during 10 μM KA application was 6.10±0.09 (n=6). Thus, even with a KA concentration (10 μM) that was just at threshold value to evoke excitotoxicity (Mazzone et al., 2010), the pH fall and the glutamate release peaked at the same time (2.7±0.7 or 2.7±0.6 min for pH or glutamate, respectively (n=5, p=0.926, t-test) with a similar rate of rise (τ value = 0.8±0.4 or 0.5±0.4 min, respectively; n=5, p=0.166, t-test). These observations suggested that these two parameters were likely interconnected, a notion compatible with the view that protons perhaps released by spinal cells might act as neuromodulators (Ruffin et al., 2014).

To further evaluate how pH changes might impact on the extent and topography of early cell damage induced by KA, we studied the topographical distribution of the fluorescent probe PI that, being a large molecule, readily accumulates inside cells with damaged plasma membrane (Brana et al., 2002). Fig. 3A shows examples of PI fluorescence signals in spinal cord slices treated with KA (10 μM; 1 h) (Fig. 3B) that, at this concentration, yielded minimal cell death (Fig. 3B) in accordance with the modest release of glutamate (Fig. 2A). Further observations suggested that this phenomenon was related to extracellular pH and the ability to activate ASIC channels. In fact, Fig. 3C, E, F compares the PI signals from WT and ASIC1a$^{-/-}$ spinal slices, indicating that, in this genotype, the toxic effect of KA was intensified (n=9-15 slices, * p≤ 0.05, t-test, vs. control). These data unexpectedly suggested that extracellular protons could exert some damage-limiting action against excitotoxicity.

ASIC channel mRNA modulation induced by KA

We next investigated whether KA mediated excitotoxicity might impact on the expression of ASIC1a, 1b, 2 and 3 by spinal cord organotypic cultures evaluated by RT-PCR. ASIC1a and ASIC2 are specific for spinal cord tissue, while ASIC1b and 3 are expressed by other structures like DRGs and non-neuronal cells (Deval and Lingueglia, 2015). In preliminary experiments we validated (by quantitative RT-PCR) the ASIC gene expression in spinal cord organotypic cultures. As described in previous studies (Baron et al., 2008), ASIC1a and ASIC2 were the predominant mRNAs in these preparations (1.26±0.01 and 1.10±0.06 change over the house keeping gene products β-actin and 18S, n=3 experiments), whereas ASIC3 and ASIC1b expression was relatively low (0.89±0.15 and 0.63±0.08, n=3) consistent with their specific location to sensory neurons (Deval and Lingueglia, 2015) and ciliated neurons of the spinal cord central canal (Jalalvand et al., 2016b).

In the current experiments on organotypic slices treated with KA (1 h) and analyzed 24 h later, the mRNA level of all four ASIC genes was significantly upregulated by 10 µM KA, while, on the contrary, only ASIC1a and ASIC2 were significantly reduced by KA (100 µM; Fig. 4, n=15 slices for each experimental condition from 3 independent experiments; **p≤0.01,*p≤0.05 vs control; Kruskal-Wallis, Dunn's Method) in line with the large toxicity evoked by such a high KA concentration (Mazzone et al., 2010). Unexpectedly, when slices were incubated with DAPI, a previously reported ASIC inhibitor (Chen et al., 2010; Alexander et al., 2011), for 24 h during KA washout, the upregulation of ASIC1a, ASIC1b and ASIC 3 was prevented (Fig. 4, n=15 slices for each experimental condition from 3 independent experiments; #p≤0.05, ##p≤0.01 vs KA 10 µM, Kruskal-Wallis, Dunn's Method). DAPI alone had no effect on any ASIC gene expression.

Cell loss mediated by ASIC inhibitors

Representative images of NeuN (neuron) and S100β (glial) positive cells in spinal cord organotypic culture treated with KA (10 µM) followed by 24 h DAPI application are shown in Fig. 5A. This data indicates that no toxic effect was apparent 24 h after washout of KA (Fig. 5A, B, C). Conversely, histograms show that the number of neurons and glial cells fell 24 h after KA application plus DAPI (Fig. 4A, B and C, respectively). While neuron numbers were unaffected by DAPI alone for 24 h, the average fluorescence signal from glia was significantly lowered after

DAPI (n=11-22 slices, *p≤0.05 vs control, #p≤0.05 vs KA, Kruskal-Wallis, Dunn's Method) in accordance with the report of its potentially toxic effect on proliferative cells via inhibition of RNA and DNA polymerase (Baraldi et al., 2004).

We next compared two ASIC inhibitors, namely DAPI and amiloride (AM, 100 µM), for their effect on KA neurotoxicity in the isolated mouse spinal cord 24 h later (Fig. 6). Fig. 6A shows representative images of regions of interest (ROIs) in spinal cord sections under control condition, after KA treatment only, or after KA application followed by DAPI or amiloride. Fig. 6A shows that, in the three ROIs examined 24 h after KA (10 µM; 1 h application), neuronal survival was excellent to confirm the lack of neurotoxicity of this low concentration of the glutamate agonist (Table 2). When DAPI or amiloride were administered for 24 h after KA washout, neuronal numbers were significantly decreased (Fig. 6 C, D and Fig. 6 B, respectively) as exemplified by quantification for the central ROI (Fig. 6E, n=9-29, *p≤0.05 vs control, # p≤ 0.05 vs AM alone, Kruskal-Wallis, Dunn's Method, $ p≤ 0.005 vs DAPI alone, Mann-Whitney Rank Sum Test) which is the one believed to contain the central pattern generator for locomotion (Kiehn, 2006, 2016). The insets to Fig. 6D indicate that DAPI administration after KA was associated with pyknosis shown as condensed chromatin appearance (Taccola et al., 2008; Mazzone et al., 2010). No change in S100β positive astrocytes was observed (Fig. 6 A-D). It is noteworthy that neither amiloride nor DAPI applied alone induced significant neuronal loss (Fig. 6 C-E).

It should be noted that there was an apparent discrepancy in the strength of the effects of 10 µM KA followed by DAPI on cell survival (Fig. 5B for organotypic slices and Fig. 6E for isolated spinal cords): we propose that the simpler bilayered structure of the organotypic slice (Cifra et. al, 2012) with comparatively fewer glial cells might have been less efficiently equipped to handle glutamate release and pH changes. Our data, thus, suggested that, regardless the type of ASIC inhibitor used, their application converted a near-threshold excitotoxic stimulus to a neurotoxic one.

ASIC inhibitors enhanced functional disruption by excitotoxicity

We next evaluated the effect of 10 µM KA, DAPI or amiloride (either applied alone or in combination) on synaptic transmission in the isolated spinal cord. Amiloride alone depressed the

polysynaptic peak amplitude and made significant decrease on mono and polysynaptic transmission following KA application (Table 3). In addition to testing synaptic activity evoked by single pulses applied to one DR, we also investigated the coherent expression of rhythmic network discharges expressed as fictive locomotion evoked by DR stimulus trains (Marchetti et al., 2001) and emerging as regular bouts of oscillations alternating amongst ventral roots (VRs; Fig. 7). This phenomenon comprises a slowly developing cumulative depolarization caused by non-linear summation of synaptic inputs (Sivilotti et al., 1993; Baranauskas and Nistri, 1996; Barbieri and Nistri, 2001) with superimposed spiking and oscillatory cycles, none of which was affected by subthreshold concentration of KA (10 μM; Fig. 7 A-D). Nevertheless, KA treatment followed by DAPI or amiloride for 24 h elicited significant decrease in the number of oscillations (Fig. 7B). Significant depression of cumulative depolarization by amiloride applied after KA was also observed (Fig. 7 C, D).

Alternating rhythmic oscillations recorded from lumbar VRs are thought to indicate the functional output of the locomotor central pattern generator (Kiehn, 2006, 2016). In the present experiments, NMDA (1.5-6 μM) plus 5HT (10 μM; see Methods) were used to generate such oscillations (Beato et al., 1997). Despite using a range of NMDA doses, oscillations expressed by mouse preparations are less stable and persistent than in other mammalian species (Zhong et al., 2012) as confirmed in our tests: this characteristic was present even after 24 h in vitro in the present experiments. In fact, only 3/11 spinal cords generated 20 consecutive cycles rhythmically alternating amongst flexor and extensor motor pools used a standard index of fictive locomotion (Kjaerulff and Kiehn, 1996) and exemplified in Fig. 8. These cycles were all expressed at low periodicity (10.3±3.6 s; n=6). The remaining preparations generated variable bouts of alternating rhythmic discharges (4 to 12). After exposure to KA (10 μM), the probability of observing 20 cycles remained similarly low (2/12 preparations), and the number of cycles in a bout varied from 4 to 12 (in 4/12 preparations) with average period of 14.6±2.3 s (n=6) and lacking alternation. DAPI or amiloride did not significantly change these patterns when they were applied after KA (2/5 or 8/12 spinal cords for DAPI or amiloride, respectively) while cycle periodicity remained slow (14.0±1.6 or 17.8±2.1 s, respectively). Cycle alternation was preserved with DAPI or amiloride per se, yet absent when these drugs followed KA.

These results could not be simply attributed to inefficient network depolarization by NMDA and 5HT because the average VR depolarization evoked by 3 μM NMDA and 10 μM 5-HT was similar in sham (0.72±0.08 mV, n=11), after KA (0.64±0.07 mV, n=12), DAPI (0.95±0.19 mV, n=7) or amiloride (0.55±0.14 mV, n=5) protocols, even when applied after KA (0.47±0.06 or 0.49±0.08 mV, n=5 or n=12, respectively).

Discussion

The present report shows that, in mouse spinal cord networks, the histological and functional outcome of excitotoxicity could depend on extracellular pH changes that likely involved activation of ASIC channels, and were important for the operation of locomotor circuitry. It was unexpected that inhibitors of ASIC channels or ASIC1a genetic ablation actually intensified the damage evoked by a borderline excitotoxic stimulus.

Protons modulate neurotoxicity

Notwithstanding the multifarious effects of acidification on neuronal receptors and channels (Holzer, 2009), the role of ASICs in the central nervous system is complex and subjected to intense regulation to modulate and transmit network signals (Krishtal, 2003) related to pathological and physiological conditions (Duan et al., 2007; Baron et al., 2008). For instance, during brain ischemia affecting mouse cortical neurons, the activation of homomeric ASIC1a channels (highly permeant to Ca^{2+}) may play a key role in acidosis-induced neuronal death (Xiong et al., 2004). Decreases in extracellular pH to 6.5 (or below) occur in several pathologies such as cerebral ischaemia, hypoxia or epilepsy due to increased lactate production by glycolysis, and, moreover, even sustained neuronal activity can lower pH (Chu and Xiong, 2012). In physiological conditions transient acidosis is normally compensated by proton uptake, for instance during glutamate release (Hnasko and Edwards, 2012). Buffering H^+ to keep the intracellular neuronal pH around 7.1 is effected by membrane located acid-base transporters (Chesler, 2003).

While the extracellular pH value is normally 7.4, intense, sustained transmitter release (when synaptic vesicles with internal pH of ~5.5 discharge their content) can significantly acidify the synaptic cleft (Hnasko and Edwards, 2012). It follows that using KA to persistently stimulate

release of glutamate stored in synaptic vesicles is likely to provide, within spinal networks, a microenvironment with acid pH to which glia might have also contributed. In fact, stimulation of astrocytes from neonate mouse brain cortex acidifies the extracellular space leading to activation of neuronal ASIC1a channels and firing of action potentials (Li et al., 2014). This finding implies a functional interaction between neurons and astrocytes for proton regulation, a process that perhaps was operative in spinal networks as well.

In the present study extracellular acidification by KA was evaluated with simultaneous recording via the glutamate biosensor and the null electrode as recently demonstrated (Llaudet et al., 2005). Although the background current attributed to pH changes faded away during KA application, we first suspected that the early fall in extracellular pH might have triggered the activation of deleterious processes that were then translated into neuronal cell death (Shabbir et al., 2015a). To our surprise, we observed that, while no significant increase in cell death was evoked by 10 µM KA as previously reported (Mazzone et al., 2010), the borderline between survival and death was readily surpassed in the presence of two different ASIC inhibitors, namely DAPI or amiloride, or with genetic ablation of the ASIC1a gene. Finally, upregulation of ASIC mRNA was observed after KA application. Altogether, these data unexpectedly indicated that a fall in extracellular pH induced by KA was somehow protecting from cell death.

ASIC expression in spinal networks

Functional ASICs are homo or heterotrimers of different subunit assembly. To date, six subunits of ASICs have been identified, namely 1a, 1b, 2a, 2b, 3, and 4 (Krishtal, 2003). Our data suggest a predominant role for the ASIC1a and ASIC2a subunits in response to KA in mouse spinal networks in analogy with previous studies (Deval and Lingueglia, 2015). A former investigation has shown that ASIC1 and 2 subtypes are expressed by the majority of small as well as large neurons in all laminae of the spinal cord (Baron et al., 2008).When 100 µM KA induced extensive neuronal cell death without large glial loss (Mazzone et al., 2010), significant reduction in ASIC1a and ASIC2 occurred attributable to cell loss. On the contrary, when 10 µM KA was applied, significant mRNA increase was observed for all subunits. To further investigate the contribution by ASICs to excitotoxicity following KA administration, DAPI, a potent ASIC

inhibitor (Chen et al., 2010), was applied during the KA washout and observed to decrease mRNA expression of all subunits.

Involvement of ASIC channels in the effect evoked by KA

In standard recording conditions DAPI had no effect on synaptic transmission or locomotor network rhythms while amiloride depressed polysynaptic activity. The latter phenomenon might be attributed to other actions by amiloride like inhibition of Cl^- transport, and of certain subtypes of Na^+ and K^+ channels (Leng et al., 2016). Although amiloride and its chemical derivatives are currently proposed as tools to develop ASIC channel blockers (Leng et al., 2016), it seems probable that the effects of amiloride on excitotoxicity could not be simply due to ASIC block. Nevertheless, in analogy to the effect of DAPI, one may posit that ASIC inhibition by DAPI or amiloride favoured excitotoxicity. Because the relatively modest acidification evoked by 10 µM KA had rather small effects on network function, it seems likely that the fall in extracellular pH evoked by KA was probably followed by reactive changes in intracellular pH as has been demonstrated in brain trauma and SCI (Huang et al., 2015b). Furthermore, early (and often overlooked) experiments had shown that moderate acidification can protect cortical or cerebellar neurons from excitotoxicity via block of NMDA receptor-mediated currents (Giffard et al., 1990; Andreeva et al., 1992) because H^+ is a strong modulator of the NMDA channel activity (Tang et al., 1990). In the present investigation ASIC inhibitors applied after KA substantially depressed network activities, especially those that required coherent oscillatory patterns. Indeed, when the ASIC inhibitors were applied after KA, the orderly recruitment of circuit elements was disrupted so that locomotor-network oscillations evoked by DR stimuli were significantly decreased. Conversely, there was much less disruption of locomotor rhythm when NMDA and 5-HT were bath applied to induce fictive locomotion. This discrepancy was due to the fact that, in the mammalian spinal cord, the ventral part of lumbar region itself can generate alternated rhythmic pattern even after ablating the dorsal horns (Kiehn and Kjaerulff , 1998; Ballerini et al., 1999) which are the areas where the strongest lesion by kainate is observed (Mazzone et al., 2010).

Mechanisms of delayed neurotoxicity by ASICs inhibitors

On the assumption that the operation of ASIC channels somehow raised the threshold for KA neurotoxicity, the question then arises how that would have been possible. Recent studies have indicated a physiological role of ASICs expressed by the central region of the lamprey spinal cord for detecting pH changes with a negative feedback to motor circuits resulting in suppression of locomotor activity (Jalalvand et al., 2016a,b). This implies that the spinal cord, at least of lower vertebrates, has a precise control system for sensing pH changes. In the mouse spinal cord an analogous system might rely on pH changes to deploy several effectors to contrast excitotoxicity at different times after the initial insult. While considering the relatively short time frame from the start of KA application, we would like to propose that ASIC activation might depress spike activity and, therefore, restrict the network excitation induced by KA (Vukicevic and Kellenberger, 2004), or that acidification may relieve the inhibitory interaction of ASIC channels with membrane K^+ channels, thereby favouring membrane potential repolarization (Petroff et al., 2008) . At later times, KA-evoked stimulation and proliferation of neuroprecursors in the central region (Mazzone et al., 2013) might be mediated by upregulation of ASIC gene activity as observed in the present study. Furthermore, ASIC1a-mediated Ca^{2+} influx with consequent ERK 1/2 and Akt activity (Su et al., 2014) may upregulate Homer1 proteins that play an important role in synaptic plasticity of the spinal cord (Miletic et al., 2009) and are neuroprotective in traumatic brain injury (Luo et al., 2014). These possibilities should be investigated in future studies.

Conclusions

Our work proposes that moderate extracellular acidification might contribute to ASIC activation in turn likely to trigger cell processes to diminish excitability and neuronal loss with a relatively short delay. One might envisage a scenario where this process perhaps operates in concert with another mechanism, namely pH-mediated opening of leak K^+ channels that can also be activated pharmacologically by a neuroprotective volatile anesthetic (Shabbir et al., 2015b).

Our earlier results indicate that contrasting an intense excitotoxic insult may require intracellular processes like HSP70-dependent inhibition of non-apoptotic cell death (Shabbir et al., 2015a). Combinatorial strategies adopted by endogenous neuroprotective processes might be the reason why excitotoxic neuronal death in the rodent and human spinal cord is often limited to the

minority of neurons (Mazzone et al., 2010) although the functional impact is suppression of locomotor-like output (Taccola et al., 2008).

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Conflict of interests

The authors declare no conflict of interest.

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Figure legends

Fig. 1 pH calibration of electrochemical experiments, pH action on cell viability and gene expression by ASIC1a knockout tissue (A) Example of organotypic mouse spinal cord culture after 22 DIV showing the ventral fissure where null and glutamate sensors were placed for detection of glutamate and pH as shown in Fig. 2. (B) Calibration curve done with Krebs solution at different pH (5.8, 6.5 and 7.4) showing the mean current change for null biosensor, expressed in nA, n=6, *p≤ 0.05, t-test. (C, D) Examples of spinal slices stained with propidium iodide in control condition or after 5 min HCl (100 µM) application. Slices show intense propidium iodide uptake after HCl application (pseudocolor scale in D). (E) Representative experiment to demonstrate gene expression profile of wildtype or ASIC1a$^{-/-}$ spinal cord samples to validate gene ablation.

Fig. 2 Analysis of proton and glutamate release by organotypic slices with electrochemical biosensors. Average glutamate current (n=6 slices) observed from slices exposed to KA 10 µM (**A**) or 100 µM (**B**; black traces). (C, D) the average current continuously monitored throughout the experiment with the null biosensor indicates pH changes detected with KA (10 and 100 µM). For sake of clarity, SEM values were omitted; SEM values are given for mean peak currents in the text.

Fig. 3 Analysis of cell death induced by KA using propidium iodide staining of spinal slices. Cross sections of spinal slices (A-D) in control condition or after 1 h KA (10 µM) application for WT animals or ASIC1a$^{-/-}$. Signals are coded by pseudocolors. (E, F) Histograms comparing the effect of KA on WT and ASIC1a$^{-/-}$ slices: data are expressed as fractional change in average propidium iodide fluorescence intensity over control; n=9-15 slices from 8 independent experiments, * p≤ 0.05, t-test, vs. control.

Fig. 4 ASIC mRNA expression in organotypic spinal cord cultures by RT-PCR. Ordinate: fractional change with respect to control condition. Cultures treated with KA (10 µM and 100 µM) demonstrated changes in the levels of mRNA transcripts of ASIC1a (A), ASIC1b (B),

ASIC2a (C), ASIC3 (D). mRNA increase was significant reduced by addition of DAPI (3.5 µM) during KA (10 µM) washout. For each ASIC gene product, amplification values were normalized with β-actin and 18S mRNA levels. Control level was set at 1 for each gene product; medians (black horizontal line) and individual data (filled circles) are shown in each panel, n=15. Different slices for each experimental condition from 3 independent experiments. For each ASIC gene, *p≤0.05, **p≤0.01, vs control; #p≤0.05, ##p≤0.01, vs KA10 µM, Kruskal-Wallis, Dunn's Method).

Fig. 5 Quantification of neuronal and glial staining in organotypic spinal cord cultures. (A) Example of neuronal and glial staining in control condition (NeuN red, S100β green, DAPI blue) in spinal cord organotypic cultures. (B) Plots show the median of NeuN positive cells (black horizontal line) calculated 24 h after the application of KA (10 µM; 1 h) in control or DAPI (3.5 µM) solution, and the distribution of individual data (filled circles), n=4-7 slices, *p≤0.05 vs control, #p≤0.05 vs KA, Kruskal-Wallis, Dunn's Method. (C) Plots show the median of fluorescent intensity data (arbitrary units, black horizontal line) together with the distribution of single measurements for S100β antibody signal detected at 24 h, n=11-22 slices, *p≤0.05 vs control, #p≤0.05 vs KA, Kruskal-Wallis, Dunn's Method.

Fig. 6 Neuronal and glial staining of mouse isolated spinal cord. (A) The upper left panel shows an example of neuronal and glial staining (NeuN green, S100β red) in mouse isolated spinal cord in control condition (left) or 24 h after the application of KA (10 µM) for 1 h (right). Three different gray matter ROIs, namely dorsal, central and ventral, are delimited by yellow boxes. (B) Example of neuronal and glial staining in control condition or 24 h after the application of KA (10 µM) followed by amiloride (AM, 100 µM) or amiloride only. (C-D) Examples of NeuN and S100β staining (low or high magnification, respectively) after the application of KA (10 µM) followed by DAPI (3.5 µM) or DAPI per se. Insets in panels D shows the condensed chromatin nucleus as marker for pyknosis. (E) Plots show the median of NeuN positive cells (black horizontal line) in the central area for control condition and 24 h after the application of KA (10 µM, 1 h) alone or followed by amiloride (AM, 100 µM) or DAPI (3.5 µM), and the distribution of individual data points (filled circles); n= 9-29 slices, 6 independent experiments were done to

include in each case 2-8 spinal cords, *p≤0.05 vs control, # p≤ 0.05 vs AM alone, Kruskal-Wallis, Dunn's Method; $ p≤ 0.005 vs DAPI alone, Mann-Whitney Rank Sum Test.

Fig. 7 Functional effect of ASIC inhibitors on mouse isolated spinal cord. (**A**) Examples of electrically evoked oscillatory patterns recorded from lumbar VRs 24 h after application of KA alone or followed by DAPI or amiloride. (**B**) Bar chart indicating the number of oscillations detected with sham, KA, KA/DAPI., DAPI, KA/amiloride or amiloride protocols. Note significant reduction in KA/DAPI and KA/amiloride responses (n=11-16 spinal cords preparations; *p≤0.05; ***p≤0.001, ANOVA, Tukey-Kramer). (**C**) Histograms showing cumulative depolarization amplitude for the above protocols; *p≤0.05; **p≤0.005, ANOVA, Fisher LSD. (**D**) Bar graph summarizing the area of DR evoked cumulative depolarization; *p≤0.05, ANOVA, Tukey-Kramer.

Fig. 8 NMDA and 5-HT evoked VR oscillations of mouse spinal cord. (A) Representative traces of oscillatory patterns evoked by excitatory chemicals applied with the protocols mentioned in Fig. 6. Traces are shown on a slow time scale on the left and records within boxed sections are also depicted on a faster time base on the right (B). (C) Histograms showing peak amplitude of the observed rhythms. Pooled data shown here are from spinal cords which produced at least 4 oscillations. Note there is no significant difference in the amplitude (p=0.14, ANOVA) (D) Bar graphs indicating the rhythm periodicity. There is no significant change in between groups (p=0.49, ANOVA, n=5-12 spinal cords preparations).

Table 1: List of primer sequences used for the quantification of specific mRNAs by real time RT-PCR in organotypic spinal cord cultures. The primer sequence was based on reliable amplification as previously reported (Baron et al., 2008; Mazzone et al., 2009; Swain et al., 2012).

Gene	Primer Forward	Primer Reverse
β-Actin[1]	CCTTCTTGGGTATGGAATCCTGTG	CAGCACTGTGTTGGCATAGAGG
18S [2]	GCCGCTAGAGGTGAAATTCT	CATTCTTGGCAAATGCTTTCG
ASIC1a[2]	GGCCAACTTCCGTAGCTTCA	ATGCCCTGCTCTGTCGTAGAA
ASIC1b[3]	AGAATCGGAAGAAGAAGAAGAGAAG	GTAGAGCAAGTCAGGGTAGCTGAG
ASIC2a	GAGGCGCTCAATTACGAGAC	ATCATGGCTCCCTTCCTCTT
ASIC3	TGAGAGCCACCAGCTTACCT	ATGTCAAAAGTCGGACTGGG

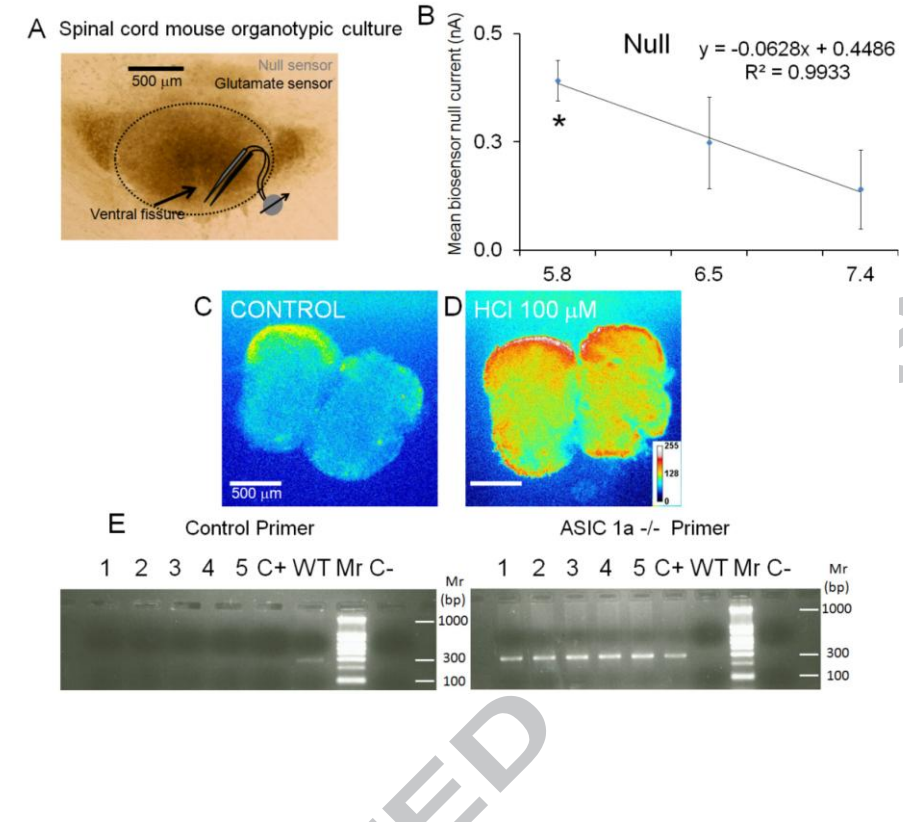
Table 2. Quantification of NeuN-positive neurons in three ROIs

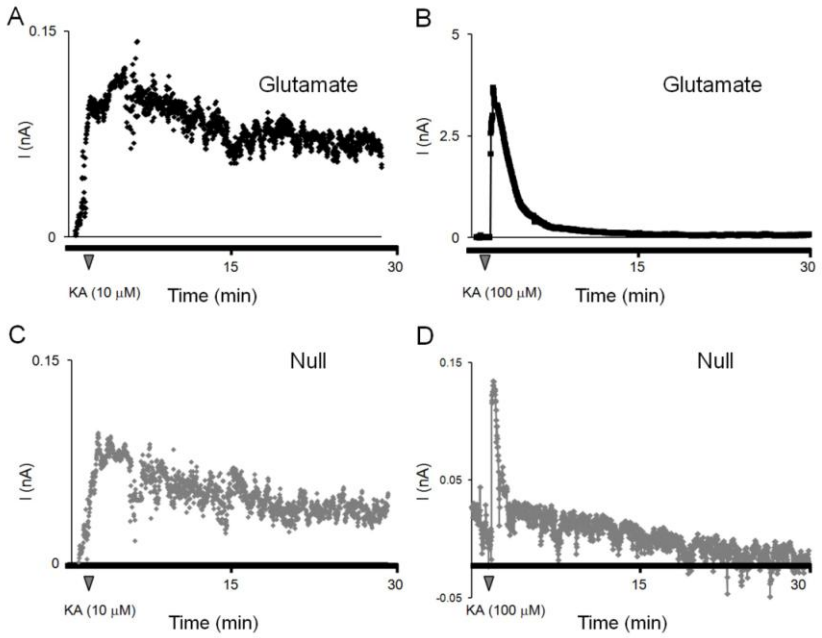
ROIs	**Sham**	**24 h after KA**
Dorsal	312.5±26.1	373.9±46.8
Central	324.4±18.5	222.2±47.9
Ventral	280.2±32.5	245.3±32.7

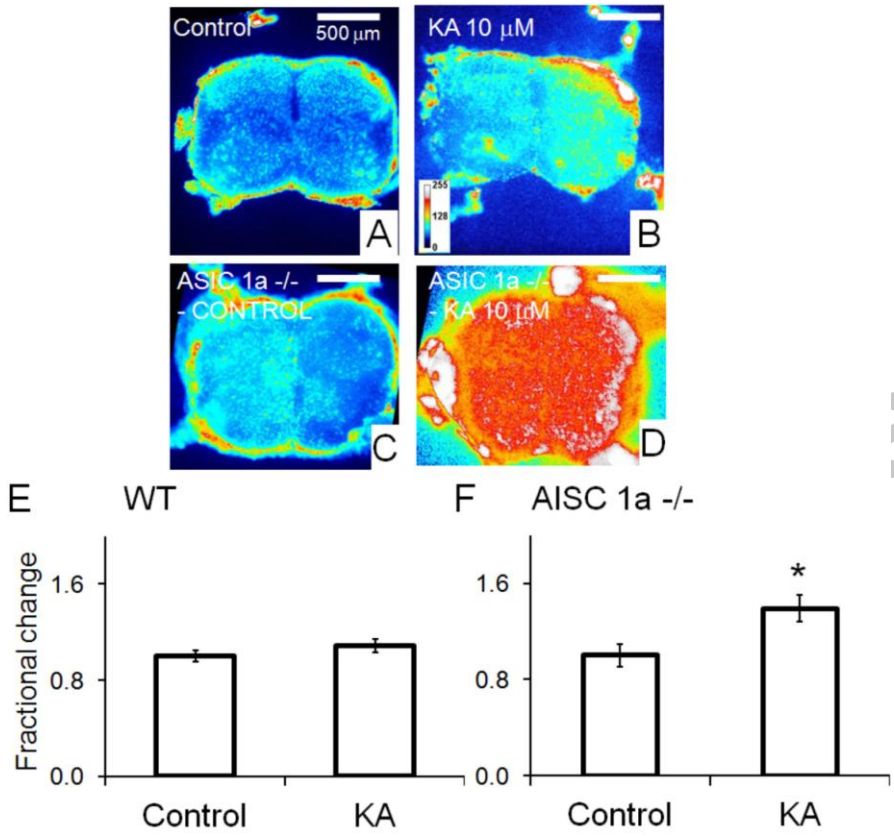
Table 3. Effects of KA, amiloride or DAPI on synaptic transmission

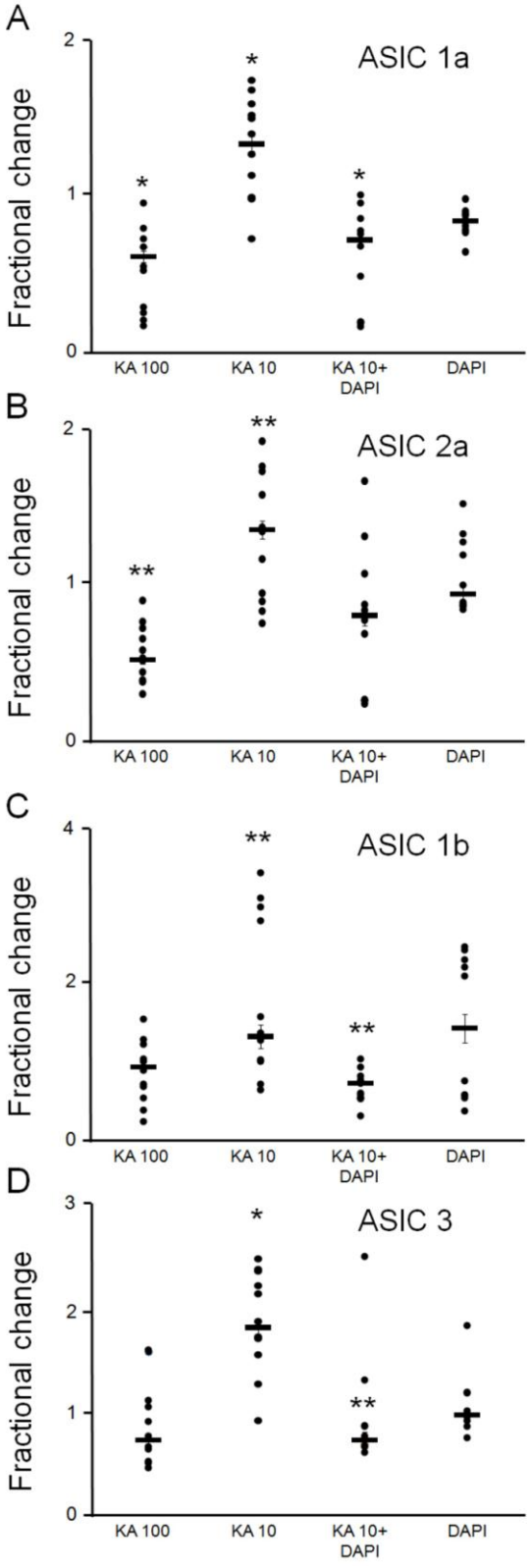
Reflex Parameters	**Sham** (n=11)	**KA**(10 µM; 1h; n=14)	**KA**(1h)/ **DAPI**(3.5 µM; 24h; n=11)	**DAPI** (3.5 µM; 24h; n=7)	**KA**(1h)/**AMILORIDE** (100 µM; 24h; n=16)	**AMILORIDE** (100 µM; 24h; n=7)
Monosynaptic amplitude (mV)	0.04±0.01	0.04±0.02	0.04±0.01	0.05±0.01	0.04±0.01	0.03±0.01
Monosynaptic Area (mV.ms)	7.47±1.98	6.39±1.46	6.19±2.01	8.48±1.11	**3.23±0.33***	5.90±1.51
Polysynaptic amplitude (mV)	0.41±0.09	0.40±0.06	0.32±0.08	0.32±0.10	**0.20±0.03***	**0.18±0.02***
Polysynaptic area (mV.ms)	1892.90±803.39	1888.57±507.25	1109.58±200.91	1357.94±398.65	1323.21±204.94	2145.08±350.69

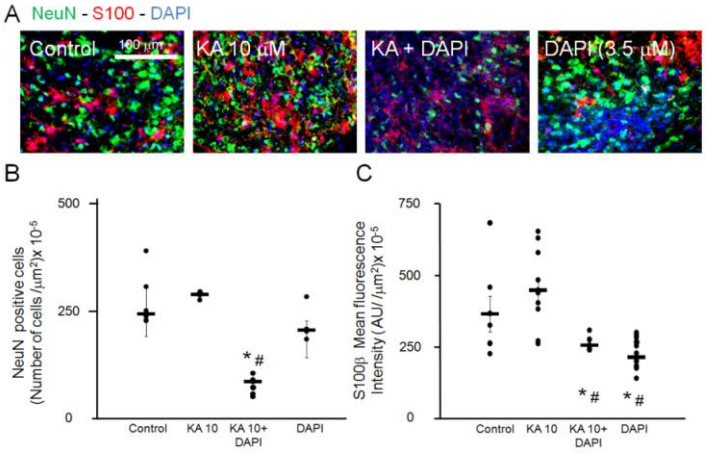
* P<0.05 vs sham data

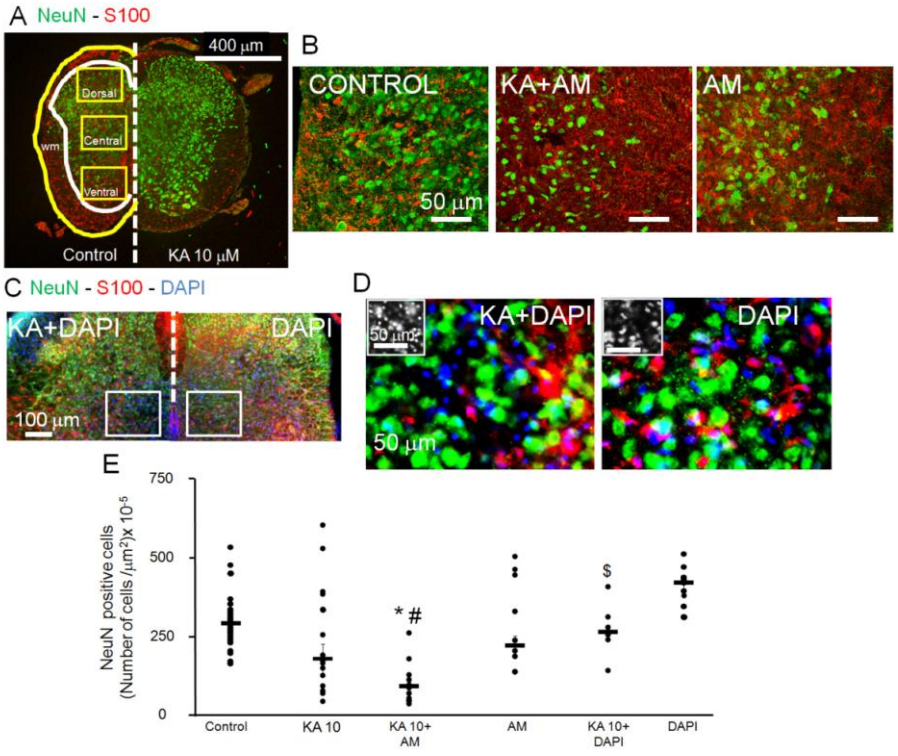


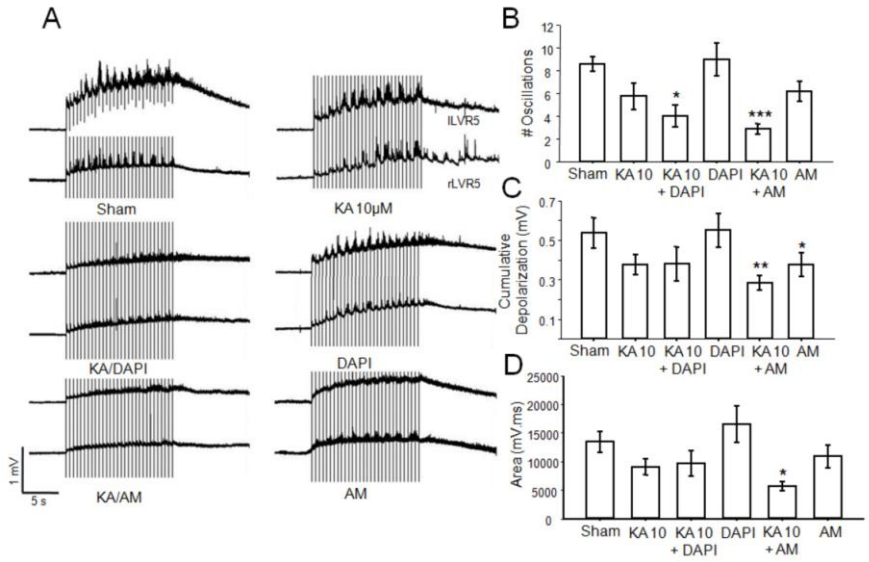


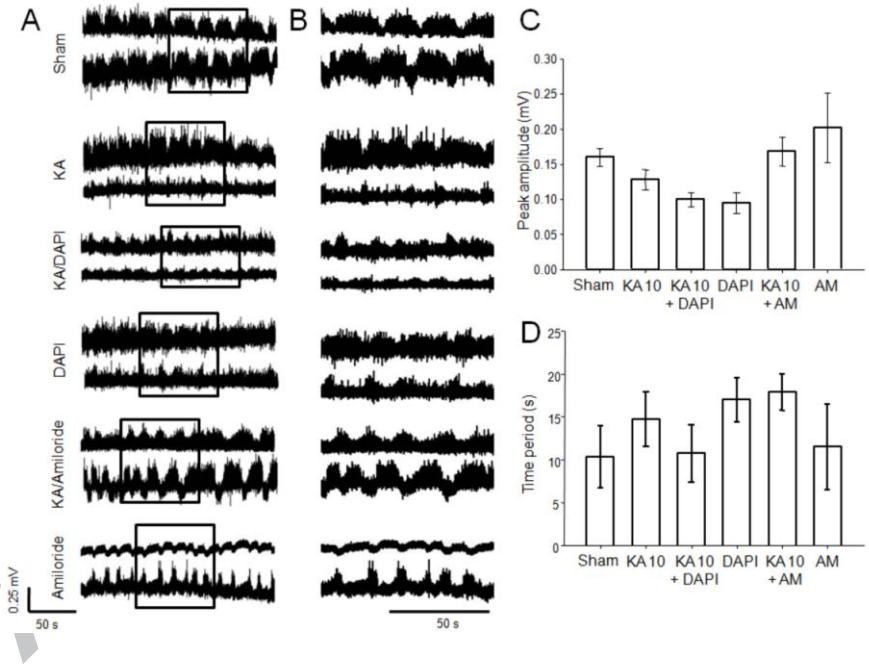












10. DISCUSSION & CONCLUSION

In summary, the following ones are the principal findings with respect to the objectives of the current study:

- CB1Rs of rat spinal cord remained functionally active after 24 h in vitro
- Chronic block of CB1Rs completely inhibited the spinal locomotor activity and changed its cell expression
- CB1Rs remained functional after SCI
- CB1R block further exacerbated SCI
- Only a limited functional and histological recovery of SCI was observed after treatment with endocannabinoids
- A low concentration of kainate (KA) could change pH in the spinal cord
- ASIC expression was modulated by KA evoked excitotoxicity
- Activity of ASICs was functionally important for the outcome after SCI

10.1. Changes in synaptic transmission by CB1Rs

Locomotor networks receive rhythmic inhibitory and excitatory inputs during the appropriate phases of each locomotor cycle. As mentioned before, various studies on hippocampus and cerebellum slices have shown that endocannabinoids are synthesized and released by membrane depolarization (caused by any ongoing network activity) and they act as retrograde messengers to depress synaptic transmission by inhibiting voltage-activated Ca^{2+} channels in presynaptic terminals (Brown et al., 2003; Chevaleyre and Castillo, 2003; Freund and Hájos, 2003; Kreitzer and Regehr, 2001 a, b; Wilson and Nicoll, 2001; Wilson et al., 2001). This effect gives the possibility to test if the endocannabinoid system is contributing to generation and regulation of locomotor

network activity when motoneurons and interneurons are cyclically active to maintain the rhythm. To support the locomotor rhythm, group I metabotropic glutamate receptors (mGluR1) are one of the factors that enhances the excitability of the neurons (El Manira et al., 2002; Nistri et al., 2006).

In the isolated lamprey spinal preparation, Kettunen et al. have shown that mGluR1 activation during ongoing locomotion induces the release of endocannabinoids from neurons which further increase the frequency of rhythmicity. This study has also shown that the increase in locomotor activity is due to the depressant effect of endocannabinoids on inhibitory synaptic transmission onto motoneurons and crossing inhibitory interneurons (leading to a decreased left-right alternation; Kettunen et al., 2005). However, studies on in vivo animal models have shown a reduction in locomotor activity after CB1R activation, although the precise mechanism and the site of action remain unclear (Bosier et al., 2010; Lee et al., 2006). Supporting this evidence, Di Marzo et al. have demonstrated that even a single dose of a CB1R agonist can reduce locomotion (Di Marzo et al., 2000).

Since many years cannabinoid ligands have been used to treat chronic pain, spasticity and obesity for an extended treatment (Clifford, 1983; Greenberg et al., 1994; Malfitano et al., 2008; Pagotto et al., 2005; Russo, 2008). Nevertheless, the report issued by National Institute of Drug Abuse (NIDA) has mentioned many short and long-term drug effects by cannabinoids starting from psychological disturbance to impaired body movement (NIDA website :https://www.drugabuse.gov/publications/drugfacts/marijuana#references). Hence, it is important to study the effect of cannabinoid ligands on mammalian locomotor network at various time points.

It is evident from earlier studies that CB1Rs are widely expressed in the rat spinal cord (Farquhar-Smith et al., 2000; Herkenham et al., 1991). In our study, it is shown that the CB1Rs are distributed throughout the lumbar dorsal, central and ventral region of neonatal (P0-P2) rat spinal cord. Their activity after 24 h in vitro is well preserved. This was confirmed by checking the levels of cAMP (which are lowered when CB1Rs are

active by inhibiting adenylyl cyclase; Howlett et al., 2004) in both basal and induced (by forskolin) conditions. Our novel result was the significant depression of the CB1R expression at the central region of spinal cord after prolonged treatment with the antagonist AM251. However, it was important to examine the functional effect of cannabinoid ligands on synaptic transmission to understand any change in complex network activity. Hence, the present study aimed to detail changes in synaptic transmission after cannabinoid ligand treatment.

Results from previous studies have shown a decline in $GABA_A$ mediated synaptic transmission (Azad et al., 2003; Jennings et al., 2001; Pernía-Andrade et al., 2009). In accordance with these results, the current study confirmed GABA depression (30%) after 30 mins of AEA application. This effect was not seen after 24 h AEA treatment even if the CB1Rs were found active. In addition, our study has also shown that there was no change in glycinergic or glutamergic synaptic transmission after short and long term treatment with the agonist AEA. Either AEA or AM251 applied up to 6 h could not produce any marked effect on monosynaptic and polysynaptic reflexes. Conversely, there was a significant decline in the polysynaptic reflex area 24 h after treatment with AM251 or rimonabant, effect not reversed even after washout. This could be due to primary afferent depression since the CB1R staining is found in all cells of the dorsal region (Herkenham et al., 1991). However, dorsal root-dorsal root potentials (DR-DRPs) after AM251 treatment were unchanged showing no alteration in primary afferent depolarization.

10.2. Modulation in network properties after CB1R ligands treatment

Short application (<3h) of AEA or AM251 showed much less modulation of network properties unlike in the lamprey spinal cord where 30-60 min application modulated the frequency of ongoing fictive locomotion (El Manira et al., 2008). In addition, prolonged treatment (12-24 h) with the agonists (AEA or 2AG) or with the uptake inhibitors (FAAH or MAGL inhibitors to increase endogenous cannabinoids (Mulvihill and Nomura, 2014; Otrubova et al., 2011) led to mild to severe modulation of spinal motor rhythm without changing reflex activities. This depression was usually observed with DR evoked fictive

locomotion, while NMDA plus 5HT induced rhythmic activity was unimpaired. Interestingly, a strong depression of network activity was observed in AM251 or rimonabant treated preparations starting from 6 h (mild effect) to 24 h (strong effect). There was a complete abolition of electrically and chemically evoked fictive locomotion after prolonged application. This finding is in line with results by Kettunen et al., as they have shown a gradual depression of locomotor activity in the lamprey spinal cord (Kettunen et al., 2005). Nonetheless, the mechanism of this depression is yet to be established. The inability to generate fictive locomotion and the depression in its components could be due to less excitation of neurons in the network.

To check for the excitatory drive (which is the initiator of locomotor rhythm) disinhibited bursts (Bracci et al., 1996a) were recorded after chronic CB1R block. In analogy with the previous data, a strong decline in the burst periodicity was observed. In the present study it is evident that there was no change in cell survival after CB1R blockers treatment confirming that any changes in synaptic transmission was due to some synaptic strength change.

Earlier studies have shown that, in CNS, CB1Rs are important during the developmental stages to ensure the correct axon guidance (Berghuis et al., 2007; Harkany et al., 2007). Thus, to avoid the possibility of development-related effects of CB1R blockers, the outcome of AM251 treatment was also studied on P4 rats that are already in the early stages of maturation (Cifra et al., 2012). The same depressive effect was observed after treating the cords with AM251 for more than 3 h. Hence, the present study clearly provides evidence that there was no maturation related change.

To summarize, the function of CB1R in the rat spinal cord in vitro was explored. The severe depression of locomotor activity after prolonged CB1R block could be due to decreased expression of CB1R in the central region where the CPG generators are present (Grillner, 2006; Kiehn, 2011). Hence, from these results it is hypothesized that CB1Rs stabilized the operational activity of the locomotor central pattern generator in the rat spinal cord in a time-dependant fashion. However, the exact molecular pathway controlling the locomotor CPG is yet to be determined.

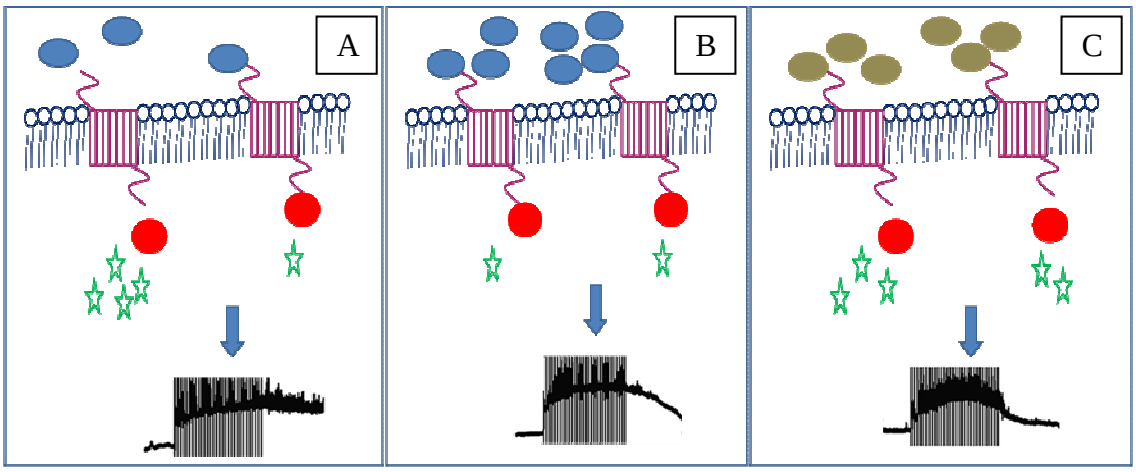


Fig 15: Graphical summary of CB1R activity in the normal locomotor function (A). Alterations in the rhythm after agonists (B) and inverse agonists/antagonists (C). CB1R, Endogenous/Exogenous CB1R agonists, CB1R inverse agonists, Gi subunit, cAMP

10.3. Expression and activity of CB1R after SCI

Endocannabinoids are used to treat inflammation, oxidative stress, spasticity, neuropathic pain, neuronal injury and several neurodegenerative diseases (Koppel et al., 2014; Di Marzo and Petrosino, 2007; Scotter et al., 2010). In a study conducted by Donat et al. it is shown that there is an increase in cannabinoid receptors 6 h after traumatic brain injury (TBI; Donat et al., 2014). In neuropathic pain and multiple sclerosis, increase in cannabinoid receptor expression reduces the disease progression (Pertwee, 2009) whereas, in other diseases, alterations in receptor expression are maladaptive (Teixeira-Clerc et al., 2006; Wang et al., 2008). Hence, the regulation of cannabinoid receptor expression is of interest from a therapeutics perspective. Apart from the cannabinoid receptors, modulation in the endogenous cannabinoids also plays a major role in determining the injury or disease progression (Garcia-Ovejero et al., 2009; Lourenço et al., 2011; Piomelli, 2014). Studies of in vivo locomotion have shown that the endocannabinoid system (increases in 2-AG and CB2, decreases in CB1) in the lesioned spinal cord changes in parallel with any spontaneous recovery (Garcia-Ovejero et al., 2009). However, the exact functional recovery (whether it is from pattern generation or pattern formation region) after the first few hours of injury has to be determined. To pursue this issue, the current study focused on in vitro neonatal rat spinal cord model

(kainate induced injury) of SCI where the secondary injury and its effects can be recorded during the first 24 h (Kuzhandaivel et al., 2011; Taccola et al., 2008).

After KA injury, a strong decline of CB1R expression in the dorsal region was observed. This depression was severely extended to the central region of AM251 treated cords after KA mediated experimental injury. This shows an injury related change of CB1R expression in the current experimental model. However, the activity of CB1R was well preserved even after 24 h of injury which was measured through examining the levels of cAMP. This data provides an anticipation that the conserved CB1R activity could respond to KA induced SCI and protect the neurons.

10.4. Limited recovery by endocannabinoids after SCI

A modest recovery in the spinal synaptic transmission and network activity was only observed after AEA treatment. This mild functional recovery corresponded to a border-line protection of central region neurons, while the FAAH inhibitor depressed the functional activity further. This could be apparently due to the fact that the inhibitor can increase the levels of endogenous glutamate (Gonzalez-Islas et al., 2012) which could expand the injury. The other non specific CB1R agonist, 2AG gave no improvement in reflex and network parameters. These results contradict earlier studies where the cannabinoids lowered the injury progression and improved the motor scores in vivo (Garcia-Ovejero et al., 2009). It is to be noted that, in the current study, the injury model is kept for 24 h (maximum time to preserve functional elements) when only a partial recovery phase can be studied.

10.5. Exaggeration of SCI after CB1R block

CB1R activation mobilizes a large number of signal transduction pathways including neurotoxic and protective ones. Because CB1Rs are largely presynaptic (Maejima et al., 2001; Wilson et al., 2001) and can inhibit Ca^{2+} influx (Mackie and Hille, 1992), their reversal of excitotoxic effects might be partly originate from reducing Ca^{2+}-dependent release of excitatory neurotransmitters, such as glutamate. Therefore, it is important to study the role of innate CB1R activity in the spinal cord after injury for any therapeutic

approach. Our current functional and histological (in the grey matter) data have shown that the SCI expanded after blocking CB1Rs with antagonist-inverse agonists. There was severe loss in dorsal and central region neurons after KA treatment followed by AM251 application. This provides evidence that basal CB1R activity is important after the injury. The improvement in spinal cord injury shown by other studies (Arévalo-Martín et al., 2003; Pacher et al., 2006) might be the result of complex later events like gliosis which occurs after a chronic time period. Nonetheless, the importance of CB1R immediately after SCI could help clinical research to provide a time dependant pharmacological intervention. Data from the current work provide basic information about the potential therapeutic application CB1R on SCI. Yet the accurate cellular mechanism and types of cells that can be manipulated by the endocannabinoid system need to be further investigated in detail.

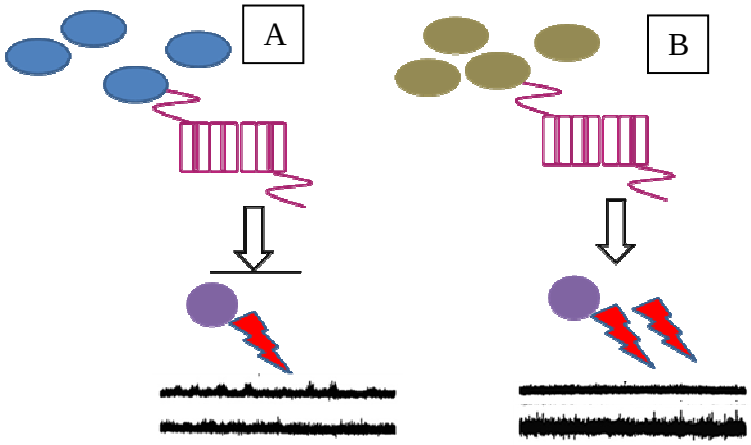


Fig 16: Graphical summary of CB1R activity in the KA induced SCI. Partial recovery of locomotor network after CB1R agonist (A). Complete depression of the activity after CB1R block (B). CB1R, Endogenous/Exogenous CB1R agonists, CB1R inverse agonists, Kainate.

10.6. Modulation of protons and ASICs after KA mediated excitotoxicity

Excitotoxic neuronal injury is known to be associated with increases in cytosolic Ca^{2+} concentrations which trigger a sequence of downstream pathways related to cell death (Arundine and Tymianski, 2003; Zhang and Bhavnani, 2006). However, it is only during the last decades that perturbations by protons associated with glutamate induced neuronal death have been studied (Hartley and Dubinsky, 1993). Studies have shown that in a few

minutes of glutamate exposure there is cumulative increase in intracellular hydrogen ions (Hartley and Dubinsky, 1993). Intracellular protons are constantly buffered by ASIC activity resulting in homeostasis to avoid an environment stressful to neurons. ASICs play an important role in physiological/pathological conditions, from sensory transmission (such as touch, taste, and nociception) to memory, retinal function, seizure, and ischemia (Wu et al., 2004). Growing evidence suggests that modulating ASICs can protect neurons from ischemia related injury (Huang et al., 2015; Xiong and Xu, 2012). Hence, in the present study the importance of ASICs on SCI was studied.

The present study has shown that even a borderline KA concentration could change the extracellular pH (from pH 7.4 to 6.1), trigger glutamate release and increase ASIC mRNA in the spinal cord.

10.7. Impact of ASIC blockade on SCI

Previous studies on cultured neurons have shown neuronal death prevention by a modest intracellular acidification induced by either lowered medium pH or inhibiting Na^+/H^+ exchange through a coupling mechanism between NMDA receptor activation and superoxide production (Lam et al., 2013). In contrast, glial alkalization through optogenetic activation of a proton pump leads to cessation of glutamate release and to relief of ischemic brain damage in vivo (Beppu et al., 2014). These studies suggest that modulation of protons in the cellular environment results in protection or magnification of injury depending upon the type of cells. Thus, the current study focused on the role of ASICs in the neonatal moderate SCI to investigate the proton flux and its impact during spinal locomotor impairment.

In physiological conditions, ASIC blockers (DAPI or amiloride) had no significant effect on the locomotor network except a mild depression of polysynaptic activity by amiloride. This depressive effect by amiloride could be due to the fact that it also inhibits Cl^-, Na^+, and K^+ channels (Leng et al., 2016). Conversely, after a moderate SCI, ASIC blockers expanded the injury by depressing the network rhythms evoked via stimulating dorsal sensory inputs. These results implied that a slight proton increase after injury was

beneficial for the recovery from the secondary phase of neuronal injury. In addition, these results were confirmed by propidium iodide (PI) fluorescence distribution based on uptake of this dye by the disrupted cell membrane. In fact, ASIC1A$^{-/-}$ spinal slices showed increased uptake of this probe after KA treatment when compared to wild type, indicating that the toxicity of KA was aggravated by ASIC deletion. Furthermore, histological results also indicated the expansion of neuronal injury (not astrocytes) after mild KA treatment followed by ASIC block. Altogether the current study shows the protective role of modulated proton levels after borderline excitotoxicity in neurons.

The present study in analogy with earlier ones (Jalalvand et al., 2016) has indicated that the spinal cord could sense even a minor change in extracellular pH. Although the precise mechanism of how ASIC block exacerbates the injury is unknown in the current experimental model, this injury expansion could be due to the fact that mild acidification can reduce NMDA receptor mediated currents (Andreeva et al., 1992; Giffard et al., 1990). Further detailed studies are needed to clarify the downstream changes after proton modulation in the present study model.

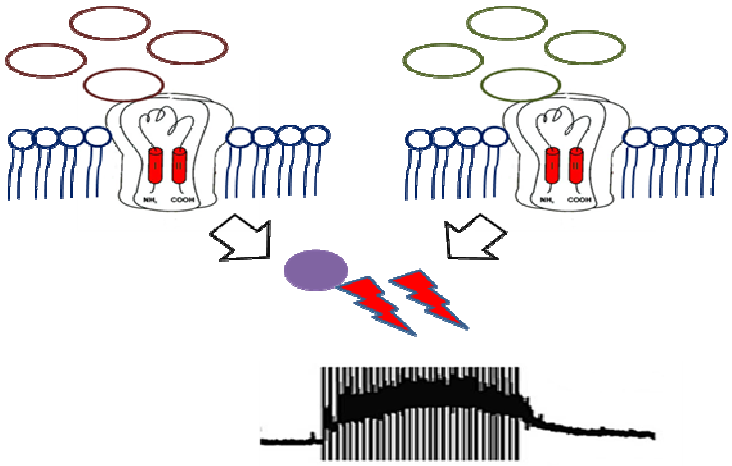


Fig 17: ASIC block on KA mediated SCI. Reduction of locomotor activity after amiloride or DAPI application following KA. ASICs, Amiloride, DAPI, Kainate.

11. FUTURE PERSPECTIVES

Based on the above findings, it is evident that ASIC ion channels and CB1Rs can modulate the spinal locomotor rhythms in both physiological and pathological conditions. On the other hand, the detailed molecular mechanisms of this modulation need to be further studied. The current observations on CB1Rs clearly depict the fact that their long term block greatly reduces in vitro locomotor activity. This depression extensively affected both cellular and functional aspects after injury. A similar depression was seen when ASICs were blocked.

Current legalization of cannabis in countries like Australia and USA has further raised the importance of studying such compounds in network systems. Before the extensive use of these compounds as therapeutics (for pain or neurodegenerative disease), there are many crucial concepts that need to be investigated. For example, Luongo et al. 2010 has reported crosstalk between glia and neurons after modulation of endocannabinoid system. Such interaction might alter the locomotor drive as well. Hence, in the present SCI model it is important to see such interaction of neurons and glia during CB1R modulation.

Recent evidence on cannabinoids has shown that there is a differential effect dependent on gender (Fattore and Fratta, 2010). Thus, the importance of male and female hormones in regulating CBR function should be taken into account when considering prescribing cannabinoid drugs. The current study showed the importance of the ASIC function after SCI, even though the range of extracellular acidification beneficial for recovery of SCI remains to be explored. Overall, the present study might provide us with useful information for improving the treatment of SCI at least at preclinical level.

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13. SUPPLEMENTARY DATA

We studied the changes in motoneuronal properties after CB1R inverse agonist (AM251) treatment to demonstrate its severe depressive effect on fictive locomotion parameters. These experiments followed the same methods from our previous study, Ostroumov et al., 2011.

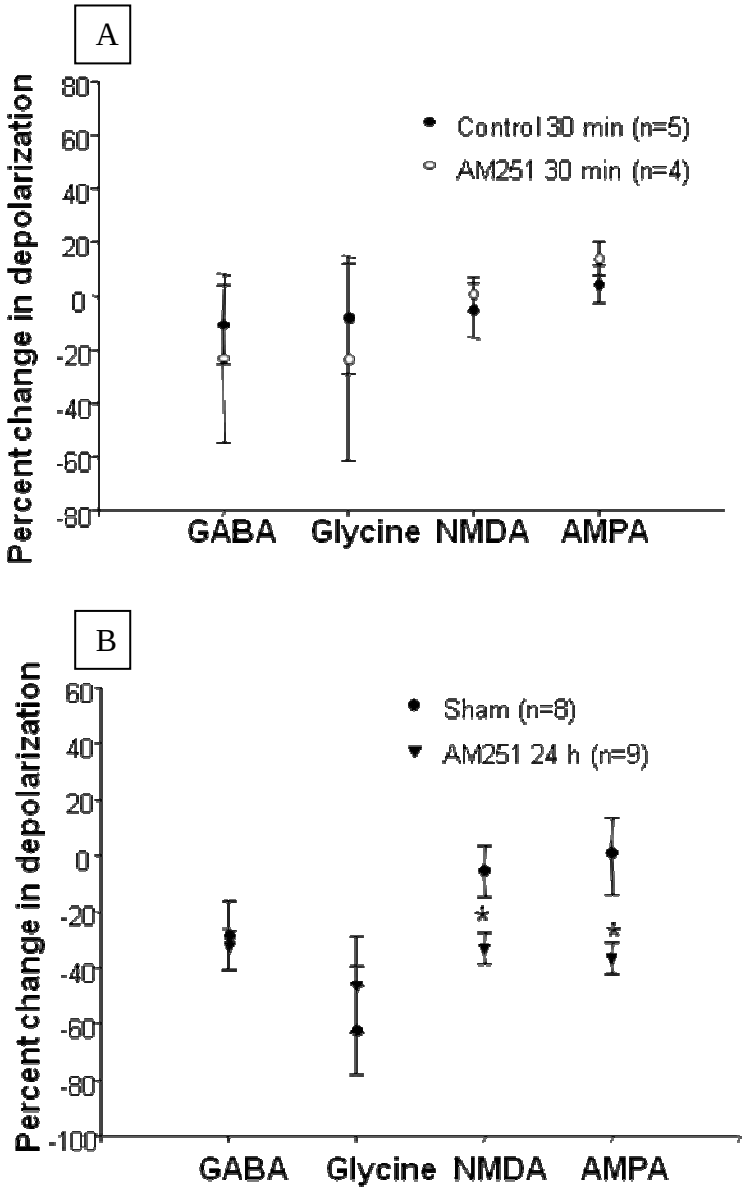

Fig 1: Changes in neurochemicals induced depolarization after AM251 (5 µM) treatment for 30 mins (A) and 24 h (B). Note the significant depression of NMDA and AMPA mediated depolarization after prolonged application of AM251 (*$p<0.05$).

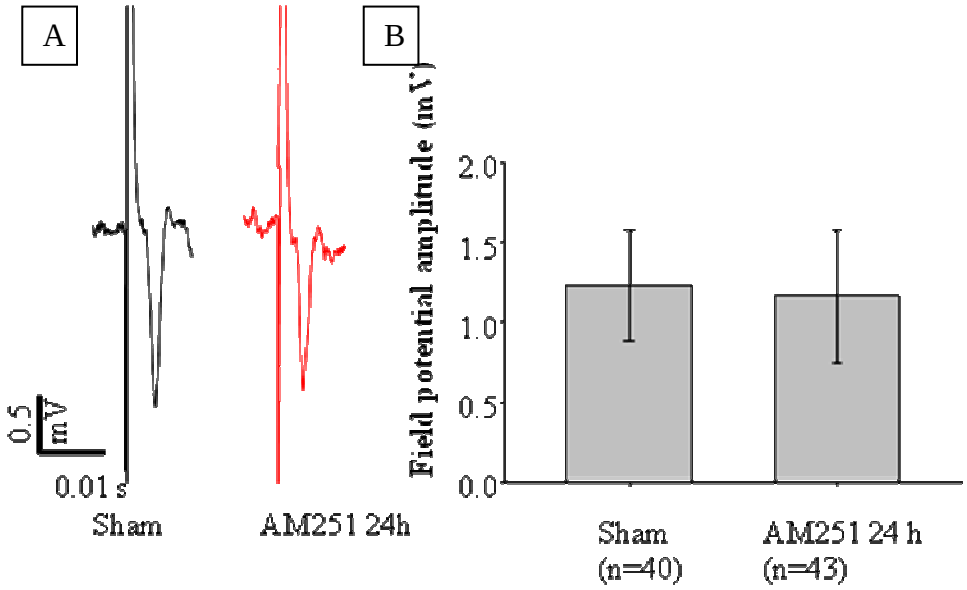

Fig 2: Field potential recorded from motoneuronal pool. (A) Representative recording of motoneuronal field in sham and AM251 (24 h) treated conditions (B) Bar graph showing peak amplitude of recorded potential.

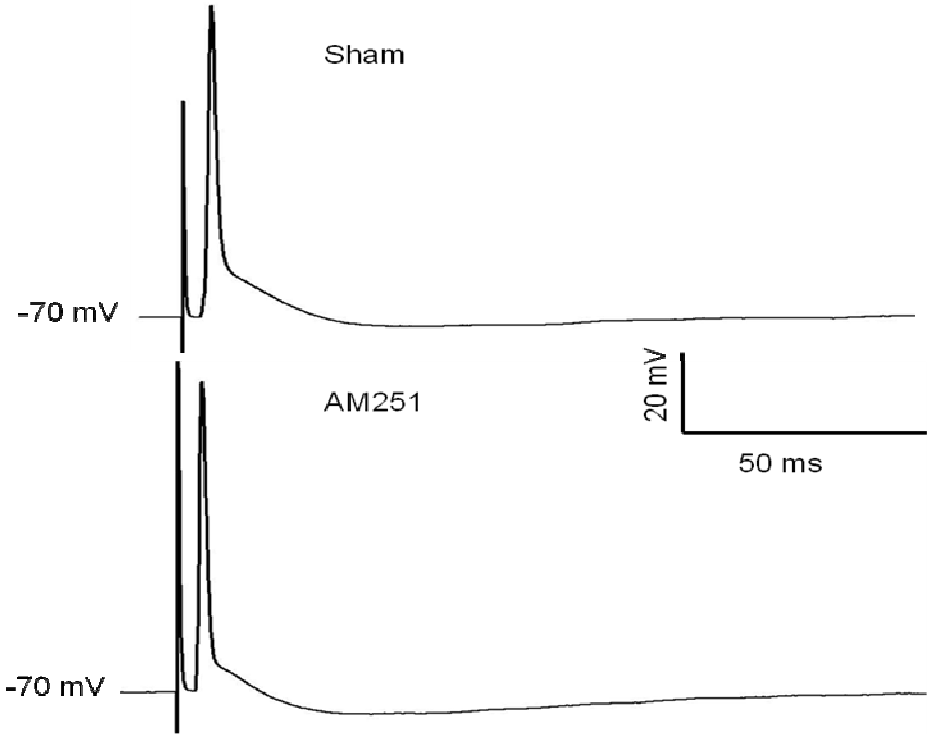

Fig 3: Antidromic action potential recorded from individual motoneurons through ventral root stimulation in sham and AM251 treated cords.

Table 1: Motoneuronal properties after AM251 (24 h) application compared to sham preparations

	Resting membrane potential (mV)	Spike amplitude (mV)
Sham (n=22)	-68.50±3.25	72.10±3.15
AM251 24h (n=19)	-67.35±6.35	70.25±7.45

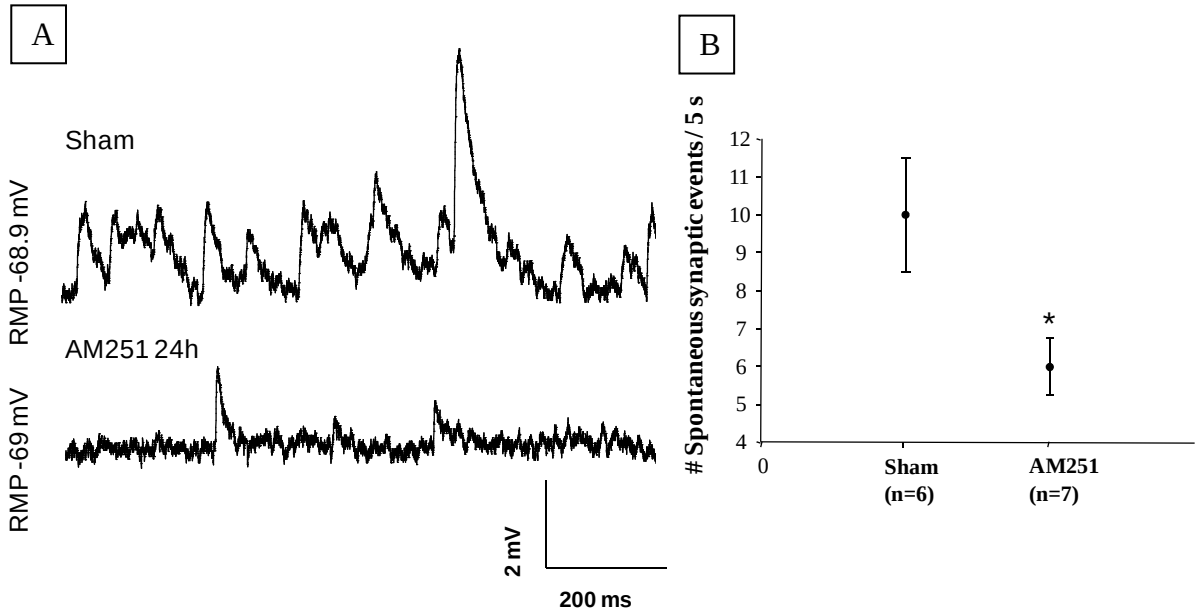

Fig 4: Spontaneous synaptic activity of individual motoneuron recorded by sharp electrodes at resting membrane potential. (A) Sample recordings from sham and AM251 treated motoneurons. (B) Plot showing the significant decline in spontaneous activity after AM251 application for 24 h (*$p<0.05$).

These preliminary results show that the motoneuron survival is not affected by continuous CB1R block. However, their excitation is slightly impaired. Further detailed studies are required to dissect out the mechanism.