Modulation of the activity of the Locomotor Central Pattern Generator in the rat spinal cord in vitro

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NOTE

Part of the data reported in the present thesis has been published in the articles listed below. In all cases the candidate personally performed the experimental work and data analysis, and contributed to paper writing.

Taccola G, Nistri A (2005) Fictive locomotor patterns generated by tetraethylammonium application to the neonatal rat spinal cord. Neuroscience *in press*.

Marchetti C, Taccola G, Nistri A (2005) Activation of group I metabotropic glutamate receptors depresses recurrent inhibition of motoneurons in the neonatal rat spinal cord in vitro. Exp Brain Res 164:406-410.

Taccola G, Nistri A (2005) Electrophysiological effects of 4-aminopyridine on fictive locomotor activity of the rat spinal cord in vitro. Acta Neurochir Suppl 93:151-154.

Taccola G, Nistri A (2005) Characteristics of the electrical oscillations evoked by 4-aminopyridine on dorsal root fibers and their relation to fictive locomotor patterns in the rat
spinal cord in vitro. Neuroscience 132:1187-1197.

Taccola G, Nistri A (2004) Low micromolar concentrations of 4-aminopyridine facilitate fictive locomotion expressed by the rat spinal cord in vitro. Neuroscience 126:511-520.

Taccola G, Marchetti C, Nistri A (2004) Role of group II and III metabotropic glutamate receptors in rhythmic patterns of the neonatal rat spinal cord in vitro. Exp Brain Res 156:495-504.

Taccola G, Marchetti C, Nistri A (2004) Modulation of rhythmic patterns and cumulative depolarization by group I metabotropic glutamate receptors in the neonatal rat spinal cord in vitro. Eur J Neurosci 19:533-541.

Marchetti C, Taccola G, Nistri A (2003) Distinct subtypes of group I metabotropic glutamate receptors on rat spinal neurons mediate complex facilitatory and inhibitory effects. Eur J Neurosci 18:1873-1883.

Taccola G, Marchetti C, Nistri A (2003) Effect of metabotropic glutamate receptor activity on rhythmic discharges of the neonatal rat spinal cord in vitro. Exp Brain Res 153:388-393.

ABSTRACT

The present study has investigated the rhythmic properties of spinal networks in the neonatal rat spinal cord *in vitro*, by means of intracellular recordings from single motoneurons (MNs) and extracellular recordings from ventral and dorsal roots (VRs; DRs).

Distinct subclasses of metabotropic glutamate receptors (mGluRs) on rat spinal neurons mediated complex facilitatory and inhibitory effects. The class I agonist DHPG evoked MN depolarization (via the mGluR1 subtype) mostly at network level and generated sustained, network-dependent oscillations (via the mGluR5 subtype). DHPG also decreased the amplitude of reflex responses induced by DR stimuli, an effect unrelated to depolarization but dependent on glycinergic transmission. Single reflex responses were insensitive to group I mGluRs antagonists, suggesting no phasic activation of group I receptors during this process. Finally, DHPG depressed the glycinergic recurrent IPSP, perhaps by impairing the cholinergic input to Renshaw cells. Thus, the cellular distribution of those mGluRs at strategic circuit connections may determine the functional outcome of the network in terms of excitation or inhibition. Activation of class II or III mGluRs had no direct action on MNs although it strongly blocked evoked synaptic transmission, presumably acting at presynaptic level.

To extend our understanding of the network-based properties, which enable a neuronal circuit to produce sustained electrical oscillations, we explored the potential contribution of mGluRs to generate rhythmic discharges.

During cumulative depolarization or fictive locomotion, spinal mGluRs were minimally activated by endogenous glutamate, although they could potently modulate these responses once activated by exogenously applied mGluR agonists. Disinhibited bursting was associated with the activation of mGluR1 receptors (facilitating network excitability) and of group II mGluRs (depressing it).

We investigated if the K^+ channel blocker 4-aminopyridine (4-AP) could facilitate spinal locomotor networks in addition to its well-known effect on motor nerve conduction. 4-AP produced synchronous VR oscillations, which did not develop into fictive locomotion. These oscillations had network origin, required intact glutamatergic transmission and were probably amplified via electrotonic coupling. 4-AP slightly increased input resistance of lumbar MNs, without affecting their action

or resting potentials. DR evoked synaptic responses were enhanced by 4-AP without changes in axon conduction. 4-AP accelerated chemically or electrically induced fictive locomotion and facilitated the onset of fictive locomotion in the presence of subthreshold stimuli, that were previously insufficient to activate locomotor patterns. Thus, although 4-AP *per se* could not directly activate the locomotor network of the spinal cord, it could strongly facilitate the locomotor program initiated by neurochemicals or electrical stimuli.

On DRs, 4-AP induced sustained synchronous oscillations smaller than electrically evoked synaptic potentials, persistent after sectioning off the ventral region and preserved in an isolated dorsal quadrant, indicating their dorsal horn origin. 4-AP oscillations were network mediated via glutamatergic, glycinergic and GABAergic transmission. Isolated ventral horn areas could not generate 4-AP oscillations, although their intrinsic, disinhibited bursting was accelerated by the substance.

Activation of fictive locomotion by either application of neurochmicals or stimulus trains to a single DR reversibly suppressed DR oscillations induced by 4-AP.

The present electrophysiological investigation also examined whether the broad spectrum potassium channel blocker tetraethylammonium (TEA) could generate locomotor-like patterns. Low concentrations of TEA induced irregular, synchronous discharges incompatible with locomotion. Higher concentrations evoked alternating discharges between flexor and extensor motor pools, plus a large depolarization of MNs with spike broadening. The alternating discharges were superimposed on slow, shallow waves of synchronous depolarization. Rhythmic alternating patterns were suppressed by blockers of glutamate, $GABA_A$ and glycine receptors, disclosing a background of depolarizing bursts inhibited by antagonism of group I mGluRs. Furthermore, TEA also evoked irregular discharges on DRs. The rhythmic alternating patterns elicited by TEA on VRs were relatively stereotypic, had limited synergy with the fictive locomotion induced by DR stimuli, and were not accelerated by 4-AP. Horizontal section of the spinal cord preserved irregular VR discharges and DR discharges, demonstrating that the action of TEA on spinal networks was fundamentally different from that of 4-AP.

INTRODUCTION

1. PHYSIOLOGY OF THE CENTRAL PATTERN GENERATOR (CPG) AND SPINAL LESIONS

For many species, the automated movements needed for walking, respiration, scratching, mastication or other rhythmic activities are generated by the remarkable organization of certain neural networks. For locomotion, one usually refers to the term "central pattern generator" to indicate a set of spinal neurons responsible for creating a motor pattern.

1.1. The CPG for locomotion

Early in past century, Thomas Graham-Brown discovered that rhythmic contractions of hind leg muscles of the spinalized cat could be generated in the absence of any sensory input from peripheral receptors in the legs. This important observation demonstrated that networks of nerve cells located entirely in the spinal cord generate the rhythm for stepping, and established the alternating pattern of activity in flexor and extensor muscles. The expression `central pattern generator` is an operational expression to designate an ensemble of neural elements whose properties and connectivity can give rise to characteristic patterns of rhythmic activity in absence of external feedback. The expression `fictive patterns` refers to these characteristic patterns themselves as expressed in paralyzed preparations or *in vitro* preparations, as opposed the full `active or real pattern` of movements *in vivo*.

General principles of the organization of these circuits and their control exerted by higher brain centers have come from the study of smaller circuits found in invertebrates and from the studies on CPGs associated with swimming in the lamprey, tadpole, leech and the molluscs Tritonia and Clione. From investigations of these networks, we learned that central pattern generation is a complex process that depends on numerous mechanisms. These mechanisms can be related to cellular properties of individual nerve cells, properties of the synaptic connections between the nerve cells and pattern of interconnections between nerve cells (Marder and Bucher, 2001).

As exemplified in Fig. 1, in all classes of vertebrates, the overall locomotor control system is designed in a similar way, which is not surprising when viewed in an evolutionary perspective.

Thus, the same mesopontine and diencephalic centres initiate locomotor activity in lampreys as in primates, via activation of lower brainstem reticulospinal neurons. These, in turn, activate the spinal CPG which generates the motor pattern, be it swimming or walking. Sensory feedback acting on the CPG is an integral part of the control system and helps to adapt the motor pattern to external events.

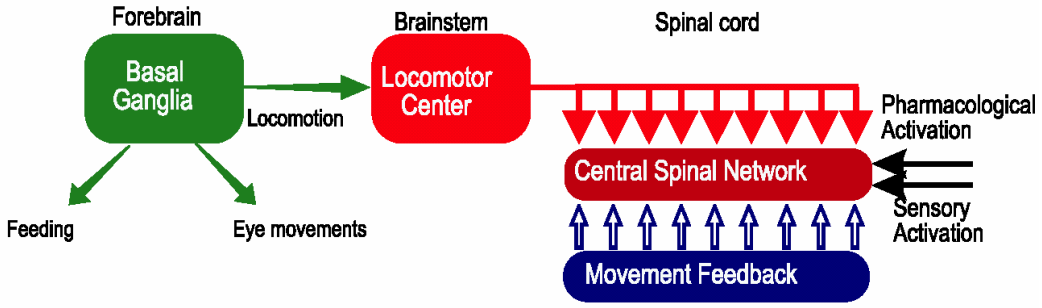


Figure 1. General control scheme for vertebrate locomotion. The basal ganglia exert a tonic inhibitory influence on different motor centres. Once a pattern of motor behavior is selected the inhibition is released, allowing in this case the locomotor centre in the brainstem to be activated. Locomotion is then initiated by an increased activity in reticulospinal neurons which activate the central spinal network, which in turn produces the locomotor pattern in close interaction with sensory feedback (from Grillner et al., *Brain Res Rev* 1998).

A remarkable feature that comes from a variety of invertebrate preparations, including the leech heartbeat system, the crustacean stomatogastric nervous system, Tritonia, and a variety of insect preparations, is that rhythmic motor patterns can be activated by a large number of different neuromodulators (Marder and Calabrese, 1996). Similarly, fictive locomotion patterns in neonatal rat spinal cord *in vitro* can be activated by several substances. Therefore, it has been suggested that these substances are responsible for configuring the spinal cord for different movements (Beato and Nistri, 1999), activating different spinal networks or switching individual neurons among distinct functional circuits.

1.2. Organization of the mammalian CPG

Recently, Lafreniere-Roula and McCrea (2005) have examined the features of spontaneous deletions of bursts of motoneuron (MN) activity that can occur within either rhythmic alternating flexor and extensor activity during fictive locomotion or scratching in adult decerebrated cats. Deletions are reductions or absences of one or more expected rhythmic bursts of activity in multiple agonist MN pools. During

rhythmic fictive locomotion and scratching, the deletions of activity in multiple agonist MN pools are presumably related to a temporary failure in the operation of the CPG.

In experiments to explore the issue, the re-emergence of the activity after spontaneous deletions during fictive locomotion and scratching, occurs as a multiple of the pre-existing cycle period without cycle resetting or frequency change. This maintenance of cycle period during deletions suggests that the failure to recruit MNs is not inextricably linked to the generation of the locomotor or scratching rhythm. This fact suggests that the organization of locomotor and scratch CPGs presents a separation between the cells generating rhythm (a sort of internal clock) and the cells distributing excitation to MNs.

A schematic representation of the proposed architecture for the CPG operating for one limb pattern is provided by Lafreniere-Roula and McCrea (2005). This consists of separate networks for rhythm generation and for pattern formation. Pattern formation modules include circuitry for reciprocal inhibition of antagonist motor pools and for controlling the activity of subsets of MNs.

The organization in Figure 2 offers an explanation for the deletions associated with the maintenance of phase of the locomotor rhythm. A spontaneous alteration of excitability within the pattern formation networks will not affect the rhythm generation network. In fact, it would simply alter MN activity, resulting in a transient, complete suppression of activity (or a reduction; partial deletion) in electroneurographic amplitude, but without producing any rhythm resetting or phase shift. When the deletion-producing episode disappears, MN activity would return precisely at the time expected if the deletion had not occurred.

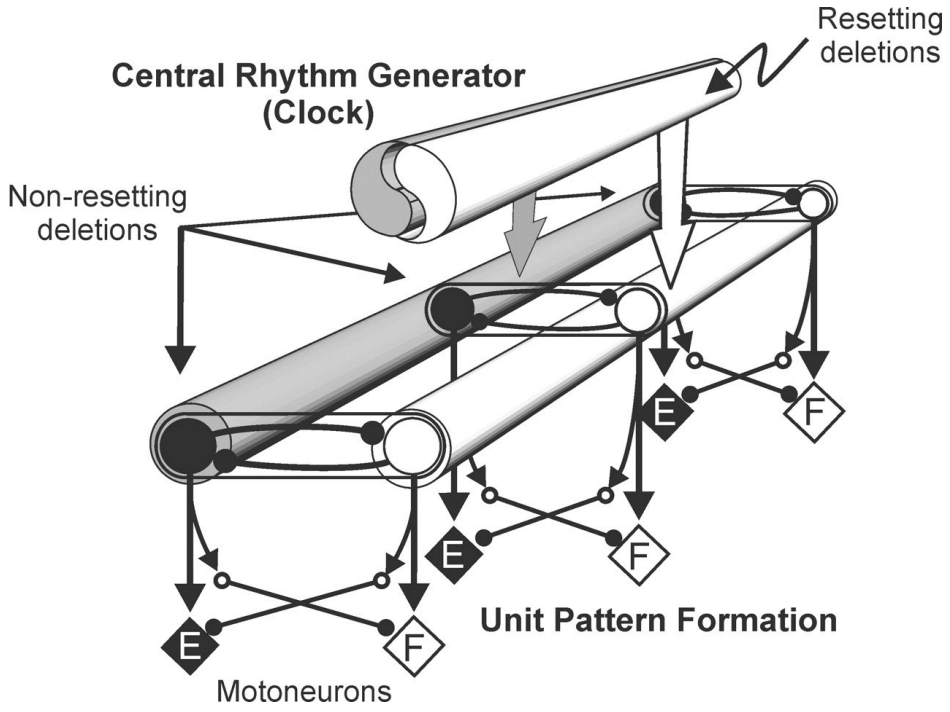


Figure. 2. Schematic representation of the proposed architecture for the central pattern generator (CPG), which consists of separate networks for rhythm generation (clock) and for pattern formation (i.e., the distribution of excitation and inhibition to MN pools; from Lafreniere-Roula and McCrea, *J Neurophysiol* 2005).

1.3. Control of locomotion

Like in other species, the function of mammalian neural networks for locomotion is dependent upon different types of external controls (Fig. 3). In the neonatal rat one is situated in the supraspinal regions, to provide the triggering system for locomotion. The second system comprising multiple segmental afferents, can modulate pattern frequency and intensity (Clarac et al., 2004). The third system, represented by propriospinal pathways, is responsible for the movement coordination among various parts of the body (Cazalets, 2005).

Supraspinal centers control locomotion via slow and fast descending pathways impinging on the spinal networks. Fast conducting reticulospinal pathways, that, in turn, become activated by mesencephalic and diencephalic locomotor regions, regulate the level of activity within the CPG and can thus induce rapid changes in locomotion. In addition, slow monoaminergic pathways affect the level of responsiveness in the spinal circuits. Both control systems appear to function in a very different way, as the monoamine system sets the level of background tone, whereas the fast reticulospinal system has the command role (Grillner, 2002).

Beside the slow and fast descending pathways controlling the locomotor CPG, other types of input are also needed for a behaviorally-adequate locomotor activity. In fact, postural adaptation involves cerebellar control via vestibulo-, reticulo- and rubro-spinal pathways, while visuomotor coordination, for instance to achieve accurate foot placement in a complex terrain, is mediated via the corticospinal pathways (Grillner, 2002).

At the same time, the brain receives feedback information from spinal locomotor networks and this appears to be a common feature of spinal pattern generation throughout phylogeny. For example, Vinay and Grillner (1992) have described the existence of neurons in the lamprey spinal cord that, with axons ascending to the brainstem, inform reticulospinal neurons about spinal CPG activity. The same organization has been previously demonstrated in mammals by Arshavsky et al. (1984) for the neurons of the ventral spinocerebellar tract. More recently, a spino-rubral pathway that directly conveys rhythmic signals has been observed in the cat by Vinay et al. (1993).

An additional way to shape and adapt the CPG operativity is represented by information coming from the periphery. In fact, during locomotion, afferent inputs determine several types of reorganization of reflex pathways, which serve not only to regulate the excitability of local groups of MNs, but also to control the basic operation of CPG. The mechanisms involved include: the suppression of interneuronal (reflex) pathways operating at rest, the emergence of new reflex pathways during locomotion, afferent actions on interneurons that form part of the CPG, presynaptic suppression of synaptic transmission from afferents and modification of MN membrane currents and firing properties (McCrea, 2001).

Many authors have confirmed that spinal locomotion can be either induced or facilitated through 'non-specific' sensory stimulation (deGuzman et al., 1991; Edgerton et al., 1991; Hodgson et al., 1994; Rossignol et al., 1996).

Already at birth, the immature afferents can modulate the fictive locomotion frequency *in vitro,* as described by Marchetti et al. (2001), who were even able to induce a rhythmic activity with strong sensory stimulation. However, in the last few years there is a growing excitement about the wealth of data on some very specific types of sensory input which are thought to have direct access to the CPG (Van de Crommert et al, 1998).

Finally, for the adequate function of the central nervous system various areas of the body must operate together to ensure either postural or propulsive behaviors during locomotion. For example, bipedal locomotion is a complex motor behavior which needs the simultaneous activation of most body parts including trunk and neck, forelimbs and hindlimbs. Thus, appropriate locomotor and postural activities in mammals result from the coordinated activation of different spinal networks. Recent results by Cazalets (2005) suggest that the mammalian spinal cord contains propriospinal pathways, including local circuits as well as long projection fibers, through which motor activity propagates along the spinal cord with a specific temporal pattern and may be involved in coordinating various parts of body.

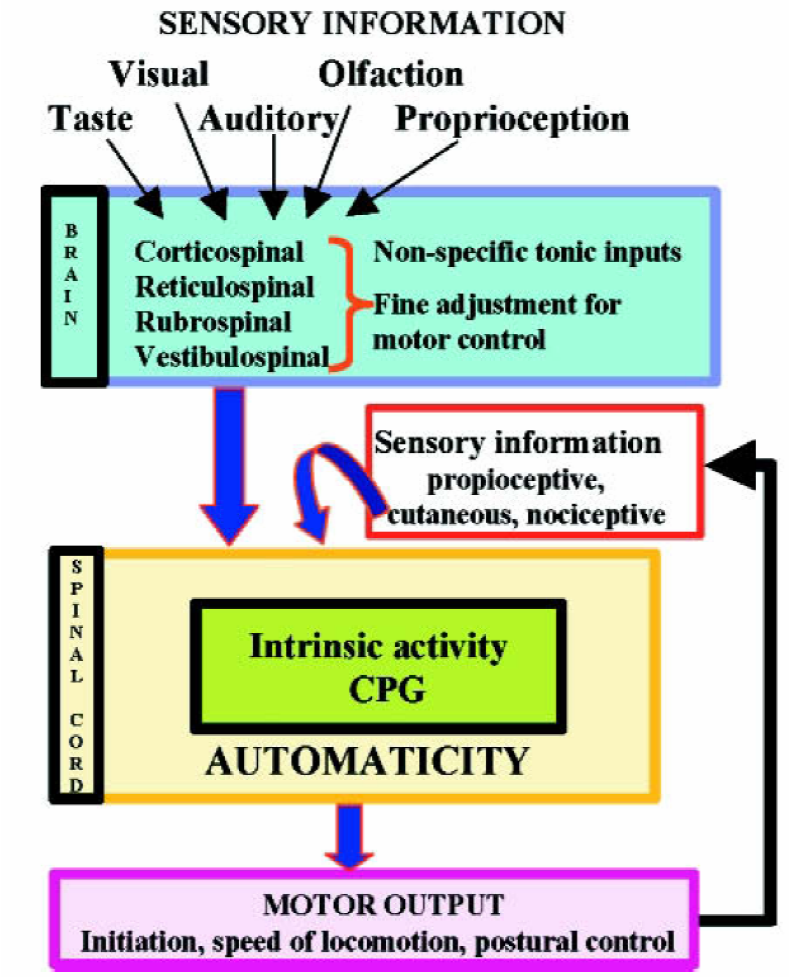


Figure 3 Automaticity is a key feature of neural motor control systems. Many sources of input to the brain (blue box) and spinal cord (yellow box) are constantly processed as we accommodate to the complexities of an ever-changing physical environment (from Edgerton et al. *Annu Rev Neurosci* 2004).

1.4. Ontogeny of spinal networks

At birth, although rodents cannot walk because of immature posture, their CPG for locomotion is already functional. In fact, in vitro preparations from newborn rodents can express alternating fictive locomotion in the presence of excitatory input as administration of NMDA plus 5-HT (Vinay et al., 2002). Similar locomotor-like patterns are also recorded from spinal cords 2 days prior to birth, showing that left-right, flexor-extensor commands are fully expressed during the last part of embryonic life (Vinay et al., 2002).

Conversely, analogous experiments done 5 days prior to birth show presence of synchronous motor patterns only (Kudo and Nishimaru, 1998, Nakayama et al., 2004). During the perinatal period, important changes occur in the spinal network neurons in coincidence with the transition from pattern synchrony to alternation. GABAergic and glycinergic interneurons located in the region dorsomedial to the lateral motor column are mainly responsible for alternation between homosegmental MNs. At this stage of development, a shift of E_{Cl^-} towards hyperpolarized values and a large growth of inhibitory connections between the two sides of the cord, convert GABA- or glycine-mediated excitation to inhibition (O`Donovan et al., 1998).

Furthermore, in the last embryonic days spinal networks undergo extensive spatial rearrangement. In fact, both in the chick embryo (Antal et al., 1994) and the rat fetus (Ma et al., 1992) the transient population of GABA immunoreactive interneurons in the ventral part of the spinal cord disappears after birth as GABAergic interneurons are mostly distributed in the dorsal horn. The fate of the ventral horn neurons remains unclear, although evidence suggests that they can switch from GABA to glycine (Gonzalez-Forero and Alvarez, 2005) while these transiently express ERG K^+ conductances typical of neuronal development (Furlan et al., 2005).

Modifications in the intrinsic membrane properties of MNs are one of the fundamental characteristics during the transition from embryonic to postnatal age. These include age-related decrease in input resistance, augmented rheobase, and the development of repetitive firing properties (Carrascal et al., 2005).

Noteworthy is that the maturation of MNs does not simultaneously proceed in all motor pools. For instance, ankle flexor MNs acquire repetitive firing properties earlier than ankle extensor MNs (Vinay et al., 2000).

The action potential properties are markedly changed during the last week of gestation and immediately after birth. Postnatal differences include a shift of the

threshold for action potential toward more negative potentials, a shortening in the action potential duration, and a decrease in the length of spike afterhyperpolarization. (Gao and Ziskind-Conhaim, 1998, Carrascal et al., 2005). The increase in MN excitability during spinal cord development is due to modifications in many ionic conductances. The threshold potential for I_{Na} activation is more negative in postnatal than in embryonic MNs. Modifications in potassium conductances involve a larger number of leak-potassium channels, a higher amplitude of I_K, a decline in current density of I_A and wider postnatal expression of $I_{K(Ca)}$ (Gao and Ziskind-Conhaim, 1998). Another potassium current whose maturation may be important with regard to rhythm generation is the hyperpolarization-activated inward current I_h (Takahashi, 1990 b; Kjaerulff and Kiehn, 1996; Kiehn et al., 2000).

Moreover, in the mammalian isolated spinal cord the locomotor rhythmic activity is blocked by antagonists of L-type Ca^{2+} channels after P7. This suggests that L-type channels can make an important contribute to mature motor behavior (Jiang et al., 1999; Perrier and Hounsgaard, 2000).

Nevertheless, intrinsic properties of mature MNs are also changeable: for instance, alterations in intrinsic properties may occur as a consequence of tissue damage, thus making the injured spinal cord closer to the conditions found in the perinatal stage (Perrier et al., 2000). Nashmi and Fehlings (2001) have reported, following spinal cord injury, an increased level of expression of channels responsible for I_A current.

In addition, during development, anatomical changes affect the membrane properties of MNs. At the embryonic stages, in fact, MN somal size increases reaching adult size at birth and similarly, the dendritic trees growth through neonatal life with consequent net enlargement of the MN surface area (Carrascal et al., 2005). Age-related modifications in the kinetics of both GABA and glutamate receptor channels have also been reported (O`Brien and Berger, 2001; Stegenga and Kalb, 2001) although it should be noted that GABA and glycine are functionally inhibitory just after birth (Marchetti et al., 2002).

A key element in the maturation of lumbar networks is the development of descending pathways in particular serotoninergic projections. At E17 pathways descending from the brainstem have just reached the lumbar enlargement and, during the first postnatal week, the growth of serotoninergic axons allows maturation of adult electrical properties in MNs (Vinay et al., 2002). Conversely, mature networks resume an embryo-like function following either pharmacological blockade of 5-HT

receptors or when 5-HT receptor activation is prevented by acute spinalization that severs descending projections (Sillar et al., 1992; Pfieger et al., 2000).

Although electrical coupling among rat spinal cord neurons decreases during the first postnatal week (Walton and Navarrete, 1991), gap junctions in the adult reappear following nerve injury (Chang et al., 2000).

In summary then, birth is associated with profound changes in the wiring properties of spinal networks as well as in the characteristics of intrinsic conductances. These modifications allow expression of locomotor patterns similar to the adult ones (Kiehn and Kjaerulff, 1996). The baby rat cannot walk because of skeletal muscle immaturity (Clarac et al., 1998), and, it can actually generates quadrupedal locomotor patterns if suspended from the ground.

These observations suggest the use of neonatal spinal cord preparations as a suitable model to investigate the physiology and pharmacology of locomotor circuits.

1.5. Intrinsic MN activity

MNs are the final convergence pathway for the CPG network, but this does not mean that they are mere transducers of the synaptic inputs received from premotor network circuits. In fact, intrinsic properties of spinal MNs determine how converging neuronal inputs are translated into the final command to muscles. In particular, in the absence of inputs from the CPG network, MNs can produce coherent synchronous rhythmic patterns as a result of the activation of some intrinsic membrane conductances. To observe this type of discharges (which is not locomotor-like because of lack of alternation) two conditions must be met. The first one is the activation of NMDA receptors that induce bursting even after pharmacological blockade of synaptic transmission mediated by action potentials. The second one is the coordination of neuronal activity across gap junctions.

Such a MN activity expressed by the activation of NMDA receptors is also dependent on the activation of 5-HT receptors (Sillar and Simmers, 1994; MacLean et al., 1998; Prime et al, 1999). A different form of sustained MN excitation (independent from CPGs) is prolonged plateau potentials during which the membrane potential remains depolarized versus the resting one. The plateau potential allows a cell to possess two stable membrane potentials: one at rest, without spike activity, and another one at a more depolarized level, that generates sustained firing. The term of bistability indicates these two stable membrane potentials (Kiehn and Eken, 1998). Functionally,

when a plateau potential is initiated, a cell can fire action potentials in the absence of continuous synaptic excitation. Such mechanism might be useful in postural muscles, where part of a continuous descending synaptic drive could be replaced by self-supporting membrane properties (Kiehn and Eken, 1998).

In spinal MNs, bistable behavior is a latent property revealed in the presence of promoting agents. This facilitation is mediated by the activation of muscarinic and group I mGluRs (Svirkis and Hounsgaard, 1997, Delgado-Lezama et al., 1997; Svirkis and Hounsgaard, 1998), but also by serotonin (Hounsgaard and Kiehn, 1989) and noradrenaline (Conway et al., 1988; Lee and Heckman, 1996).

Bistability is originated by a non-inactivating or slowly inactivating inward current. In spinal MNs this current is predominantly mediated by the influx of Ca^{2+} through L-like calcium channels, as it is blocked by the calcium blocker nifedipine (Hounsgaard and Mintz, 1988; Hounsgaard and Kiehn, 1989; Alaburda et al., 2002). The activity is terminated when Ca^{2+} influx activates calcium dependent potassium channels (K_{Ca}) that, in turn, causes a progressive hyperpolarization (Grillner et al., 2000).

It has been proposed that the voltage sensitive persistent inward current in MNs is normally present, but masked by outward currents (Schwindt and Crill, 1982). Thus, after pharmacological block of the outward potassium conductance by neuromodulators, the inward current is capable of generating plateau potentials (Housgaard and Kiehn, 1985; Hounsgaard and Mintz, 1988). Neuromodulators can activate intracellular pathways, that target several types of ion channels and, in this way, regulate outward and inward current generators in parallel.

In addition to the persistent inward current mediated by L-type Ca^{2+} channels, other persistent inward currents have been identified for generating plateaux in spinal MNs.

For bistable behavior generation in turtles MNs, many studies have proposed the involvement of a calcium-activated voltage-dependent sodium current referred to as I_{CAN} (Rekling and Feldman 1997; Perrier and Hounsgaard, 1999). However, the functional significance of I_{CAN} and its presence in the spinal MNs of other species remain unknown.

Furthermore, the inward current I_h, that carries a mixture of K^+ and Na^+ ions in response to hyperpolarization and that is strongly enhanced by 5-HT (Kjaerulff and Kiehn, 2001), has been suggested to play a role in generating plateaux (Kiehn and Eken, 1998). On the contrary, more recent evidence has suggested that $I_{h,}$ rather than

contributing to bistability, acts as a tonically depolarizing leak conductance (Kiehn et al., 2000).

The voltage- and tetrodotoxin- sensitive sodium persistent current I_{NaP} can initiate and maintain tonic or burst firing which, in some cases, is sufficient to evoke a plateau potential (Harris-Warrick, 2002). It has been reported that the pre-Botzinger complex for respiratory rhythmic activity incorporates an insipiratory pacemaker (kernel of neurons) possessing a high I_{NaP} (Del Negro et al., 2002a). In further support of the data, blocking I_{NaP} with the relatively selective blocker riluzole abolishes nauronal bursting (Del Negro et al., 2002b).

1.6. Evidence for the human CPG

In contrast to the abundance of data in animals, leading to the general assumption of a CPG underlying the central control of locomotion, little is known about spinal networks acting like CPGs in primates in general and in humans in particular. Hence, in the context of human locomotion, an important question arises: is there a CPG in humans?

Spontaneous, involuntary and alternating stepping-like movements in the lower extremities have been repeatedly observed by Calancie and his colleagues (1994) under certain conditions in a subject with a chronic history of neurologically incomplete spinal cord injury (SCI). These authors pointed out that many of the recorded movements had the characteristics of the stepping movements seen in a variety of animal studies, in which the CPG operation has been implicated.

Further evidence suggesting that neural networks responsible for generating rhythmic locomotor activity can be located in the human spinal cord, comes from experiments in which specific sites of the spinal cord were electrical stimulated. Dimitrijevic et al. (1998) have demonstrated that tonic electrical stimulation of human completely injured spinal cord induces patterned, locomotor-like activity (Fig. 4).

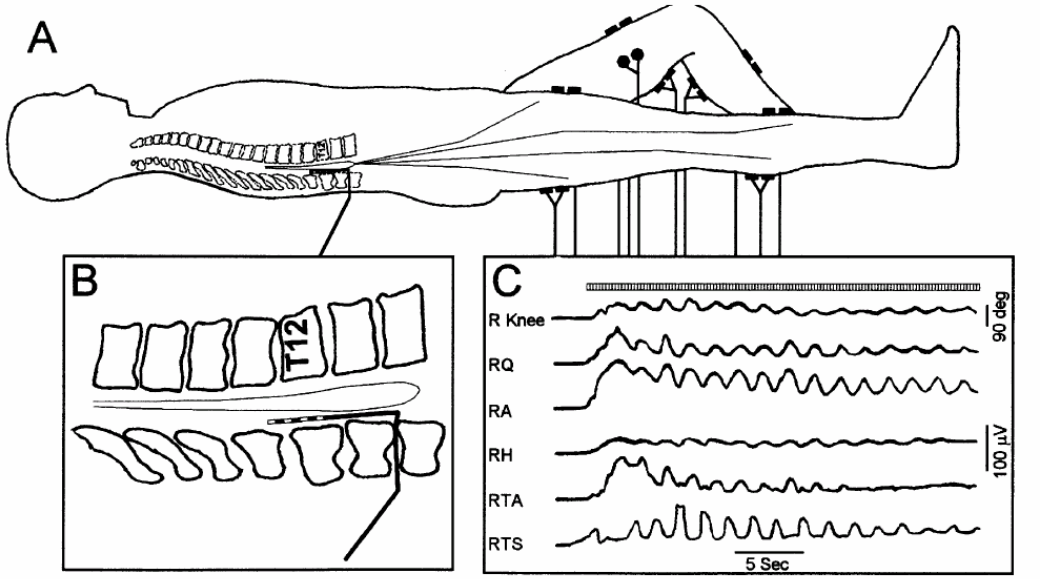


Figure 4. Diagrammatic sketch of the experimental design of this study. In (A), the subject under examination is in the supine position with the stimulating epidural electrode above the lumbar cord. Pairs of surface electrodes for electromyographic (EMG) recording are placed over both quadriceps, adductors, hamstrings, tibial anterior, and triceps surae muscle groups. Diagram of the quadripolar epidural electrode placed within the spinal canal above the posterior lumbar cord structures (B); EMG recording of rhythmic activity from the right lower limb during stimulation of the upper segments of the lumbar cord, with position sensor trace recording movement of the knee during flexion and extension of the lower limb (C); (from Dimitrijevic et al. *Ann N Y Acad Sci* 1998)

Gerasimenko et al. (2002) have also used spinal cord stimulation in paraplegic patients (lesioned at thoracic or cervical level) and found that stimulation of the ventral surface of the cord initially produces tonic activity in the motor pools of the leg, eventually giving way to rhythmic activity.

Another example of involuntary stepping movements is given by sleep-related periodic leg movements. These are stereotypic, periodic, movements involving one or both lower limbs. As reported by Bixler et al (1982) such movements are not disease-specific and can also appear in healthy subjects without voluntary trigger.

Moreover, the possible existence of intrinsic spinal networks in humans is shown by the presence of primitive step-like movements in the externally supported newborn infants whose stepping is less likely to be under strong cortical control than in the adult (Forssberg, 1985).

Recently, Yang and his collaborators (2005) showed, for the first time, that infants can step in a coordinated manner on a split-belt treadmill when the belts run at remarkably different speeds and when the belts are running in opposite directions. Since different types of coupling and opposite directions of stepping are simultaneously possible in both legs, these authors have concluded that the pattern generator for each leg has some autonomy, even in infants.

The innate character of the CPG is further supported by the well-known presence of coordinated movements during the prenatal phase. Rayburn (1995), monitoring fetal movements, has shown that coordination of the whole-body movements is very similar to the one seen in the newborn infant.

1.7. Spinal cord injury (SCI)

SCI may be defined as a lesion to the spinal cord that compromises, either completely or incompletely, its major functions (motor, sensory, autonomic and reflex).

Following the initial impact after SCI, there is a cascade of downstream events termed 'secondary injury', which evolves in degenerative events in the spinal cord. 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 (Park et al., 2004).

Nearly all spinal-cord injuries damage both upper and lower MNs. Segmental lower MNs are damaged or lost in the central gray matter at the injury site and in several segments above and below the lesion, resulting in flaccid paralysis at the injury level. Variable injury to the surrounding white matter affects the long tracts, and interrupts descending pathways coming from supraspinal motor centers. In all lesions, apart from complete transections, a small doughnut-like rim of white matter remains at the injury site. This outer white matter allows some axons to
remain intact, but many others cease to function because of segmental demyelination.

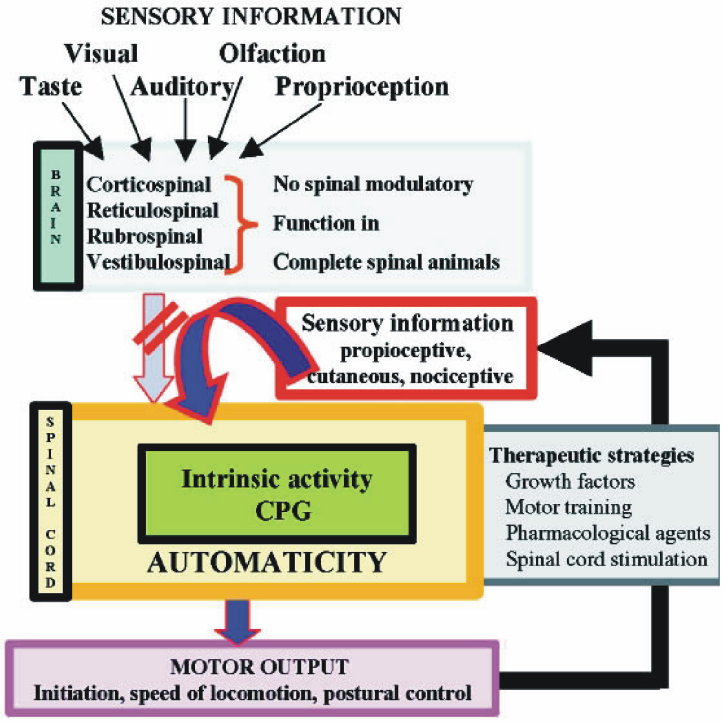
While motor function is severely disrupted following SCI, the spinal circuitry, however, exhibits a great degree of automaticity and plasticity after an injury (Edgerton et al., 2004). Automaticity describes the concept by which spinal circuits are able to carry out complex motor tasks without “conscious” supraspinal input. Plasticity indicates changes at the cellular level in the spinal cord induced in an activity-dependent manner. These characteristics should be strongly considered as

advantageous in developing therapeutic strategies to assist the recovery of locomotor function following SCI.

These concepts are important and necessary for the design of locomotor rehabilitation strategies in patients with spinal cord injuries, especially when different approaches such as locomotor training, pharmacoterapy and functional electrical stimulation are combined (Barbeau and Rossignol, 1994).

Pain and spasticity are common sequelae of SCI (Burchiel and Hsu, 2001). Chronic pain is a major complication to SCI. Epidemiologic studies indicate that approximately two-thirds of all SCI patients suffer from chronic pain, one-third have severe pain. Spasticity is defined as both abnormal increase in tone (hypertonus) and velocity-dependent increased resistance to muscle stretch.

An understanding of these disorders and the therapeutic means to achieve functional restoration from these complications of SCI are particularly crucial to allow individuals with para- and quadriplegia the opportunity to live a full, productive, and comfortable life. Fig. 5 shows diagrammatic scheme concerning modification of spinal networks induced by SCI. It is noteworthy that after lesion sensory input becomes greater than normal, whereas the direct supraspinal influence on locomotion is greatly reduced.



(B) **Figure 5.** After a complete SCI, where the supraspinal connections are severed, the spinal circuitry adapts to its altered combination of inputs to facilitate motor output. The locomotion and standing patterns that can be exhibited after training illustrate not only a high level of spinal automaticity but also the spinal cord's capacity to learn and perform motor tasks. The gray box lists various therapeutic strategies by which functional motor recovery may be enhanced after SCI (from Edgerton et al. *Ann Rev Neurosci* 2004).

2. STUDYING THE SPINAL PATTERN GENERATION: THE ISOLATED SPINAL CORD PREPARATION

2.1. The *in vitro* isolated spinal cord of the neonatal rat

Many experimental preparations from various types of animal have been used to study the physiology of spinal networks.

Mouse preparations are preferable for defining genetic markers for spinal interneurons (Carlin et al., 2005; Hinckley et al., 2005), while mouse as much as rat or chicken preparations are equally suitable for studying the neonatal development of spinal networks (O`Donovan et al., 1998; Vinay et al., 2002). The turtle spinal cord provides particular advantages (stability, maturity, etc) for analyzing the properties of various adult neurons (Alaburda et al., 2002). Lamprey, frog and rat spinal cords have been traditionally employed for analyzing neuronal networks subserving locomotion (Grillner et al., 1998; 2000; Clarac et al., 2004). Feline spinal cord preparations have long been used to describe networks responsible for spinal reflexes because of they resemble those of the human interneuronal systems involved in mediating simple and complex reflex responses (Jankowska and Hammar, 2002). Of course, feline spinal cord preparations in vivo can generate locomotor-like patterns after i.v. injection of excitatory neuromodulators (Nielsen et al., 2005), thus demonstrating close similarities with the fictive locomotion process recorded from in vitro rodent preparations.

In the present project, recording from the rat isolated spinal cord has shown many analogies with the data available from feline preparations, such as the modulation of chemically-induced fictive locomotion (Zangger, 1981), the appearance of synchronous discharges recorded from both ventral and dorsal roots in the presence of 4-AP (Dubuc and Rossignol, 1989 a; b), and MNs plateau properties (Lee and Heckman, 1999). Furthermore, a recently-developed robotic device has been able to improve locomotion in spinalized rats after intense training (De Leon et al., 2002) suggesting that the rodent CPG for locomotion can be reactivated, after lesion, by training as demonstrated with feline locomotor networks (Rossignol, 2000).

Among them, the most widely adopted is the neonatal rodent spinal cord (Kerkut and Bagust, 1995; Clarac et al., 2004). Such a mammalian preparation can be used routinely for several hours when maintained at room temperature in an appropriate physiological medium, that reproduces the composition of the cerebrospinal fluid and

that is continuously bubbled with a O_2/CO_2 mixture, setting the extracellular pH at 7.4.

There is a number of important advantages to justify the widespread use of this preparation. The first among them is that preparative surgery can be done rapidly and more easily compared to other mammals. Moreover, all chemical parameters of the medium are controlled and drugs can be simply added to perfusion bath.

Finally, this mammalian model provides the opportunity to study the operational mechanisms of the locomotor CPG, which may be important to provide new approaches to neurorehabilitation of SCI patients.

Because of its accessibility and long-term stability, such a preparation represents a very advantageous model to evaluate the pharmacological action of drugs proposed for the symptomatic treatment of spinal cord injured persons.

From the isolated spinal cord of the neonatal rat it is possible to record different types of rhythmic discharge, namely spontaneous firing, locomotor-like alternated oscillations, and synchronous bursting.

2.2. Spontaneous activity recorded from ventral roots

Ventral roots (VRs) exhibit ‘‘spontaneous’’ activity, whose occurrence seems to be inversely proportional to the degree of maturation of the preparation.

Evidence coming from studies on chick embryos has suggested that spontaneous activity can arise from networks built of either $GABA_A$ and glycinergic connections or excitatory amino acid (EAA) and cholinergic connections (O’Donovan et al., 1998 a; b). In embryos, spontaneous VR discharges can alternate between flexor and extensor MN pools, suggesting the engagement of the spinal circuitry later responsible for locomotion in adult life. Alternation is ensured by a selective depolarizing shunt of action potential generation in flexor MNs during extensor activity (O’Donovan et al., 1998 a, b). Behavioral and electromyographic (EMG) experiments have shown that the basic synergies of muscle activation that characterize locomotion, (alternation of antagonists and co-activation of synergists), emerge early in development and become progressively refined thereafter.

These observations have led to the idea that the circuitry controlling locomotion is assembled quite early in development. Several important differences exist between the mechanisms of embryonic motor activity and the production of adult locomotion. The most important distinction between the two types of behavior is the way in which

the networks are activated. In the adult, locomotion is initiated and controlled by descending commands from the brain. In the embryo, motor activity is generated autonomously by spinal circuits in the absence of descending inputs (Landmesser and O`Donovan, 1984).

Conversely, Nishimaru et al. (1996) have stated that the function of spontaneous patterns does not seem to be related to the establishment of the specific neuronal connections necessary for the operation of adult networks. They have proposed that the presence of these spontaneous patterns could be due to transient synaptic connections simply involved in spreading excitation within the immature spinal cord.

Nevertheless, spontaneous activity appears to be fundamental in supporting the development of muscles and joints (Jarvis et al., 1996), the neurochemistry and gene expression of spinal neurons (Spitzer et al., 1995), the intramuscular branching patterns of MNs (Dahm and Landmesser, 1998) and the survival of MNs during development (Pittman and Oppenheim, 1978).

Even the postnatal mouse shows spontaneous rhythmic motor activity as observed in *in vitro* spinal cord preparations (Bonnot et al., 1998; Whelan et al., 2000). Such a spontaneous pattern is characterized by several patterns of VR discharge including synchronous slow potentials, unilateral rhythmic discharges restricted to one or more segments of the cord, relatively fast oscillatory depolarizations synchronized over many segments and also spontaneous bouts of rhythmic alternating discharges which could be considered as a 'locomotor-like' activity.

Using rat preparations, Nishimaru and his colleagues (1996) have been the first to notice spontaneous movements in fetuses at E15 *in utero*. Similar synchronous spontaneous burst activity has been obtained from VR recordings from the *in vitro* isolated fetal rat spinal cord.

In the neonatal rat spinal cord, we have often observed brief episodes of spontaneous alternating activity, recorded from VRs during control conditions. Figure 6 shows examples of alternating activity in the rat isolated lumbar cord (top traces) as well as in the brain stem spinal cord preparation (bottom traces).

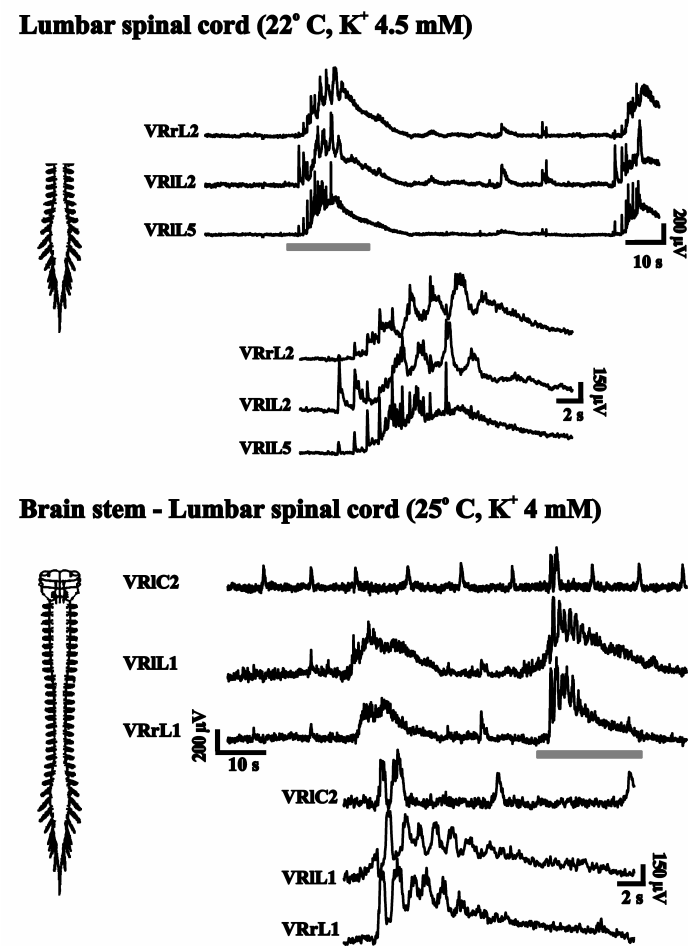


Figure 6. Spontaneous activity recorded from neonatal (P1) rats. In both isolated spinal cord (top traces; G Taccola unpublished data) and brainstem-spinal cord preparation (bottom trace) there are episodes of alternating activity between left-right L2 and left L2-5 (top insert) and between left-right L1 (upper trace; G Taccola and K Ballanyi unpublished data).

2.3. Fictive locomotion

The CPG is responsible for generating locomotor signals, that are electrical discharges alternating between flexor and extensor motor pools and between left and right MN within the same segment.

As Figure 7 shows, analogue motor signals are also recorded from isolated rat as well as mouse spinal cord and are characterized by oscillations alternated between left and right sides and between lumbar 2 and 5 (L2-L5) pairs of VRs. This phenomenon is called Fictive Locomotion and is manifested as rhythmic oscillations exclusively from VRs.

Some studies have left the preparation of the dissected spinal cord still connected to one or two hind limbs to correlate neuronal activity with muscular output. In these

cases, when a locomotor rhythm is pharmacologically induced, it has been possible to visualize real alternating movements of the hind limbs (Atsuta et al., 1988; Cazalets et al., 1992). By keeping one leg attached to the spinal cord, Kiehn and Kjaerulff (1996) have demonstrated that the rostral lumbar VRs (L1, L2 and L3) contain mostly axons innervating flexor muscles, whereas the L5 segment contains MNs that determine the contraction of extensor muscles.

Fictive locomotor signals are evoked by perfusing the isolated mammalian spinal cord with excitatory agents or by repeated stimuli applied to one DR.

2.4. Chemically induced Fictive Locomotion

Many studies have shown that limb alternation or VR alternated discharges can be induced by application of a variety of neurotransmitter agonists.

Glutamate and aspartate as well as the EAA agonists NMDA and kainate, induce locomotor activity with a left-right, alternating pattern (Kudo and Yamada 1987; Cazalets et al., 1992). AMPA cannot induce fictive locomotion in rats probably due to desensitization of this receptor class (Dingledine et al., 1999). Moreover, either NMDA or non-NMDA glutamate receptors are activated during locomotor-like activity since, when NMDA and non-NMDA receptors are simultaneously blocked, there is no fictive locomotion (Kudo and Yamada 1987; Cazalets et al.1992). However, Beato and his colleagues have been able to elicit stable locomotor activity in the presence of, NMDA or non-NMDA receptor antagonism respectively, showing that the operation of one glutamate receptor class is sufficient to express locomotor activity (Beato et al., 1997).

The literature contains other examples of locomotor network operation occurring independent of NMDA receptor activation. Recently, Cowley and his colleagues (2005) have demonstrated that, although NMDA receptor activation may appear essential for locomotor network operation under some experimental conditions, locomotor-like rhythms can, nevertheless, be generated in the presence of AP5.

5-HT is also able to induce a locomotor pattern: the intensity of the bursts is stronger, suggesting an activation of a larger pool of MNs, while the rhythm frequency is much slower (Cazalets et al.1992). The serotonin-induced rhythm can be blocked by a 5-HT_1 antagonist (propranolol) and by 5-HT_2 antagonists (kentanserin, myenserin and cyproheptadine), whereas 5-HT_3 antagonists are ineffective (Cazalets et al.1992).

In contrast, if 5-HT and NMDA are applied together, the discharges are much more regular and the rhythm has a frequency which is intermediate between those obtained with each substance applied separately: this activity remains very stable for a long time (Fig 7., Sqalli-Houssaini et al.1993).

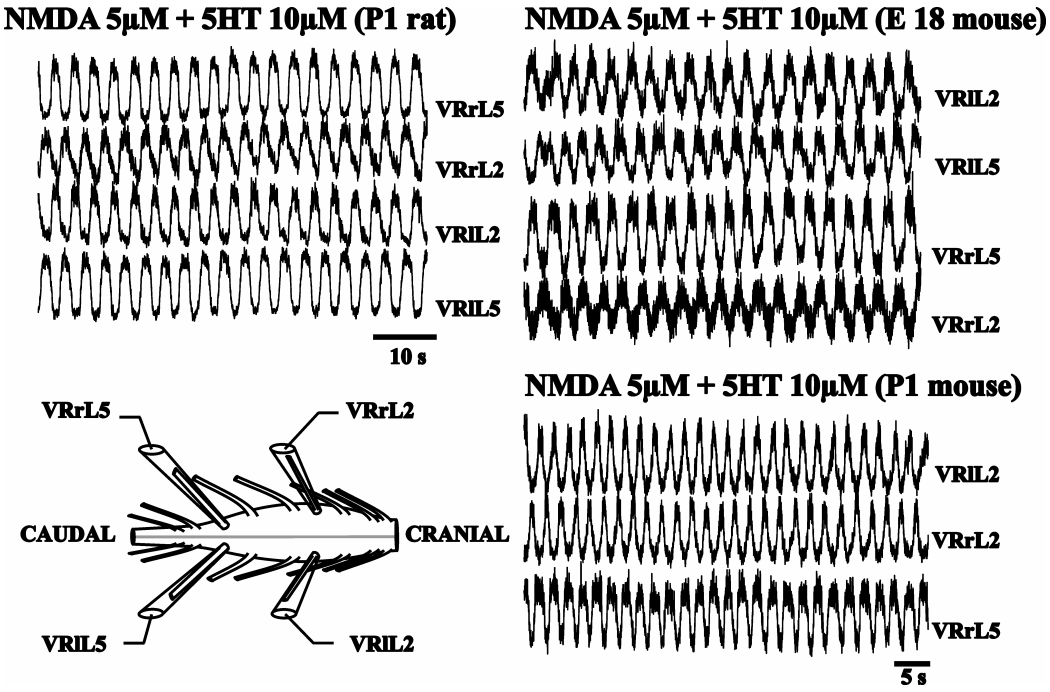


Figure 7. Chemically induced fictive locomotion in rat (right) and in mouse (left) preparations (left bottom). Stable rhythmic oscillations alternated between left and right lumbar roots (L2 or L5) and between L2 and L5 homolateral roots are recorded in the presence of NMDA (5µM) and 5HT (10µM; from G Taccola, unpublished data).

Sqalli-Houssaini and Cazalets (2000) have described how bath application of noradrenaline (NA) elicits an extremely slow alternating motor pattern. The alternation is clear between right and left sides, but not between flexors and extensors on the same side. The role of NA could be mimicked by the α receptor agonists methoxamine and phenylephrine, whereas α and β receptor agonists alone do not elicit any activity. Kiehn and Kjaerulff (1996) have characterized the fictive rhythms induced by 5-HT and dopamine. The dopamine-induced rhythm is more irregular and slower. Kiehn and Kjaerulff (1996) have shown that 5-HT has a tendency to facilitate flexor drive, whereas dopamine may exaggerate extensor activity.

Acetylcholine (ACh) combined with the acetylcholinesterase inhibitor edrophonium, usually elicits sustained rhythmic activity characterized by left-right alternation and

non-locomotor co-activation of ipsilateral flexor and extensor discharges (Cowley and Schmidt, 1994). However, more recent experiments have shown that, if low levels of cholinergic stimulation are used, transient episodes of rhythmic activity with a locomotor-like pattern can be evoked (Jordan and McVagh 2004).

The role of GABA has also been studied (Cazalets et al.1994; Cowley and Schmidt 1995; Kremer and Lev-Tov 1998). Bath-applied $GABA_A$ or $GABA_B$ agonists decrease the frequency of the rhythm and, depending upon the dose used, they can completely suppress activation previously obtained with the NMDA-5HT mixture. Similarly, the GABA uptake inhibitors nipecotic acid or guvacine, suppress the rhythmic pattern. The nipecotic response can be reversed by bicuculline or phaclofen.

Bracci and co-workers (1998) have demonstrated that high external K^+ induces alternating motor patterns only within a very narrow range of concentrations. The alternated pattern can be evoked also in the presence of NMDA, non-NMDA and 5-HT antagonism suggesting that, in the neonatal rat spinal cord, the CPG operation seems to require a widespread increase in neuronal excitability rather than selective activation of a distinct subgroup of spinal neurons. A clear answer to this question is complicated by the fact that the CPG has not been precisely identified in anatomic terms as discussed below.

2.5. Localization of CPG

Regarding the localization of the CPG responsible for locomotor activity recorded from *in vitro* preparations of the mammalian spinal cord, there are conflicting and contrasting results. Some bath-partition studies point out that the hindlimb locomotor CPG is not segmentally distributed, but confined to a restricted upper lumbar region (Cazalets et al., 1995). Nevertheless, the current consensus arising from spinal ablation experiments, indicates that the rhythmogenic network controlling leg movements is distributed within all lumbar segments (Fig. 8; Kjaerulff and Kiehn, 1996). The latter view is in accordance with that of Bracci et al. (1996 a) who have found that the mechanisms for burst triggering require a relatively small neuronal network, confined to a ventral quadrant, though distributed among all segments.

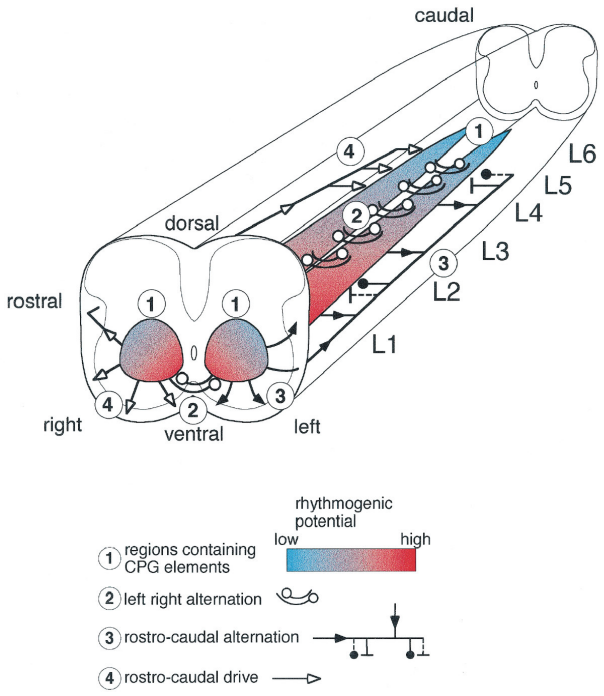


Figure 8. Summary of CPG localisation. The rhythm-generating network in L1–L6 is shown distributed along the cord as two medial columns (1). The taper and the color gradient indicate the high rostral and lower caudal ability to generate rhythmic activity. The localization of the pathways mediating left/right alternation in the ventral commissure is indicated (2). The pathways mediating rostrocaudal alternation are shown widely distributed in the lateral and ventral funiculus on the left side of the preparation (3 The rostrocaudal drive is indicated on the right side of the cord (4); from Kjaerulff and Kiehn *J Neurosci*, 1996)

In the cat and in the rat, activity-dependent neuronal labeling has suggested that the medial part of the intermediate gray and the area around the central canal are important for rhythm-generation during locomotion (Viala et al., 1991; Kjaerulff et al., 1994; Kjaerulff and Kiehn, 1996).

A key element in all locomotor CPGs is the left–right coordination, that ensures that limbs on opposite sides of the body alternate (walking) or are activated in synchrony (hopping). The CPG elements controlling left–right coordination necessarily include commissural interneurons (CINs): these neurons send axons to cross the midline to form synapses onto MNs and/or other interneurons (including CINs) situated in the contralateral hemicord.

In the neonatal spinal cord *in vitro*, Butt and Kiehn (2002) have shown that in the medial VII, VIII, X laminae of the lumbar cord there is a significant concentration of interneurons with diverse projections crossing the midline. In a later study, using an electrophysiological approach, the same authors have identified such neurons as functionally responsible for left-right coordination of hindlimbs (Butt and Kiehn, 2003).

2.6. Electrically evoked Fictive Locomotion

In the neonatal rat spinal cord, rhythmic locomotor-like patterns can be elicited by bath-applied excitatory substances like NMDA (Kudo & Yamada, 1987), 5-HT (Cazalets et al., 1992; Beato et al., 1997) or high potassium (Bracci et al., 1998). Although this approach has yielded important insights into the mode of operation of the fictive locomotor network, persistent bath application of these excitatory substances to the entire spinal tissue represents a non-physiological condition.

Marchetti and co-workers (2001) have suggested that, in the neonatal rat spinal cord *in vitro*, locomotor-like patterns of activity can be reliably evoked by trains of stimuli applied to one DR (Fig. 9). This condition might thus mimic physiological activation of the CPG by sensory inputs. The oscillatory activity induced by DR stimulation presents the typical phase alternation at segmental and intersegmental level which is a hallmark of fictive locomotion evoked by chemical substances (Kiehn *et al.*, 1997).

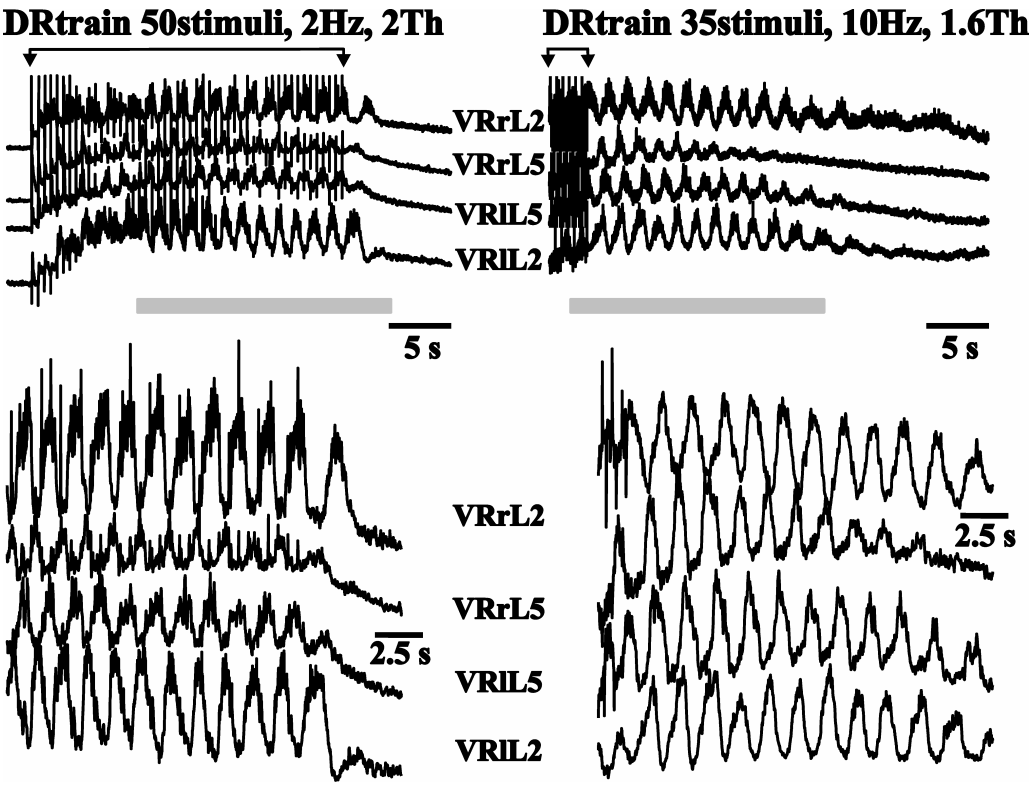


Figure 9. Each panel shows DC records of responses from pairs of VRs (left or right of L2 and L5). Oscillations are in alternation between contralateral VRs and between intersegmental VRs of the same side. In two different preparations, alternating oscillations outlasting the last stimulus are induced by two different protocols of electrical stimulation (number of stimuli, frequency and intensity) applied to L5 right DR (from G Taccola, unpublished data).

Such alternating discharges of MNs are likely to be driven by the CPG and can be regarded as a locomotor-like pattern turned on by the activation of dorsal afferents which are known to project to the CPG itself (Hultborn et al., 1998).

Unlike the persistent and stable fictive locomotor pattern elicited by neurochemicals (Kiehn et al., 1997), the DR-induced one is usually transient. Loss of rhythmicity might be due to either fatigue in the pathways upstream of the CPG (as observed with afferent impulses in the presence of strychnine and bicuculline; Bracci et al., 1997) or stimulus-dependent release of transmitters which inhibit the CPG operation.

2.7. Disinhibited Rhythm

In the neonatal rat spinal cord, network-driven rhythmic bursting arises spontaneously after applying saturating concentrations of strychnine and bicuculline, which block GABA and glycine receptors respectively (Fig. 10). This rhythm, called disinhibited rhythm, is unphysiological; however, blocking fast, chloride-mediated inhibition largely simplifies the rhythmogenic network. Furthermore, disinhibited rhythmicity, by using excitatory connections only, resembles the early type of collective network bursting (Sernagor et al., 1995).

After dorsal horn removal from the neonatal spinal cord of rat, the remaining network, localized to the ventral horn, can still perform rhythmic activity in the absence of synaptic inhibition (Bracci et al., 1996 a).

To comprehend the role of inhibition in the genesis of the fictive locomotor pattern, it is important to answer the question of whether disinhibited rhythm and fictive locomotion are generated by the same network (Bracci et al., 1996 a; Nishimaru & Kudo, 2000). Evidence favors this hypothesis, as Bracci et al. (1996 a; b) have found that disinhibited bursting shares with locomotor patterns the same sensitivity to block of either class of ionotropic glutamate receptors (Beato et al., 1997; Bracci et al., 1996 a) and the same anatomical location (Bracci et al., 1996 a; Kjaerulff & Kiehn, 1996). In addition, this rhythm can be accelerated by 5-HT (Bracci et al., 1996 a), known to activate fictive locomotor patterns (Cazalets et al., 1992), and a strong synergy between disinhibited rhythm and locomotor pattern is demonstrated with the use of the split bath configuration (Beato & Nistri, 1999).

Observations made on the organotypic spinal slice culture have further supported the possibility that a common network is involved in generation of the two rhythms. In fact, it has been shown that bursting induced by disinhibition or by increased

excitability are composed of similar waves and involve the same areas of the slice (Tscherter et al., 2001).

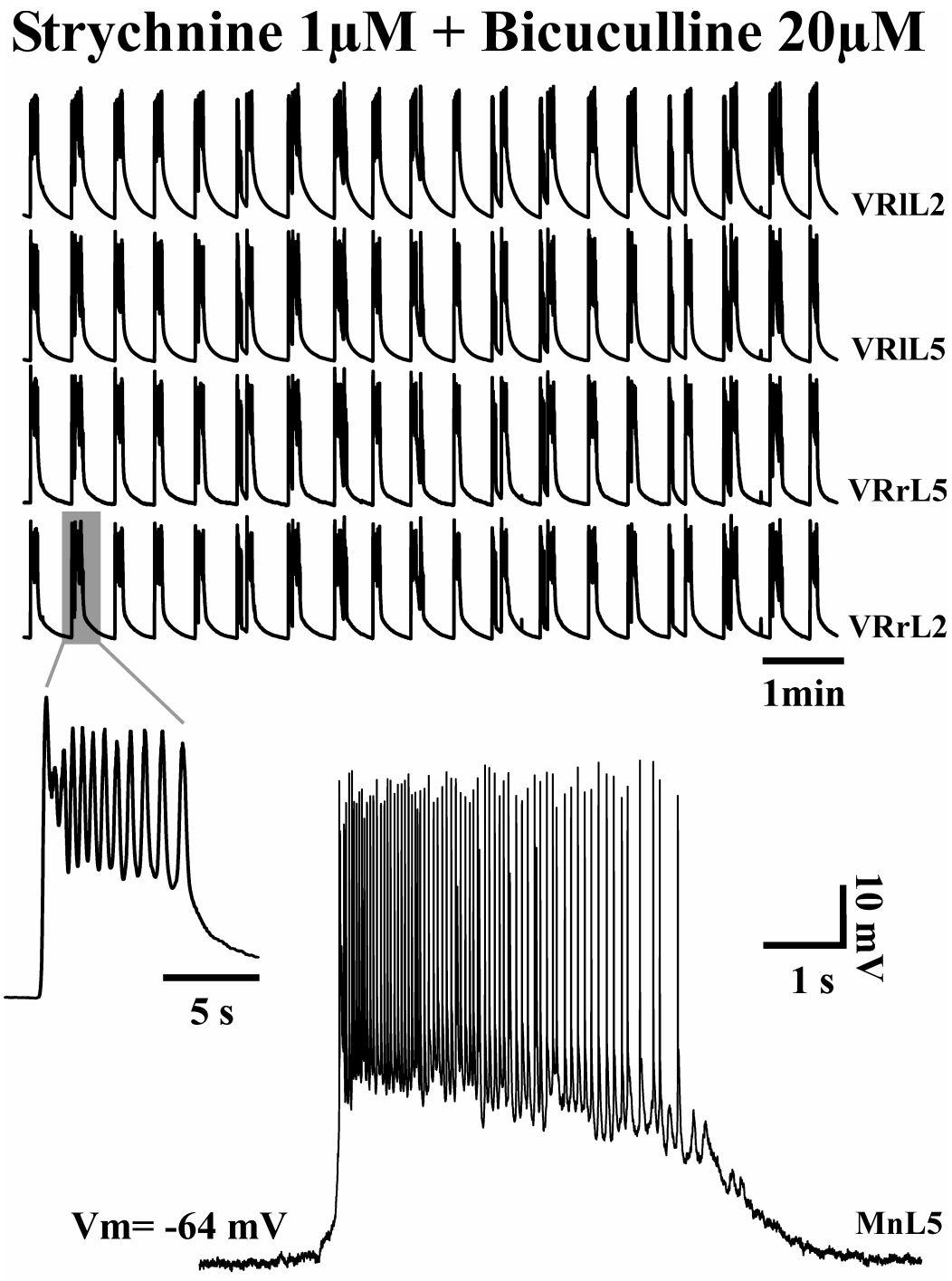


Figure 10. Synchronous rhythmic bursts evoked by pharmacological blockade of synaptic inhibition could be recorded from all lumbar VRs (left, right L2 and L5; top traces). DC-coupled extracellular recording of single burst (left insert) presents similar bursting pattern and structure as observed from intracellular recording of lumbar MN (bottom trace; from G Taccola, unpublished data).

3. MODULATING SPINAL CIRCUITS: A TARGET FOR REHABILITATIVE PURPOSES

3.1. Modulation of neural networks

The existence of intrinsic rhythmicity in the isolated spinal cord suggests the possibility to use spinal activity to restore functional movements by the spinal cord of SCI patients. Extending our knowledge of CPG modulation by endogenous factors could improve therapeutic interventions designed to select and increase functionally useful motor behaviors generated by spinal cord.

In animal models, activation or blockade of different receptors or conductances could participate in various aspects of network output control, such, for instance, its initiation and the modulation of timing and amplitude characteristics.

Modulation can occur at the level of the central pattern generating circuit itself, on the motor neurons directly, or on the preMNs that bring the rhythmic drive to the MNs.

Studies in invertebrates show that similar motor patterns can be produced by different mechanisms (Thoby-Brisson and Simmers, 1998) and that neuromodulation can switch on/off different motor patterns, corresponding to a wide range of behaviors. These facts suggest that networks are not hard-wired, but are reconfigured by the modulatory environment. It is likely that, like in invertebrates (Getting and Dekin, 1989; Dickinson et al., 1990; Wu et al., 1994), also in the mammalian CPG, individual neurons can be switched among functional circuits via either endogenous and/or exogenous interventions.

Neuromodulatory control can also act on the intrinsic properties of spinal MNs, by influencing a palette of ionic currents that are expressed by each neuron and, thus, shaping network activity. For example, the transient potassium current I_A modulates the output from the pyloric network of the crustacean STG. When this current is reduced by 4-aminopyridine, the cycle frequency increases, neuronal bursts are phase advanced, and the cells fire at higher frequencies (Tierney and Harris-Warrick, 1992).

Moreover, in both vertebrate and invertebrate preparations, modulatory inputs can strongly influence the pattern of activity in a neuron, by modifying its ability to produce plateau potentials (Marder and Calabrese, 1996). In fact, many neurons of motor circuits present the common feature of supporting rapid transitions between two relatively stable membrane potentials. When such a neuron is at a hyperpolarized resting potential, a short pulse of depolarizing current will trigger a sustained

depolarization (plateau) that will long outlast the stimulus. Numerous reports have appeared showing clearly that modulatory substances can elicit plateau properties in MNs (Hounsgaard and Kiehn, 1993). In addition, the modulation of plateau properties may influence the specific phase relationships in a rhythmic motor pattern or may alter its frequency (Nagy et al., 1988). Recent work has showed that motor network activity facilitates the persistent inward current responsible for plateau potentials and it increases excitability of coactivated MNs, pointing out to the modulation as an intrinsic component of spinal motor output (Alaburda and Hounsgaard, 2003).

Neuromodulation can also affect the response given by neurons to synaptic inputs.

In fact, the co-release of different transmitters, stored either in the same vesicles or in different ones, could represent an additional way to modulate the final output. For example, from studies on the CPG of the crab stomatogastric nervous system, it has been proposed that some of the differential actions of distinct neurons containing the same neurotransmitter can be attributed to their diverse cotransmitters (Blitz et al., 1999).

Since sensory neurons play crucial roles in initiating and modifying rhythmic movements, the modulatory control of sensory responses will strongly influence movement. At presynaptic level, the excitability of primary afferent fibers can be modulated so that a given stimulus can produce a different strength output.

Recent studies (Kettunen et al., 2005) show that, in the lamprey spinal cord, the activation of postsynaptic mGluR1 can induce the release of endocannabinoids from MNs, that act as retrograde messengers to depress inhibitory synaptic transmission within the locomotor network. This conditional release of endocannabinoids can transform MN and crossing interneurons into modulatory neurons, by enabling them to regulate their inhibitory synaptic inputs and, thus, to contribute to the control of the locomotor burst frequency.

The roles that neuromodulatory inputs could play in the development and maturation of CPG circuits have been studied in numerous preparations.

It has been shown that neuromodulation is required for correct maturation of the networks. In fact, gestational depletion of neurotransmitters determines an alteration in network functions in the adult life (Viemari et al., 2004).

All these findings could be transferred to clarify how the CPG works and they could be used to improve gait rehabilitation in spinal injured patients. In fact, one hope for functional recovery subsequent to lesions is the replacement of the neuromodulatory

substances found in descending modulatory projections with exogenous substances, thus activating the CPG networks below the lesion. On the other hand, exogenous drugs could restore locomotion by switching on some accessory networks spared by trauma and able to replace CPG functions.

Different approaches can be undertaken to modulate motor networks by external intervention. First of all, it should be possible to facilitate the operation of the CPG by increasing the total excitability of spinal cord cells, for instance by increasing the extracellular potassium concentration in the tissue (Bracci et al., 1998). In fact, even if many patterns are induced, once the locomotor program starts, it could suppress all other sources of spinal output (Jing and Gillette, 2000).

Second, when working with selective channel blockers, it should be possible to increase responsiveness of strategically localized conductances, which represent crucial elements for generating and sustaining motor pattern.

Finally, one should modulate motor networks by interfering with synaptic pathways involved in controlling locomotion. By taking advantage of the selectivity of receptor mechanisms, it should be possible to facilitate excitatory transmission in an even more restricted region of spinal cord.

Through these different strategies, it might even be feasible to activate alternating motor commands from accessory networks to express the basic features of locomotion.

3.2. Rehabilitative approaches

There is convincing evidence from spinal animals that training for any motor task provides sufficient stimulation to initiate a reorganization of neural networks within the spinal cord and, for example, to generate locomotion (Edgerton et al., 1997; Pearson, 2000). A considerable degree of locomotor recovery in mammals with a SCI can be attributed to the reorganization of spared neural pathways (Curt et al., 2004; Edgerton et al., 2004). In cats, it has been estimated that, if as little as 10% of the descending spinal tracts is spared, some locomotor functions can recover (Basso, 2000; Metz et al., 2000). Even if the loss of supraspinal inputs to the spinal cord is complete, following intense training, the neuronal networks existing below the level of the lesion eventually adapt to generate locomotor activity (De Leon et al., 1998 a, b; Wirz et al., 2001). Moving on from these observations in animals, plasticity to SCI patients can be exploited for rehabilitative purposes using specific training approaches

(Fig. 11). This is achieved by partially unloading (up to 60% of their weight) the patients who are standing on a moving treadmill (Dietz et al., 1994, 1995, 1998; for review, see Dietz, 1997). In severely affected patients, leg movements usually have to be assisted externally, especially during the shift from stance to swing. These training effects lead to a greater weight bearing function of the extensors, so that body unloading during treadmill locomotion can be reduced during the course of training. This indicates that the isolated human spinal cord has the potential not only to generate a locomotor pattern but also to show some plasticity. However, only persons with incomplete paraplegia benefit from the training program as far as they can learn to perform unsupported stepping movements on solid ground (Dietz et al., 1994, 1995). Unfortunately, patients with complete or almost complete paraplegia do not, yet, profit from locomotor training for their mobility: the associated muscle size increase and the training effects on leg muscle activation actually get lost after training is stopped (Wirz et al., 2001).

One strategy that may assist the rehabilitation of patients with SCI is drug therapy aimed at enhancing the activity in CPGs.

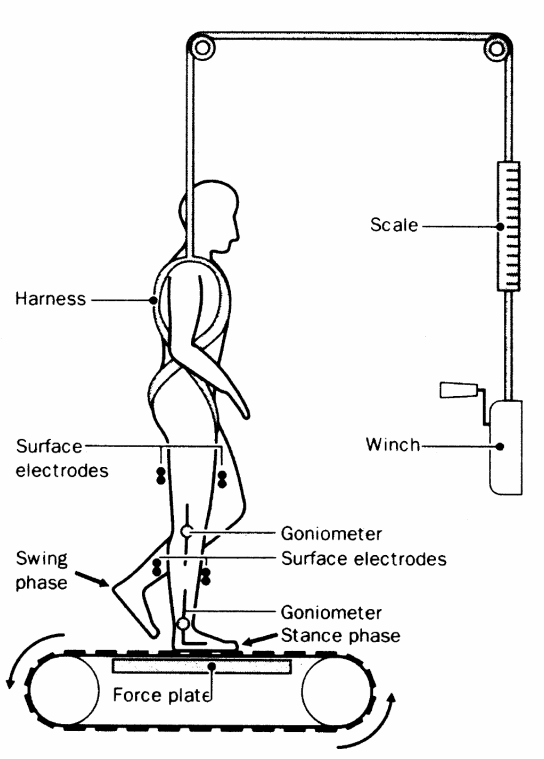


Figure. 11. The experimental set-up as used during treadmill training for humans, (from Van de Crommert et al. *Gait and Posture* 1998).

The most effective drugs augment locomotor abilities either by reducing the hyperactive reflexes that cause spasticity (for example, baclofen, a $GABA_B$ agonist, and cyproheptadine, a $5HT_2$ antagonist) or by directly activating spinal locomotor patterns (for example, clonidine, a noradrenergic (α_1, α_2) agonist; Norman et al., 1998; Rossignol et al., 2001, Barbeau and Norman, 2003). Blockade of K^+ channels with 4-aminopyridine (4-AP) has been reported to restore some sensorimotor functions in patients with sub-total spinal lesions, presumably by increasing action potential conduction along any spared axonal projections (Grijalva et al., 2001; Waxman, 1993).

One further strategy is the electrical stimulation of muscles to mimic the normal pattern of contractions. This technique is called functional electrical stimulation (FES). FES in combination with locomotor training and drug therapy may prove to be the best approach for enhancing functional recovery of these patients in the immediate future.

4. THE ROLE OF METABOTROPIC GLUTAMATE RECEPTORS (mGluRs)

4. mGluRs in the spinal cord

A pharmacological approach to modulation of locomotor network should consider the role of mGluRs. In fact, while in vertebrate spinal cord preparations locomotor CPG rely on interplays between co-activation of AMPA and NMDA receptors activated by glutamate release both from descending fibers such as the reticulospinal system (Dale and Roberts, 1984; Brodin et al., 1985) and from local glutamatergic spinal interneurons (Dale, 1986; Buchanan and Grillner, 1987), mGluRs may also be activated by synaptic release of glutamate.

mGluRs are G-protein coupled receptors that trigger intracellular signaling cascades to modulate neuronal signalling. mGluRs are divided into three groups, depending on sequence homology, transduction mechanisms and pharmacological profiles (Table 1). The group I comprises mGluR1 and mGluR5, while group II consists of mGluR2 and mGluR3, and group III is made up by mGluR4, mGluR6, mGluR7 and mGluR8.

The group I mGluRs increase intracellular Ca^{2+} levels to activate protein kinase C. Both group II and group III mGluRs are coupled to inhibition of adenylyl cyclase. For reviews of mGluR electrophysiology, pharmacology, structure and second messengers

mechanisms see Anwyl (1999), Pin et al. (1999), Schoepp et al. (1999), Cartmell and Schoepp (2000) and Schoepp (2001).

		AGONISTS	ANTAGONISTS	Mechanism of Action
GROUP I	**mGlu1**	DHPG, t-ACPD	CPCCOEt, AIDA, MCPG	**PLC ↑**
	mGlu5		MPEP, MCPG	
GROUP II	**mGlu2,3**	DCG-IV, t-ACPD	EGLU, MCPG	**AC ↓**
GROUP III	**mGlu4,6-8**	L-AP4	CPPG	**AC ↓**

Table 1. Summary of mGluR subclasses and of compounds used for this study

mGluRs have a profound effect on locomotor pattern generation in lamprey, but their role and mechanism of action in vertebrates remain largely unexplored. Given the limited data available on locomotion, and that this work has been performed exclusively on the lamprey, it is straightforward to separate their pharmacological effects into two broad categories. Activation of group II and/or III receptors inhibits glutamatergic synaptic transmission and fictive locomotion (Krieger et al., 1994, 1996; Cochilla and Alford, 1998). Conversely, activation of group I receptors facilitates locomotion both pre- and postsynaptically by release of Ca^{2+} from internal stores and augmenting glutamate release during locomotion (Cochilla and Alford, 1998; Kettunen et al., 2002; Takahashi and Alford, 2002).

It seems likely that the longlasting changes in neuronal biochemistry induced by mGluRs confer these receptors with the potential to induce sustained alterations in the excitability of CPGs. Hence, the location of mGluRs in the spinal cord becomes a strategic issue.

Group I mGluRs in the spinal cord represent the largest group in terms of expression. The majority of mGluR immunoreactivity is found on the perisynaptic and extrasynaptic plasma membrane of neurons, and partly on glia.

mGluR5s are densely expressed in laminae I-III of the dorsal horn with gradual decrease in deeper laminae. About half of vesicle-containing profiles stained for mGluR5 are also positively stained for GABA (Jia et al. 1999). mGluR1s are mostly distributed throughout laminae III-X (Berthele et al. 1999; Alvarez et al. 2000) with patchy immunoreactivity of varying intensity in somata and dendrites of spinal motor

nuclei including MNs and interneurons of the ventral horn and in presynaptic axon terminals (Alvarez et al. 2000). Group II and III are scattered throughout the spinal cord with predominance in the dorsal horn (Berthele et al. 1999) and even some glial labeling (Ohishi et al. 1995; Jia et al. 1999; Azkue et al. 2000, Azkue et al. 2001). In addition, group II mGluR3 mRNA is expressed in the small cells surrounding MNs, while group III mGluR4 mRNA is strongly present in the spinal MNs (Berthele et al. 1999).

Similar results have been obtained by Aronica and his colleagues working on human spinal cord (Aronica et al., 2001). Immunocytochemical analysis has demonstrated an expression of mGluR1, mGluR5 and mGluR2/3 throughout the human spinal cord. mGluR1 has the highest level of expression in ventral horn neurons (laminae VIII and IX), whereas intense mGluR5 immunoreactivity is observed dorsally in the superficial laminae I and II. The neuronal cell expression of group-II mGluRs (mGluR2/3) is low and mainly concentrated in the inner part of the lamina II. In another study, (Tomiyama et al., 2001) mRNA coding for II group of mGluRs has been shown to be completely absent in human lumbar spinal neurons.

Physiologically, laminae I and II of the spinal cord contain primary afferent inputs coming from small myelinated and unmyelinated fibers that bring information from nociceptors and thermoreceptors. The deeper laminae (III–V) receive inputs from laminae I and II and from larger myelinated fibers which mediate information deriving from muscle, skeletal, cutaneous, and subcutaneous mechanoreceptors. The differential mGluRs localization in the dorsal horn proposes that different groups of mGluRs carry different sensory modalities.

4.2. Neuroprotective effects of mGluRs in the central nervous system

The involvement of these receptors in excitotoxicity is ambiguous, since both toxic and neuroprotective effects have been reported. A role for mGluRs in neurodegeneration arises from the fact that injections of the agonist of group-I mGluRs, (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (trans-ACPD), determine neurotoxic consequences in the rat hippocampus and caudate nucleus (McDonald and Schoepp 1992; Sacaan and Schoepp 1992; McDonald et al. 1993) and in experimental models of ischemia (Henrich-Noack and Reymann 1999; Pellegrini-Giampietro et al. 1999). Further proof of the role of group-I mGluRs in neurotoxicity comes from the effect of selective mGluR1 antagonists, which show neuroprotective

effects in *in vitro* and *in vivo* models of excitotoxic degeneration (Bruno et al. 1999; Mills et al. 2000).

However, several authors have indicated a possible survival advantage for those neuronal populations expressing high levels of group-I mGluRs. For instance, Anneser and his colleagues (1999) have processed for *in situ* hybridization the spinal cord of the rat, and found that autonomic MNs express higher levels of mGluR5 than somatic MNs. It is suggested that the presence of mGluRs 5 has an important neuroprotective role responsible for sparing sacral sympathetic neurons in amyotrophic lateral sclerosis (ALS). Similarly, it has been shown that spared motor neurons in ALS have abundant mGluR 5, while vulnerable ones do not express this mGluR subtype (Tomiyama et al., 2001).

Group-II and III mGluRs may prevent neuronal death (Bruno et al. 1994, 1995; Buisson and Choi 1995; Orlando et al. 1995; Buisson et al. 1996). As demonstrated by Bruno et al. (1994), the group-II agonists inhibit the neurotoxicity mediated by kainate and NMDA in cortical neurons. Moreover, activation of group-II mGluRs helps functional recovery following traumatic brain injury (Allen et al. 1999), and is protective against ischemia (Bond et al. 1999). Using a combined approach, Schiefer and his colleagues (Schiefer et al., 2004) have demonstrated that chronic administration of a selective mGluRs 5 antagonist plus an mGluRs II selective agonist, increases survival and attenuates loss of motor coordination ability in a transgenic mouse model of Huntington`s disease (Schiefer et al., 2004). Group-III mGluRs activation attenuates post-traumatic neuronal death *in vitro* (Faden et al. 1997).

4.3. Group I mGluRs and SCI

Many experimental studies have shown how inhibition of group-I mGluRs helps functional recovery following SCI, thus proposing group I mGluRs as therapeutic targets for SCI intervention strategies (Millis et al., 2000, 2002 a). Following contusive SCI, acute actions via different subtypes of group-I mGluRs have different behavioral outcomes on such as locomotor recovery, development of chronic pain and survival of spinal tissue.

mGluR1 antagonism causes an initial improvement in locomotor activity, mitigating the development of mechanical allodynia and promoting grey/white matter neuroprotection. On the other hand, the use of mGluR5 specific antagonism does not

affect locomotor recovery and has a neuroprotective efficacy is restricted to the gray matter only, although it decreases the development of thermal hyperalgesia (Mills et al., 2002 a).

Following SCI, several subtypes of mGluRs are differently expressed (Mills et al., 2001 a). Hence, after injury, even though there is no modification in mGluR5 expression in segments rostral or caudal to injury, there is an increase in mGluR1 expression in I-IV laminae leading to hyperexcitability of dorsal horn nociceptive neurons and the development of mechanical and thermal allodynia (Mills et al., 2002 a).

Gwak and Hulsebosch (2005) indicate that SCI induces a significant increase of mGluR1 and mGluR5 expression in both spinal dorsal horn neurons and astrocytes. It is important to note that group-I mGluRs (mGluR1 and mGluR5) are upregulated faster in astrocytes than in neurons.

Thereafter, a persistent, mGluR-sustained positive feedback loop between astrocytes and neurons appears after SCI which is then followed by neuronal changes.

Individual subtypes of mGluR regulate in different ways the extracellular EAAs concentrations, occurring after SCI. Mills et al. (2001 b) have shown that administration of the mGluR5 specific antagonist MPEP, but not of the mGluR1 specific antagonist LY 367385, determines a marked fall in glutamate and aspartate extracellular concentrations immediately following SCI. These results propose that mGluR5 plays a greater role in augmenting extracellular EAAs concentrations, when compared to mGluR1.

Since the neuroprotective effects of both mGluR1 and mGluR5 and their action on the EAAs release are independent from each other, the observed neuroprotective effects following SCI are not simply due to the decreased release of glutamate but it could arise from the inhibition of some group-I mGluR-mediated intracellular pathways that lead to cell death.

Increasing evidence support the hypothesis that group-I mGluR receptors are also involved in the pathophysiology of traumatic injury to the spinal cord white matter. In fact, in the isolated dorsal white matter, Agrawal et al. (1998) have indicated that pharmacological blockade of group-I mGluR receptors by the selective antagonist AIDA attenuates the conduction impairment following clip compression injury. The selective group-I agonist DHPG exacerbates posttraumatic reduction in action potential conduction.

4.4. Group II and III mGluRs and SCI

There are conflicting results on the mGluR II expression after spinal trauma. This scenario is probably due to the differences in the experimental techniques used to inflict spinal injury (hemisection versus contusion), and its magnitude. Gwak and Hulsebosch (2005) have observed that mGluR2/3 expression is significantly increased in the chronic phase and is restored to control in the chronic phase after hemisection injury. However, following contusive impact to the cord, Millis et al. (2001 a) have reported a significant decrease in mGluR2/3 expression in laminae II, III, IV, and V.

According to the latter authors, since group-II mGluRs function primarily as presynaptic autoreceptors to decrease synaptic transmission, the reduced expression of group-II mGluRs on primary afferents may result in sustained or enhanced neurotransmitter release, thereby prolonging nociceptive inputs to the dorsal horn.

Unfortunately, we lack information on group-III mGluR expression following spinal trauma. However, activation of group-III, but not group-II, mGluRs following SCI reduces extracellular EAAs levels (Millis et al., 2001 b).

One *in vivo* study (Millis et al., 2002 b) has shown that activation of group-II and -III mGluRs following SCI reduces the development of mechanical allodynia and changes in exploratory behavior associated with chronic central pain, without affecting locomotor recovery or onset of thermal hyperalgesia.

4.5. Chronic central pain: a finely tuned interplay between mGluRs.

Interactions between different types of mGluR could sustain neurogenic pain that often represents one of the most disabling consequences of SCI. It seems that by PKC may have a crucial role in interplaying with mGluRs.

In fact, activation of PKC induces phosphorylation of group-II and -III mGluRs, which inhibits their coupling to GTP-binding proteins. The increased expression of mGluR1 may lead to higher levels of PKC and thus to a greater inhibition of group-II and -III mGluRs. Therefore, the reduction in mGluR2/3 expression combined with an increase in mGluR1 expression may exacerbate the hyperexcitability of cells in the pain pathways. The combined effects of an increase in mGluR1 and a decrease in mGluR2/3 could contribute to the development and/or maintenance of dorsal horn neuron hyperexcitability, leading to chronic central pain following SCI (Mills et al., 2002 b).

5. THE ROLE OF K^+ CONDUCTANCES BLOCKERS IN THE SPINAL CORD

5.1. Pharmacological blockade of K^+ conductances: 4-aminopyridine (4-AP)

Another approach to increase the operativity of CPG is to augment excitability of spinal neurons. Since Bracci et al. (1998) have been able to induce fictive locomotion with higher concentrations of extracellular K^+, it seems promising to study the action of potassium blockers on locomotor networks. In particular, it could be interesting to evaluate whether different K^+ channels play a similar role in modulating the CPG or or if there is some selectivity in their action when tested against various K+ conductances. Unfortunately, it is not always possible to assign every K^+ current to a particular class of channels on the basis of its pharmacology (Rudy, 1988).

Classical pharmacological blockers of voltage-gated K^+ channels are 4-aminopyridine (4-AP) and tetraethylammonium (TEA).

4-AP is a relatively non specific blocker of the transient outward K^+ current, termed I_A. This current is fast compared to other K^+ currents and is also quickly inactivated. Steady-state inactivation is complete near resting potential (-50 mV; Takahashi, 1990 a) and the threshold for activation is close to potentials, which are slightly depolarized with respect to resting potential (McLarnon, 1995). In most neurons the time constant of inactivation is around 50 ms (Rudy, 1988).

Thus, I_A operates in the subthreshold region for action potential generation, opening transiently with small depolarizations which start from hyperpolarized potentials.

The hyperpolarization that follows an action potential removes the steady-state inactivation of A current channels and the resultant transient outward current slows down the return of the membrane potential toward action potential threshold. As a result, the interspike interval is prolonged. When this current is reduced by 4-AP, the cycle frequency increases and the cells fire at higher frequencies (Tierney and Harris-Warrick, 1992).

At presynaptic level, 4-AP prolongs the presynaptic action potential, increasing calcium concentration in the nerve terminal and, thus, the transmitter release.

In fact, it has been demonstrated that small doses of 4-AP are capable of enhancing synaptic transmission in the spinal cord (Jankowska et al., 1982) and at the neuromuscular junction (Molgo et al., 1977). For this reason, 4-AP has limited clinical application for restoring neuromuscular transmission following the use of

neuromuscular-blocking agents, or in disorders of neuromuscular transmission such as myasthenia gravis.

Recently, 4AP has been proposed to offer symptomatic treatment to patients affected by chronic SCI and multiple sclerosis. A sustained release form of this substance, Fampridine-SR, is, at the moment, under phase III clinical trial for its therapeutic efficacy on chronic SCI. One suggested mechanism underlying the effect of 4-AP is the increased conduction in demyelinated axons together with the facilitation of synaptic transmission. In fact, I_A potassium current is present in the internodal axon membrane under the myelin. Therefore pharmacological blockade of K^+ channels might enhance conduction in injury-demyelinated axons (Nashmi and Fehlings, 2001).

Controversial experimental data have been published regarding the facilitation of fictive locomotion induced by 4-AP. While Zangger (1981) found that in the presence of 4-AP, reduced doses of DOPA were sufficient to evoke fictive locomotion, Cazalets et al. (1999) showed that addition of 4-AP destroyed fictive locomotion induced by 5HT plus NMDA. Additional experiments are needed to clarify the exact role of 4-AP in modulating motor networks.

5.2. 4-AP evokes rhythmic antidromic discharges from dorsal roots

Perfusing the isolated mammalian spinal cord with low concentrations of 4-AP induces spike activity that can be recorded from dorsal roots (DRs). This activity reflects depolarization of the terminal arborizations of primary afferent fibers, which is then antidromically conducted along the DRs (Al-Zamil et al., 1988).

It is interesting to note that repeated discharges are not exclusive to the spinal cord. In fact, at concentrations of 50-100 µM, 4-AP is a widely used model for epilepsy (Aram et al., 1991; Traub and Jefferys, 1994).

In decerebrated cats, Dubuc and Rossignol (1989 a, b) have recorded synchronous discharges from pheripheral nerves and from DRs following intravenous injection of 4AP.

In order to dissect out the minimal spinal circuitry for 4-AP-dependent rhythmic activity, Dubuc and Rossignol (Dubuc and Rossignol, 1989 a, b) have used a reduced preparation consisting of two spinal cord segments only. In this condition the rhythm persists, indicating that the recorded activity may result from the activation of multiple, segmentally-coupled oscillators.

In *in vitro* preparations, application of 4-AP generates rhythmic activity from dorsal horns even in small segments of the spinal cord. Ruscheweyh and Sandkuhler (2003), using transverse slices with an attached DR, have found that 4-AP, in a dose dependent manner, evokes rhythmic activity in nociceptive superficial dorsal horn neurons. Discharges are not prevented by cutting off the ventral horns and the controlateral side of the slice, showing that activity seems to have a focus in the medial superficial dorsal horns, but it then spreads to all regions of the dorsal horn.

Since epileptiform activity of nociceptive spinal dorsal horn neurons may contribute to paroxysmal forms of neuropathic pain, Ruscheweyh and Sandkuhler (2003) speculate that 4-AP induced discharges represent a model to investigate the mechanisms underlying the central pathophysiology of hyperalgesia and allodynia. In fact, such an activity is prevented by anticonvulsants like phenytoin, carbamazepine and valproate clinically used to relieve pain attacks. In contrast to the anticonvulsants, neither μ-opioid agonists nor α_2–adrenergic agonists block the spinal dorsal horn activity induced by 4-AP.

More recently, Asghar et al. (2005) have demonstrated that high extracellular potassium induces field oscillations in the neonatal rat substantia gelatinosa neurons of the lumbar spinal cord in vitro. The pharmalogical profile of such discharges is similar to that of epileptiform activity induced in the same preparation by 4-AP, since both activities need intact non-NMDA excitatory transmission and functional inhibitory neurons. Since in this study there is a preservation of potassium-evoked rhythmic activity in dorsal horn quadrants, Asghar et al. (2005) have suggested the dorsal horn contains the basic circuitry for rhythmogenesis. In summary, the exact mechanisms by which 4-AP induces rhythmic discharges from DRs is not yet clear. The action could be exerted on interneurons, which in turn activate primary afferent terminals, or directly at the level of primary afferents terminals, or on their impulse conduction. Dorsal root potentials (DRPs) similar to the ones antidromically recorded from a primary afferent fiber following application of 4-AP can also be seen spontaneously on lumbar DRs (Ryan et al., 1984; Kerkut and Bagust 1995). DRPs can occur in the isolated cord in the absence of supraspinal structures and afferent inputs, suggesting that this spontaneous activity is generated within the spinal cord. This activity is blocked by high extracellular Mg^{2+} demonstrating that it is mediated by synaptic processes (Ryan et al., 1984; Kerkut and Bagust 1995).

In the isolated rat spinal cord preparation, rhythmic spontaneous potentials, simultaneously occurring in homolateral DRs, are completely abolished by picrotoxin or low concentration of bicuculline, demonstrating that they are supported by GABAergic mechanisms (Fellippa-Marques et al., 2000).

5.3. Synaptic mechanisms of primary afferent depolarization

DR discharges can be also evoked by electrically stimulating a neighbour DR. Such an antidromic activity is triggered in primary afferent fibers by the depolarization that occurs following the arrival of peripheral nerve volleys in the spinal cord (Rudomin and Schmidt, 1999; Vinay et al., 1999; Willis, 1999).

The mechanism proposed for electrically induced primary afferents depolarization (Fig.12) is useful to explain how antidromic activity can be generated.

A primary afferent fiber (pink trace on the left) coming from a dorsal root ganglion (DRG), before making synaptic contact with a MN, receives inputs at presynaptic level from spinal interneurons. Although a single GABAergic interneuron is represented, several populations of GABAergic interneurons may be responsible for presynaptic inhibition. By electrically stimulating the primary afferent of a neighbour DR, a glutamatergic interneuron becomes activated, which will in turn increase transmitter release from GABAergic interneuron to the MN.

The resulting depolarization of the intraspinal afferent terminals, which is due, at least partly, to the activation of $GABA_A$ receptors, may be large enough to reach threshold and evoke action potentials that can be antidromically recorded.

The bottom-right insert of the Fig. 12, depicts the cellular mechanisms by which GABA application can depolarise primary afferent fibers. In DRGs, just like their synaptic terminals, GABA and chloride equilibrium potentials are at a more depolarised level than the resting potential. Therefore, when chloride channels are opened by GABA, efflux of chloride depolarizes the membrane.

Anatomical support for the neuronal pathways shown in Fig. 12 has been provided by the work of Conradi (1969), who has described the microanatomy of the large Ia boutons, which synapse onto motoneuronal somata and proximal dendrites.

Maxwell et al. (1995) have used gold immunostaining of GABA, glutamate and glycine terminals to show that the presynaptic boutons contacting the afferent fibers are immunoreactive to GABA, thus presumably mediating presynaptic inhibition.

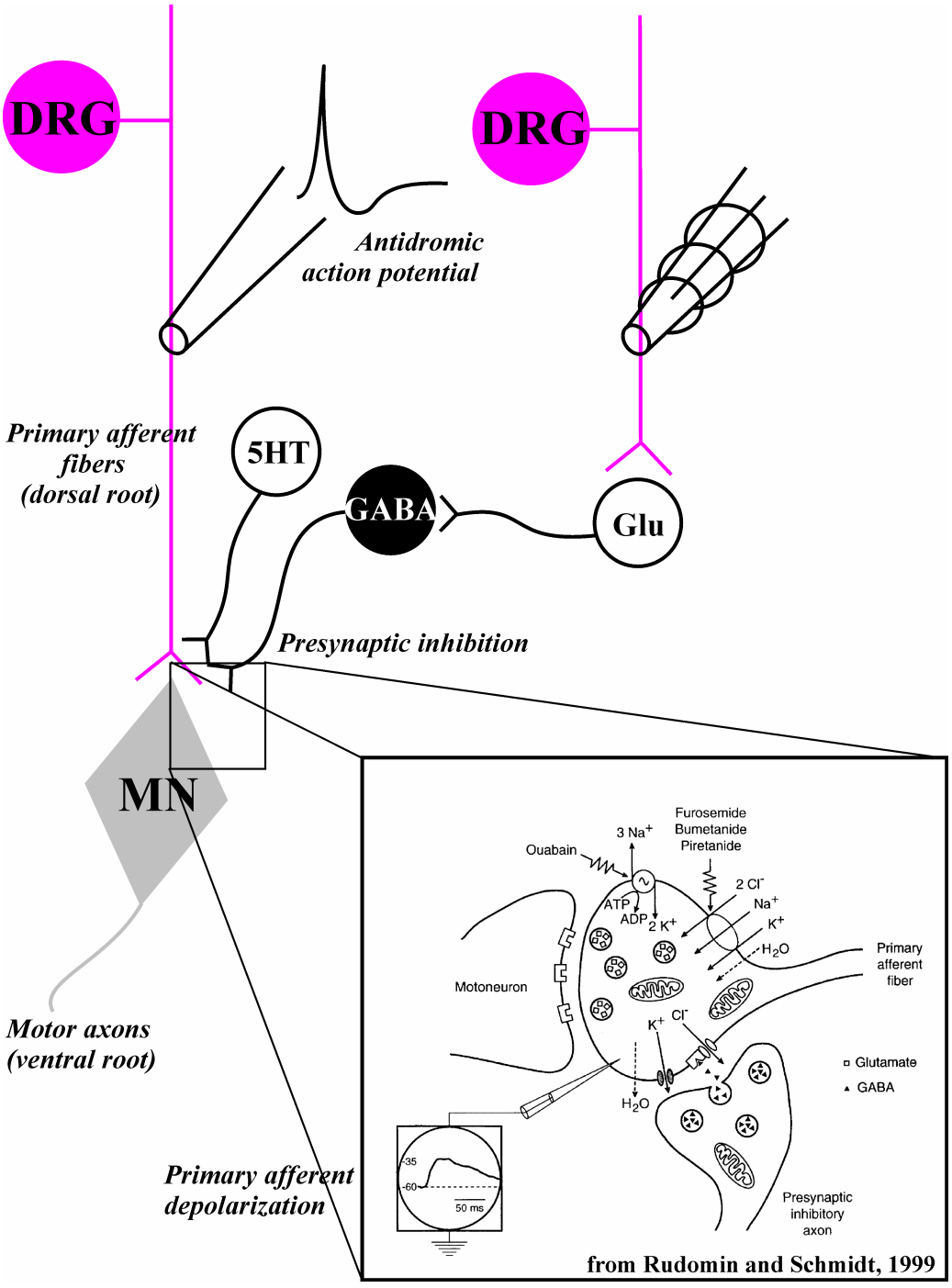


Figure 12. Neuronal pathways mediating presynaptic inhibition and primary afferent depolarization (see text). The insert (from Rudomin and Schmidt, *Exp Brain Res*, 1999) depicts a schematic representation of a presynaptic inhibitory axon establishing synaptic contacts with a primary afferent terminal, which in turn makes synaptic contacts with a motoneuronal dendrite. A microelectrode inserted in the terminal of the primary afferent records the depolarization produced by GABA. This depolarization is due to Cl^- efflux. The depolarization activates K^+ channels with consequent K^+ efflux. The outwardly directed Cl^- gradient is reestablished by the operation of the Na^+ -K^+ -$2Cl^-$ co-transporter. Osmotic water fluxes are indicated with dashed arrows.

5.4. Modulation of CPG by other voltage dependent K^+ channel blockers: TEA

In order to ascertain the selectivity of the pharmacological blockade of K^+ conductances, it could be interesting to test if other K^+ blockers can regulate CPG operativity. In this sense, it seems noteworthy to investigate the role of TEA in inducing rhythmicity from lumbar spinal cord and to find out if TEA can modulate locomotor patterns. Traditionally, TEA has been indicated as a blocking agent for delayed rectifier channels and is thought to be much less effective in blocking I_A current (Table 2).

Pharmacological properties (IC_{50}) of TEA or 4-AP [mM]	**Subunit of voltage-gated K^+ channels**	**Biophysical features**
≈ 0.5	**K_v 1.1**	Delayed rectifier
1.7/7	**K_v 1.6**	Delayed rectifier
≈ 8	**K_v 2.2**	Delayed rectifier
0.02/0.6 0.15/0.2	**K_v 3.1**	Delayed rectifier and A-current (I_A)
≈ 0.15	**K_v 3.2**	Delayed rectifier
≈ 0.14	**K_v 3.3**	A-current (I_A)
0.09/0.3	**K_v 3.4**	A-current (I_A)
≈ 1	**KCNQ 2**	Slow delayed rectifier

Table 2. Functional properties of voltage-activated K^+ channels blocked by TEA (blue) and 4-AP (red) in the range of concentrations used in our experiments (from Coetzee et al., *Ann NY Acad Sci*, 1999).

The delayed rectifier is a slowly inactivated voltage-dependent K^+ current responsible for the repolarization of the action potential (Rudy, 1988). TEA application results in an evident prolongation of action potential repolarization (McLarnon, 1995).

However, at the level of spinal fibers, TEA shows no significant difference on injured and noninjured axons (Nashmi and Fehlings, 2001). In fact, since TEA sensitive K^+ channels are clustered with a nodal distribution (Eng et al., 1988), injured and noninjured axons produce similar responses to these blockers.

A previous study of the effects of TEA on locomotor networks has shown that TEA *per se* cannot induce alternating locomotor-like rhythms (Cazalets et al., 1999). However, the fictive locomotion elicited by 5-HT and NMDA is accelerated by adding TEA (Cazalets et al., 1999). Unfortunately, since only low concentrations of TEA (<4 mM) were used, we cannot rule out that augmenting the concentration of TEA to completely block the delayed rectifier (Coetzee et al., 1999; table 2) is sufficient to start fictive locomotion.

Several neurotransmitters can modify neuronal excitability by modulating the function of delayed rectifiers. This channel is an integral membrane protein that can be modulated via second messengers activated as a result of neurotransmitter-receptor interaction (Rudy, 1988). In different experimental protocols, comprising elevation of cAMP by treatment with the adenylate cyclase activator forskolin (Strong and Kaczmarek, 1986), bath perfusion with ATP or its analogues (Brown and Dale, 2002), or application of dopamine (Gruhn et al., 2005), it has been shown that neurotransmitters can alter motor network by modifying the delayed rectifier conductance.

We have tried applying Forskolin (Fig. 13) and the agonist of subtypes D_1 dopamine receptors, SKF 81297 (Fig. 14). In both cases we recorded an alternating rhythm with rhythm frequency and periodic occurrence of deletions similar to the ones seen with TEA.

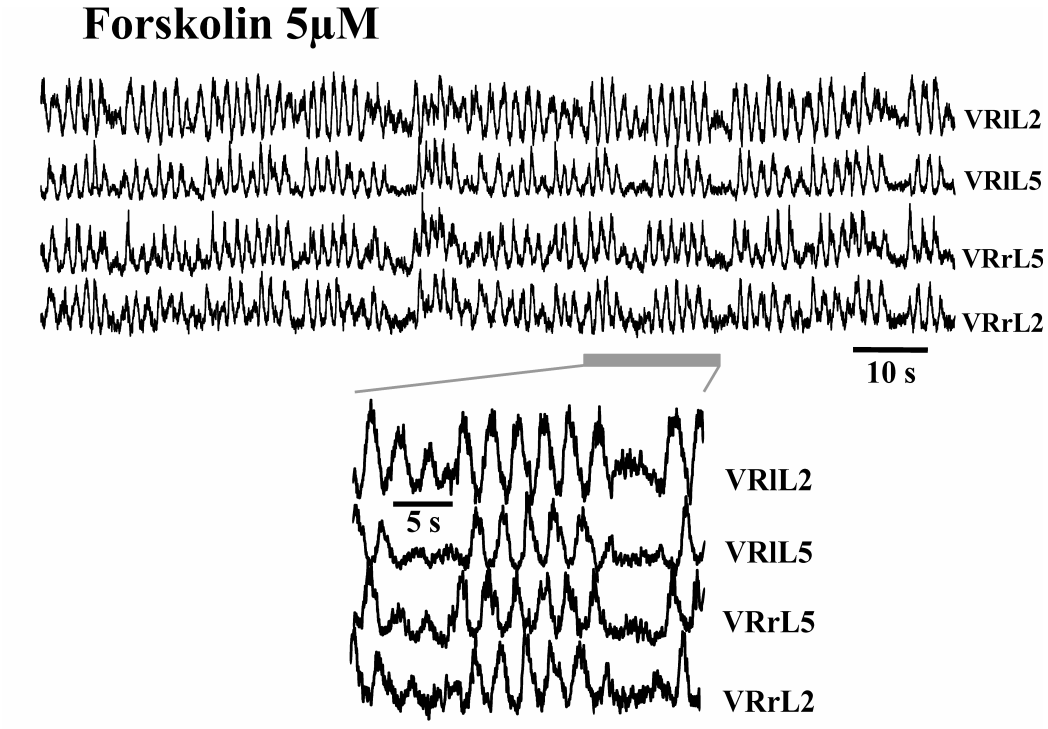


Figure 13. Bath-perfusion with the adenylate cyclase activator forskolin induced cycles of fictive locomotion (G. Taccola and K. Ballanyi, unpublished data).

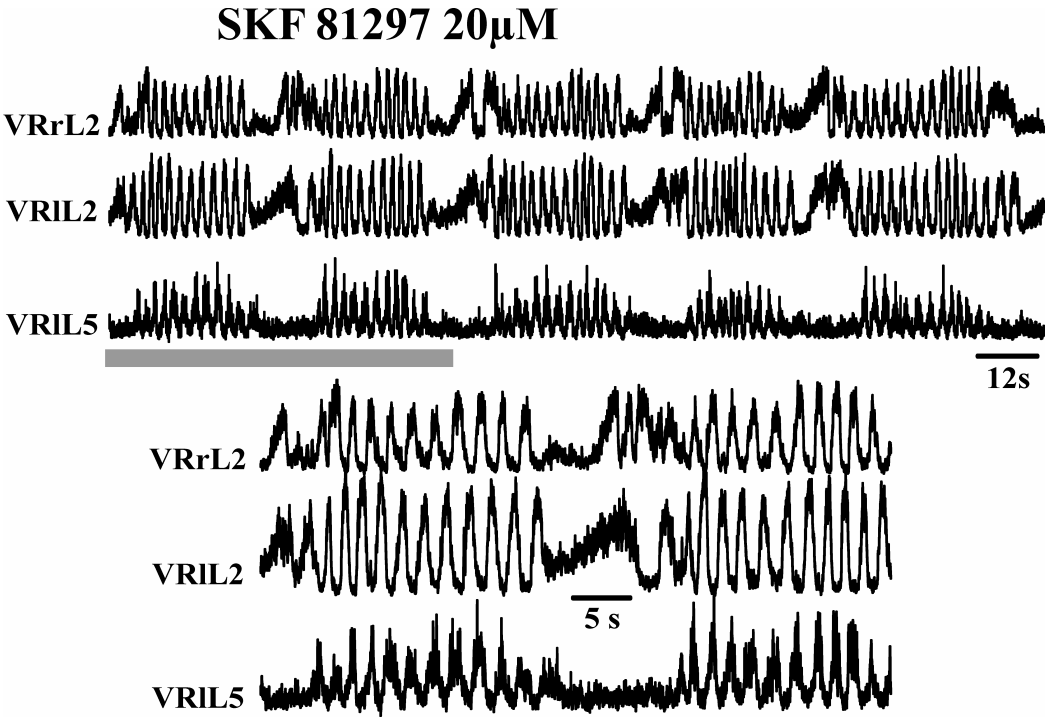


Figure 14. The D1 agonist SKF 81297 elicited cycles of locomotor-like pattern discharges (G. Taccola and K. Ballanyi, unpublished data).

6. AIMS OF THE PRESENT STUDY

Several issues concerning the mechanisms of activation and modulation of the CPG are yet to be explored. A better knowledge of the basic elements regulating locomotor networks should allow us to devise potentially therapeutic strategies to obtain recovery of function in SCI people.

The aim of this thesis is to give an answer to the following questions:

1. What is the functional characterization of the mGluRs present in the neonatal spinal cord? Particular attention will be given to:
   - their functional location in spinal networks
   - their role in synaptic transmission
   - their ability to elicit rhythmical activity in relation with other known patterns of fictive locomotion and/or disinhibited bursting
2. What is the role of 4-AP sensitive K^+ conductances in spinal networks? The study will focus on the following topics:
   - effects of 4-AP on synaptic transmission and action potential
   - rhythmogenic properties of 4-AP
   - spinal topography of the 4-AP induced oscillations
   - identification of the minimal neuronal structure responsible for generation of oscillations
   - the contribution by 4-AP sensitive K^+ conductances to Fictive locomotion (FL)
   - interaction of oscillations induced by 4-AP with fictive locomotion.
3. Which are the electrophysiological effects of TEA on the neonatal spinal cord? We will consider such points:
   - action on synaptic and firing properties of MNs
   - rhythmic activity induced by TEA
   - spinal topography of TEA-dependent oscillators
   - discharge patterns elicited by co-application of TEA plus 4-AP
   - interaction between TEA-evoked oscillations and fictive locomotion

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FICTIVE LOCOMOTOR PATTERNS GENERATED BY TETRAETHYLAMMONIUM APPLICATION TO THE NEONATAL RAT SPINAL CORD *IN VITRO*

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Abstract—Intrinsic spinal networks generate a locomotor rhythm characterized by alternating electrical discharges from flexor and extensor motor pools. Because this process is preserved in the rat isolated spinal cord, this preparation *in vitro* may be a useful model to explore methods to reactivate locomotor networks damaged by spinal injury. The present electrophysiological investigation examined whether the broad spectrum potassium channel blocker tetraethylammonium could generate locomotor-like patterns. Low (50–500 μM) concentrations of tetraethylammonium induced irregular, synchronous discharges incompatible with locomotion. Higher concentrations (1–10 mM) evoked alternating discharges between flexor and extensor motor pools, plus large depolarization of motoneurons with spike broadening. The alternating discharges were superimposed on slow, shallow waves of synchronous depolarization. Rhythmic alternating patterns were suppressed by blockers of glutamate, $GABA_A$ and glycine receptors, disclosing a background of depolarizing bursts inhibited by antagonism of group I metabotropic glutamate receptors. Furthermore, tetraethylammonium also evoked irregular discharges on dorsal roots. Rhythmic alternating patterns elicited by tetraethylammonium on ventral roots were relatively stereotypic, had limited synergy with fictive locomotion induced by dorsal root stimuli, and were not accelerated by 4-aminopyridine. Horizontal section of the spinal cord preserved irregular ventral root discharges and dorsal root discharges, demonstrating that the action of tetraethylammonium on spinal networks was fundamentally different from that of 4-aminopyridine. These results show that a potassium channel blocker such as tetraethylammonium could activate fictive locomotion in the rat isolated spinal cord, although the pattern quality lacked certain features like frequency modulation and strong synergy with other inputs to locomotor networks. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: motoneuron, burst, central pattern generator, spinal network, 4-aminopyridine.

Recent approaches to neurorehabilitation after spinal cord injury have proposed to exploit accessory spinal networks to perform the generation of locomotor programs damaged by lesions to interneurons (collectively termed central pattern generator, CPG) responsible for initiating and maintaining locomotion (Barbeau and Fung, 2001; Grasso et al., 2004; Parker, 2005). Emerging concepts in the field of spinal plasticity applied to locomotion suggest that propriospinal circuits accessory to the CPG may be activated by pharmacological modulators: hence, appropriate training and coincidence of afferent signals and pharmacological agents can produce a concerted interaction to re-activate, at least in part, the lost locomotor function (Edgerton et al., 2004).

Within this framework, a few studies have been focused on the use of the potassium channel blocker 4-aminopyridine (4-AP) that has been suggested to improve the outcome of rehabilitation programs (Grijalva et al., 2003). Initially (Hansebout et al., 1993) it was supposed that 4-AP could mainly act by blocking potassium channels of axons unmasked by a demyelinating lesion, thus restoring conduction of action potentials. Recent studies have indicated that low concentrations of 4-AP facilitate the operation of the locomotor CPG, though 4-AP alone cannot activate the locomotor program (Taccola and Nistri, 2004). These observations actually raised the question whether other potassium channel blockers could mimic the action of 4-AP or even improve upon it by direct stimulation of the CPG activity.

Former studies have shown that tetraethylammonium (TEA), a broad spectrum potassium channel blocker, could unmask bursting by spinal motoneurons and interneurons by promoting the activation of various depolarizing conductances and by augmenting excitatory synaptic inputs (Schwindt and Crill, 1980; Takahashi, 1990). These results suggested to us that TEA could be an interesting agent to investigate its ability to induced locomotor-like patterns. A previous report has suggested that low concentrations of TEA can speed up the cyclic activity of the locomotor CPG activated by NMDA and serotonin (5-HT; Cazalets et al., 1999). In the present study we examined how a wide range of TEA concentrations could affect the ability of spinal networks to evoke rhythmic discharges, whether these had the characteristics of fictive locomotion, the contribution of motoneurons to this activity and the locus of its origin within the spinal cord *in vitro*.

EXPERIMENTAL PROCEDURES

All methods have been recently described (Taccola and Nistri, 2004, 2005). In brief, spinal cords were removed from neonatal

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Abbreviations: ANOVA, analysis of variance; APV, D-amino-phosphonovalerate; CCF, correlation coefficient function; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CPCCOEt, 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester; CPG, central pattern generator; CV, coefficient of variation; DR, dorsal root; l, left; NMDA, *N*-methyl-D-aspartate; r, right; S.D., standard deviation; TEA, tetraethylammonium; Th, threshold; TTX, tetrodotoxin; VR, ventral roots; 4-AP, 4-aminopyridine; 5-HT, serotonin; Φ, mean phase.

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Wistar rats (0–5 days old) under urethane anesthesia (0.2 ml i.p. of a 10% w/v solution) and isolated from mid-thoracic level to cauda equina. The experiments were run in accordance with the National Institutes of Health guidelines and the Italian act Decreto Legislativo 27/1/92 n. 116 (implementing the European Community directives n. 86/609 and 93/88): all efforts were made to reduce the number of animals used and to minimize animal suffering.

Cords were superfused (6.5 ml min^{-1}) with oxygenated Krebs solution of the following composition (in mM): NaCl 113, KCl 4.5, $MgCl_2$ 1, $CaCl_2$ 2, NaH_2PO_4 1, $NaHCO_3$ 25, glucose 11, pH 7.4, at room temperature. Drugs were applied via the Krebs solution. Horizontal lesions to separate dorsal from ventral parts of the spinal cord were performed using a surgical razor blade as indicated by Bracci et al. (1996).

Ventral and dorsal root (VR and DR, respectively) activity was recorded by means of suction electrodes, and stored on digital cassette. Fictive locomotor patterns were obtained from electrical recordings of the activity from left (l) and right (r) L2 VRs (mostly flexor motor-pool commands to the hindlimbs) and l and r L5 VRs (mostly extensor motor-pool commands to the hindlimbs) (Kiehn and Kjaerulff, 1998). A typical indicator of fictive locomotion (see Butt et al., 2002) is the double alternation between homosegmental l and r VRs, and between L2/L5 on the same side.

Single or trains of electrical stimuli (0.1 ms duration) were delivered to one DR via a bipolar miniature suction electrode. Intensity of stimulation was referred in terms of VR response threshold (Th).

For intracellular recordings, antidromically identified lumbar (L4 or L5) motoneurons (Fulton and Walton, 1986) were impaled with 3-M-KCl-filled microelectrodes under current-clamp conditions. The input resistance of motoneurons was obtained by delivering hyperpolarizing current steps (0.05–0.55 nA). Current/voltage plots were linear within the voltage range recorded.

The analysis of rhythms was carried out as indicated by Taccola and Nistri (2004, 2005). The discharge period (T) was measured as the time between the beginning of two subsequent oscillations (calculated as average of 20 responses). The coefficient of variation of the period (CV=standard deviation (S.D.)×$mean^{-1}$) was used to quantify the regularity of bursting. Signal correlation between pairs of roots was expressed as the correlation coefficient function (CCF) using pCLAMP 9.2 software (Molecular Devices, Union City, CA, USA). While CCF $>+0.5$ indicates synchronicity between two roots, CCF <-0.5 shows alternation. As for the analysis of fictive locomotor-like rhythm induced by 10 mM TEA on VRs, 10 min traces were analyzed by fast Fourier transform that gave two different mean frequencies, corresponding to synchronous and alternated rhythm. All data were expressed as mean±S.D., with "*n*" as the number of experiments. Rayleigh test and circular statistic were used to represent phase coupling between roots (Drew and Doucet, 1991) as previously reported (Taccola and Nistri, 2004). The length of vectors in the polar plots indicates the strength of signal coupling, while the direction shows the concentration of phase values around the mean phase (Marchetti et al., 2001). The Φ value, expressed in angular degrees, corresponds to the time span from the onset of a cycle in one root to the onset of the corresponding cycle on the other root, divided by the period. A $\Phi=180°$ means that the two cycles are completely alternated, while a Φ value of 0° or 360° describes a fully coincident phase (Kjaerulff and Kiehn, 1996).

After performing a normality test to distinguish between parametric or non-parametric data, different statistical approaches

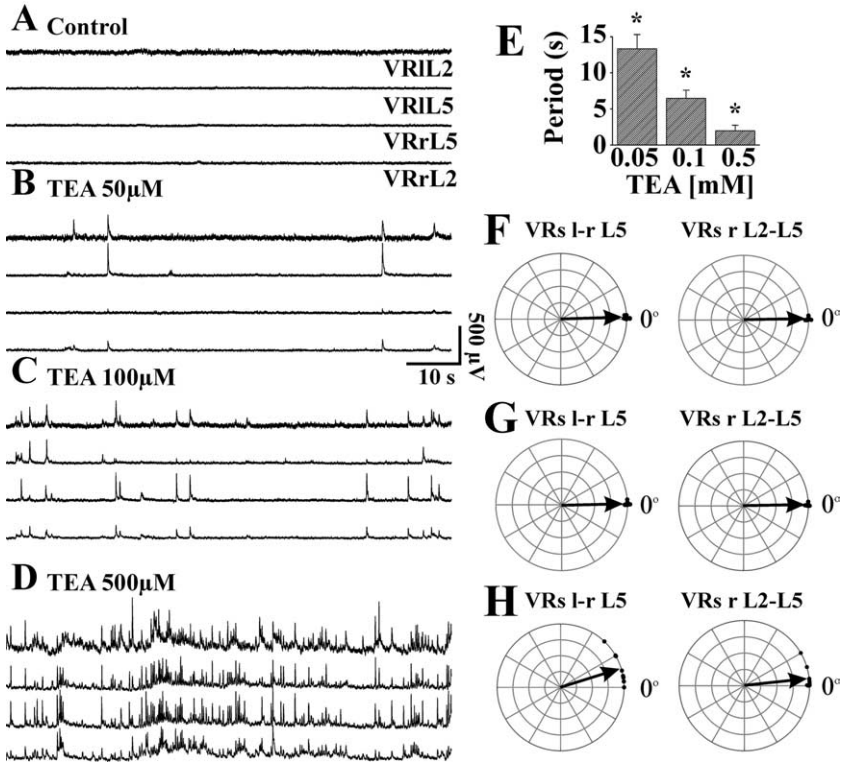

Fig. 1. Synchronous oscillations induced by 50–500 μM TEA. (A) Control records taken from four VRs (identified by their abbreviations at the end of the corresponding trace); note lack of activity under resting conditions. (B) Synchronous discharges of short duration and irregular period appear on all VRs. (C) TEA at 100 μM increases synchronous discharges. (D) Intense oscillatory activity observed in the presence of 500 μM TEA. A–D are from the same preparation. Scale bars apply to A–D records. (E) Histograms indicating period values for three concentrations of TEA, $n=6$. Asterisks=$P<0.05$. (F–H) Polar plots of VR oscillations shown in B, C and D. Clustering of data points around 0° to indicate synchronicity. Length of signal vector indicates strength of phase coupling between VRs.

were carried out to compare sets of data. For parametric values, we used the Student's *t*-test (paired or unpaired) for comparison between two groups of data, and ANOVA (analysis of variance) for more than two groups. For non-parametric values, the tests used were Mann-Whitney one if groups to be compared were two, and ANOVA on ranks, followed by a post hoc test (Tukey test) when groups were >2. Results were considered significant when $P<0.05$.

Tocris (Bristol, UK) was the commercial source of the following chemicals: 4-AP, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), D-amino-phosphonovalerate (APV), *N*-methyl-D-aspartate (NMDA), tetrodotoxin (TTX), 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt). Sigma (Milan, Italy) was the commercial source of the following drugs: TEA, 5-HT, carbenoxolone, bicuculline and strychnine.

RESULTS

The present study is based on 67 spinal cord preparations, from which root responses were systematically recorded. Furthermore, we also obtained data from 24 intracellularly-recorded motoneurons (-76 ± 5 mV initial resting potential, 48 ± 24 MΩ input resistance). These values are typical of spinal motoneurons recorded with sharp microelectrodes (Fulton and Walton, 1986) and accord with those measured in our recent investigations (Marchetti et al., 2003; Taccola and Nistri, 2004; Taccola et al., 2004).

Low concentrations of TEA induced synchronous rhythms in spinal networks

Fig. 1A–D shows that TEA (50–500 μM) induced electrical oscillations recorded from four lumbar VRs (L2, L5) of the same preparation. Concentrations lower than 50 μM were ineffective. The polar plots of Fig. 1F–H indicate that these oscillations were synchronous between pairs of VRs because data points for homosegmental and homolateral discharges were clustered around similar Φ values (near 0°, thus indicating coincidence of activity). Strong coupling between these roots is demonstrated by the length of the vectors (arrows in Fig. 1F–H). Inspection of raw data in Fig. 1B–D, however, suggested that these oscillations were very irregular, a fact corroborated by the large value of their period CV (0.82 ± 0.44 for 50, 0.80 ± 0.27 for 100, and 0.60 ± 0.21 for 500 μM TEA, respectively; $n=6$). Analysis of cross-correlation function for a pool of preparations in the presence of 100 μM TEA further supported oscillation synchronicity: in fact, the CCF was 0.70 ± 0.11 ($n=6$; homolateral L2–L5 VRs) and 0.72 ± 0.02 ($n=4$; homosegmental L2–L5 VRs). The oscillation periodicity was also dependent on the TEA concentrations as shown in the histograms of Fig. 1E.

High concentrations of TEA evoked alternating patterns of VR discharges

The effect of TEA on spinal networks changed dramatically with larger concentrations. In fact, once concentrations equal or above 1 mM were used, cycles of alternating VR patterns emerged, an effect most reliably observed with 10 mM and lost with 20 mM TEA when irregular firing replaced any rhythmic activity. One example is provided by the records of Fig. 2A, in which, following application of

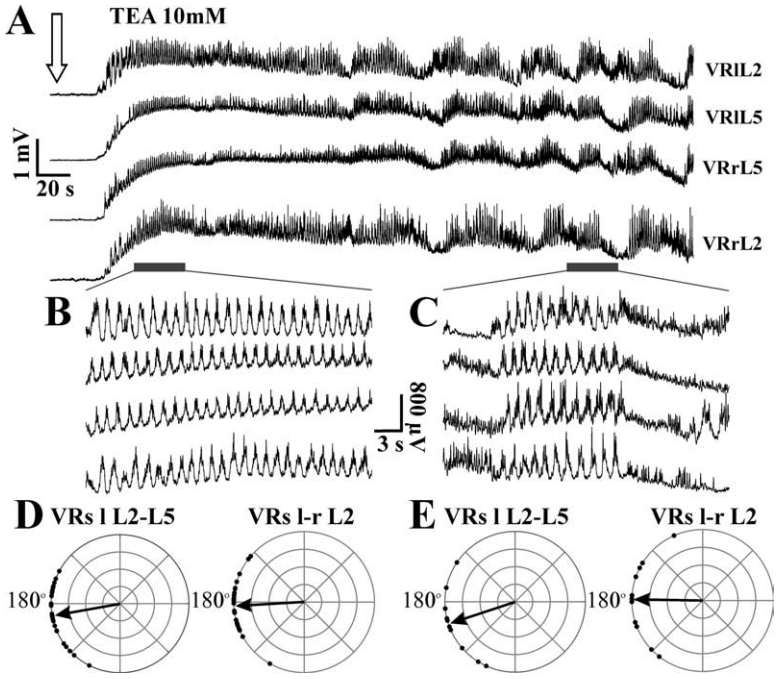

Fig. 2. TEA (10 mM) evokes rhythmic alternating oscillations on VRs. (A) Application of TEA (vertical open arrow) depolarizes VRs (upward shift of baseline) and generates fast oscillations. During continuous application of TEA, the VR depolarization declines and there is appearance of slow waves with superimposed cycles of fast, alternating oscillations. Scale bars apply to all four VRs. (B) Expanded traces of fast oscillations recorded during the time corresponding to early phase of TEA application (horizontal gray bar in A). (C) Expanded traces of fast oscillations during late phase of TEA application (below gray bar on the right). Scale bars apply to B and C. (D, E) Polar plots of VR oscillations displaying alternation as data are clustered around 180°. For further details see legend to Fig. 1. All data are from the same preparation.

10 mM TEA (open vertical arrow), there was a baseline shift indicative of VR depolarization (1.3 mV on average for the four VRs) with superimposed alternating oscillations (see Fig. 2B, C for expanded time base traces corresponding to records indicated by the gray bars; 1.3±0.3 s period). The polar plot of Fig. 2D shows that for l L2–l L5 (left panel) and l, r L2 (right panel), the Φ data were clustered around 180° (190±24° and 183±22°, 24 cycles). Similar values (198±28° and 178±34°, respectively; Fig. 2E) were obtained for same root pairs at later times of TEA application, even though the VR depolarization evoked by TEA slowly waned. At this stage, VR oscillations typically emerged in association with slow waves of depolarization.

On a random sample of eight preparations bathed with 10 mM TEA solution, the early oscillations alternating between homolateral and homosegmental VRs had 1.6±0.3 s period with 0.37±0.10 CV. The VR depolarization recorded by means of suction electrodes was 1.2±0.5 mV (n=8), while using an intracellular microelectrode the depolarization of single motoneurons was 39±8 mV (n=5).

Spinal cord preparations did not fully repolarize to baseline during sustained application of TEA: the average residual depolarization was 0.7±0.1 mV (n=8). Rhythmicity was stably expressed with alternating events at the top of slow waves (42±12 s, CV=0.34±0.10, average duration=59±28 s; n=8) which were synchronous among all VRs (CCF=0.74±0.14). For each waveform there were 17±3 alternated cycles (mean period=1.6±0.4 s, CV=0.33±0.07). Alternation of oscillations recorded from VRs was also confirmed with cross-correlation analysis applied to the first 10 min of each trace (n=8) which yielded an average CCF value of −0.60±0.11.

Washing out 10 mM TEA after a standard 25 min application was followed by a gradual disappearance of slow waves and fast oscillations, with return to baseline activity after about 1 h.

With lower concentrations of TEA (1–5 mM), the period of alternated events (1.7±0.2 and 1.6±0.1 s for 1 and 5 mM respectively; n=4) at the top of each slow wave was not different from the one observed with 10 mM TEA. Nevertheless, the time spent for alternating activity (as percent of the whole recording) was significantly increased from 33±4%, (1 mM TEA), to 54±9% (5 mM TEA) and 88±4% (10 mM TEA; P<0.05; n=3).

Pharmacology of TEA evoked rhythmic discharges

The Na^+ channel blocker TTX (1 μM) completely abolished (within 6±1 min) all TEA-induced rhythmicity recorded from VRs and single motoneurons (n=5).

Application of 10 mM of TEA, after 50 min exposure to the gap junction blocker carbenoxolone (100 μM), induced VR depolarization (1.4±0.4 mV) not different from the one shown in the presence of 10 mM TEA alone, and left alternating oscillations unchanged (CCF =−0.66±0.12; n=4). Carbenoxolone (100 μM) per se did not change motoneuron membrane potential (0.3±0.6 mV, n=4), input resistance (110±18%, n=3) or the peak amplitude of the DR-evoked reflexes (107±39%, n=4). All parameters of the motoneuron antidromic spike (peak 100±5%, area 95±9%, rise time 115±8%, decay time 106±32%, half-width 114±8%) were unaffected by 100 μM carbenoxolone.

The ionotropic glutamate receptor antagonists CNQX (10 μM) and APV (50 μM) blocked the alternating oscillations induced by 10 mM TEA as exemplified in Fig. 3A–B, leaving, however, the slow wave activity which became shallow and asynchronous (period=39±10 s; CV=0.47±0.13; duration=16±4 s; CCF=0.25±0.28; n=8). When TEA was applied in the presence of CNQX and APV, it, however, retained its ability to depolarize motoneurons (14.5±5.9 mV, n=6). Further ap-

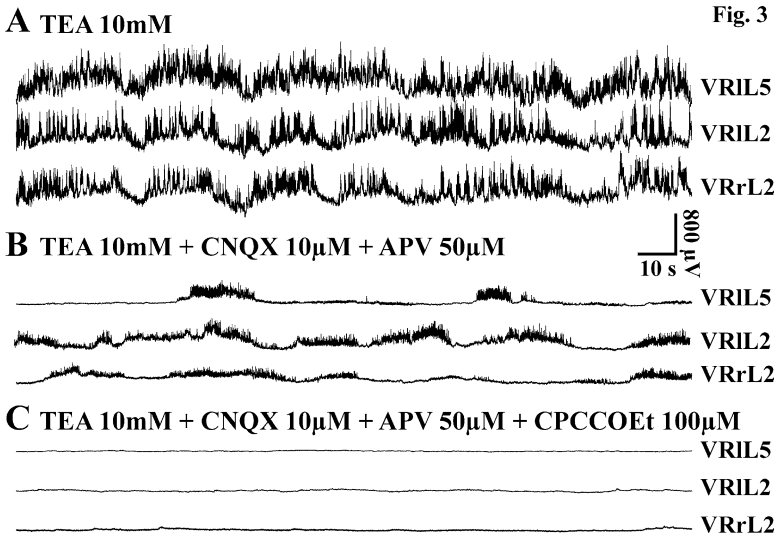

Fig. 3. Pharmacology of rhythmic patterns evoked by 10 mM TEA. (A) VR responses comprising slow waves with superimposed fast, alternating oscillations recorded from three VRs as indicated alongside the records. (B) On the same preparation application of CNQX and APV eliminates fast alternating oscillations, leaving shallow slow waves of very irregular nature with superimposed spikes. (C) Further application of the group I mGluR antagonist CPCCOEt abolishes spontaneous electrical discharges. Scale bars apply to all traces.

plication of the glycine antagonist strychnine (1 μM) and the $GABA_A$ antagonist bicuculline (20 μM) failed to suppress the un-coordinated slow wave activity (45±17 s, CV=0.38±0.07, n=4) and strongly enhanced event amplitude (325±39% with respect to those in CNQX plus APV).

It was possible to suppress fully the slow wave activity by applying the group I mGluR antagonist CPCCOEt (100 μM; Schoepp et al., 1999) as shown in Fig. 3C. This observation was consistently obtained with 10 preparations after an application time of 344±117 s. It is noteworthy that, when ionotropic receptor blockers were not applied, co-application of 10 mM of TEA and 100 μM CPCCOEt induced VR depolarization (1.8±0.6 mV; n=3) with typical superimposed oscillations alternated between homolateral and homosegmental VRs (period=1.4±0.23 s, CV=0.38±0.01). Thus, the ability of CPCCOEt to suppress slow waves depended on the presence of ionotropic glutamate receptor blockers.

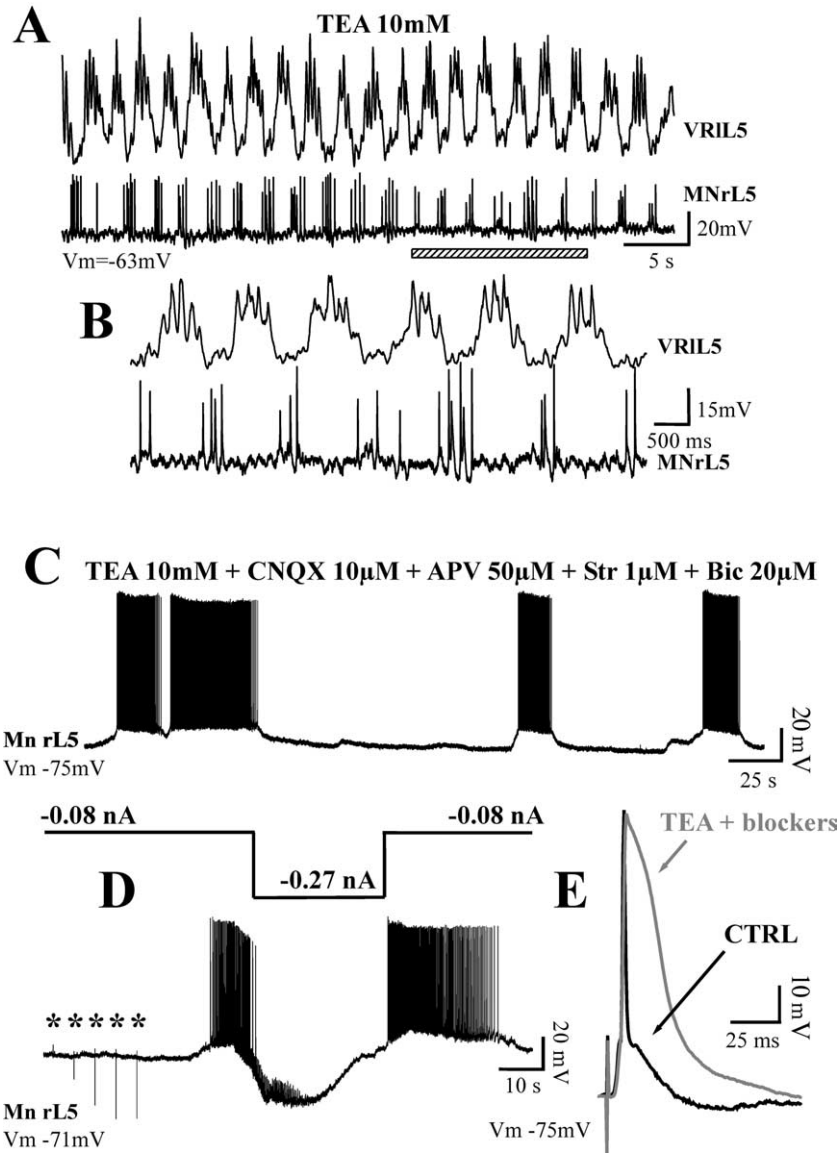

Fig. 4. Effects of 10 mM TEA on VRs and motoneurons. (A) Simultaneous record of lL5 VR oscillations in anti-phase with spike discharge of rL5 motoneuron (MN) during application of 10 mM TEA. MN membrane potential is repolarized to −63 mV membrane potential (Vm) by intracellular current injection (−0.35 nA). For the top trace recorded extracellularly (VR) the vertical bar is equal to 450 μV. (B) Fast time-base records of events indicated by horizontal bar in A. The vertical scale bar=400 μV for the VR record. (C) Bursting of motoneuron in the presence of 10 mM TEA plus the ionotropic receptor blockers CNQX, APV, strychnine (Str) and bicuculline (Bic). The cell was kept at −75 mV by steady intracellular current injection (−0.05 nA) and displayed depolarizing bursts with superimposed spike firing. (D) Strong membrane hyperpolarization (see top trace) suppresses (in a reversible fashion) burst and spike firing. Asterisks show that DR stimulation (see artifacts indicated by downward deflections) fails to generate synaptic responses, confirming that pharmacological blockers shown in C have abolished synaptic inputs to motoneuron. Different motoneuron from the one shown in C. (E) Superimposed antidromic spike in control (CTRL) or 10 mM TEA solution plus ionotropic receptor blockers (gray trace). Records are averages of five spikes.

Propensity of motoneurons to oscillate in the presence of ionotropic blockers

When 10 mM TEA was added to the standard control solution, motoneurons fired in alternation with their contralateral VR as exemplified in Fig. 4A, B for one rL5 motoneuron and lL5 VR. In this example the motoneuron membrane potential was depolarized by 42 mV and was repolarized by injection of hyperpolarizing current. When 10 mM TEA plus CNQX, APV, strychnine and bicuculline were co-applied, motoneurons were depolarized by 16.6±6.2 mV (n=5). Cells were then repolarized by current injection and displayed recurrent, slow depolarizations (as depicted in Fig. 4C) with a superimposed barrage of broad spikes (Fig. 4E). On average, there was no change in the antidromic spike peak (102±15%) and rise-time (111±17%), while the half-width was increased 10-fold (1107±137%; P<0.02; n=4). The input resistance of motoneurons was increased to 119±21% of control (n=6; P<0.02). Injection of hyperpolarizing current during one slow oscillation, suppressed spike activity (Fig. 4D), while electrical stimulation of DR fibers failed to generate synaptic responses (asterisks in Fig. 4D) confirming synaptic transmission block.

Interaction of TEA with fictive locomotor patterns

Fictive locomotor cycles can be elicited by trains of electrical stimuli applied to a single DR (Marchetti et al., 2001) as exemplified in Fig. 5. In this example a series of 35 impulses (2 Hz; 1.5×Th) evoked cumulative depolarization (recorded from three VRs) associated with homosegmental (L2) and homolateral (L2–L5) discharge alternation (see

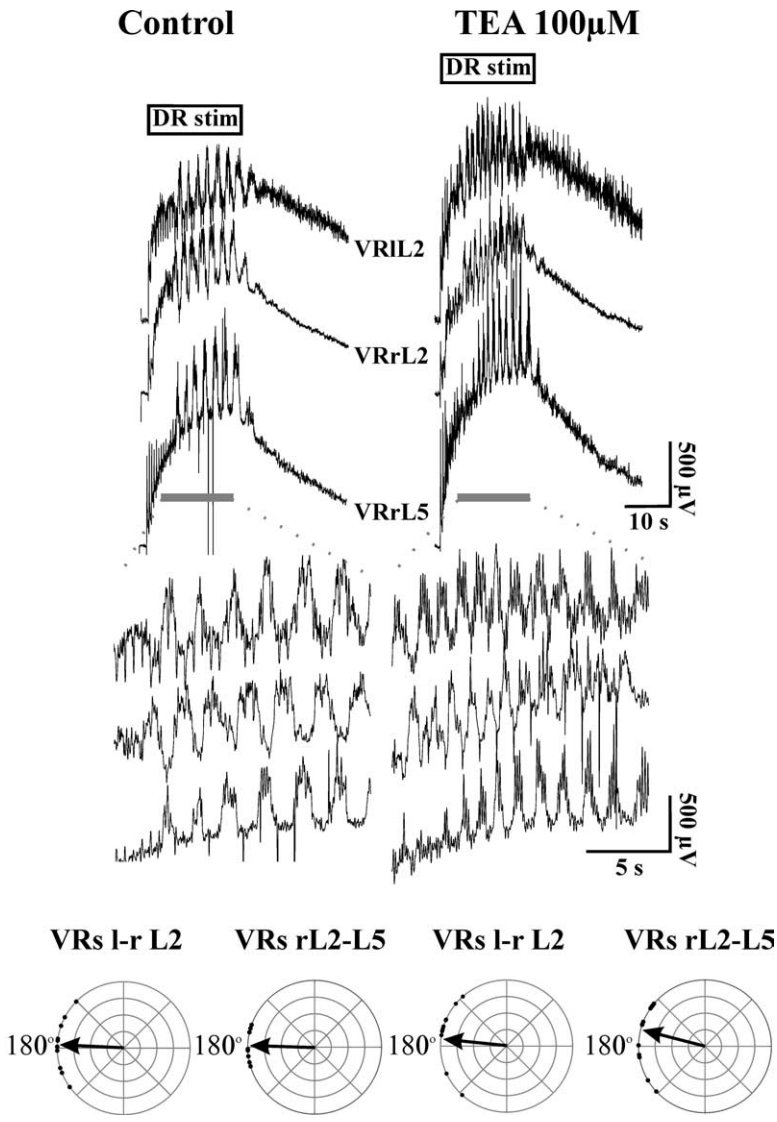

Fig. 5. Oscillations produced by stimulus trains in the presence of 100 μM TEA. In control solution a train of 35 DR stimuli (DR stim; 2 Hz; 1.5×Th) evokes cumulative depolarization with alternating oscillations (see also expanded traces below horizontal gray bar). When the protocol is repeated in the presence of 100 μM TEA the cumulative depolarization peak is larger (25% rise) and associated with a higher number of oscillations with lower (64%) period. The polar plots (bottom) show that VR data for the indicated roots are clustered around 180°, indicating anti-phase of fast oscillations (left hand plots for control, right hand plots for TEA). In each panel scale bars apply to all records.

expanded records taken from traces underlined with horizontal gray bars). When the protocol was repeated in the presence of 100 μM TEA, cumulative depolarization amplitude was increased by 25% and was accompanied by a larger (138%) number of alternating discharges (see expanded records). Stimulus trains either in control solution or in the presence of 100 μM TEA generated alternating VR discharges with anti-phase signals between homosegmental and homolateral VR (see polar plots at the bottom of Fig. 5 with $\Phi = 177° \pm 24$ and $179° \pm 14$, $n = 10$ cycles in control, and $174° \pm 33$ and $165° \pm 31$, $n = 13$ cycles in TEA solution).

Fig. 6 shows pooled data obtained with stimulus trains in the presence of various concentrations of TEA, either below Th for generating alternating patterns (50–500 μM) or above it (1–5 mM). It is noteworthy that the TEA-evoked facilitation of the size of cumulative depolarization (area and amplitude; Fig. 6A, B) was an evanescent phenomenon, because it disappeared when the concentrations of TEA were ≥500 μM, actually turning into inhibition at 5 mM. Likewise, oscillations were more numerous as long as the concentration of TEA was below the one triggering alternating patterns, and suppression of alternating discharges occurred with large doses of TEA (Fig. 6C, D).

When stimulus trains were below Th for generating fictive locomotion, repeating this protocol in the presence of 50–500 μM failed to bring responses to Th ($n = 7$). When the area and the peak amplitude of VR responses evoked by single stimuli applied to one DR were measured in the presence of 100 μM TEA, they were significantly increased (178±47% and 129±29%, respectively, $n = 7$; $P < 0.001$).

Fictive locomotion brought about by standard co-application of NMDA (5 μM) and 5-HT (10 μM) was accelerated by 500 μM TEA. On average, the period of the rhythm was reduced to 67±12% (control was 2.6±0.3 s; $n = 5$; $P < 0.005$), while the discharge amplitude was unchanged (100±9%).

Co-application of 4-AP and TEA

Our previous observations have indicated that 4-AP per se generated synchronous discharges which were replaced by faster fictive locomotion when the locomotor CGP was activated chemically or electrically (Taccola and Nistri, 2004). In the present study we also observed that application of 10 mM TEA in the presence of 4-AP (5 μM; a maximally effective dose to induce synchronous rhythmic discharges) could still evoke VR depolarization accompanied by alternating discharges in place of the synchronous rhythm produced by 4-AP (Fig. 7A). This phenomenon is clearly demonstrated with fast VR records in Fig. 7B, C and was preserved even later during the TEA

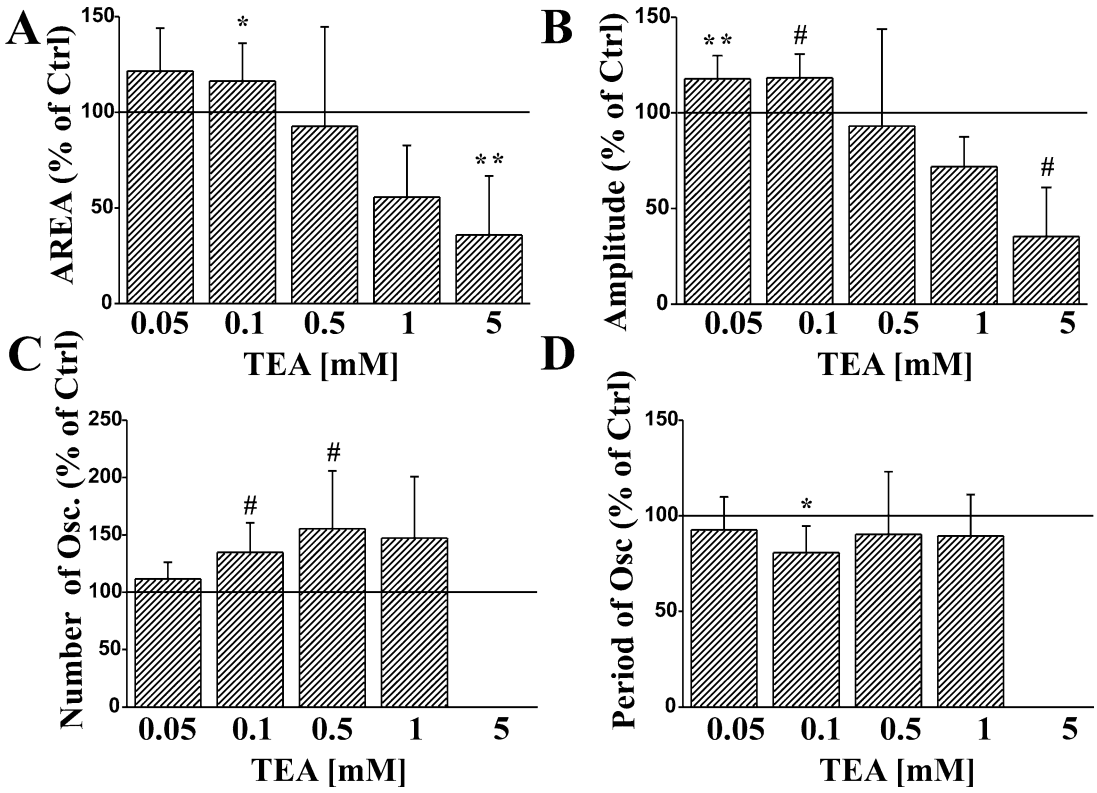

Fig. 6. Summary of effects of TEA on responses evoked by DR trains. Histograms represent mean effects of various concentrations of TEA and responses ($n = 4–9$) have been normalized with respect to control (CRTL). (A) Slight increase in cumulative depolarization area with 100 μM TEA and significant reduction with 5 mM TEA. At 10 mM there is no cumulative depolarization. * $P < 0.05$; ** $P < 0.02$. (B) The peak amplitude of cumulative depolarization is significantly increased with 50 and 100 μM TEA, and decreased at 5 mM TEA. ** $P < 0.02$; # $P \leq 0.005$. (C) Increment in the number of alternating fast oscillations in the presence of 100 and 500 μM TEA. # $P \leq 0.005$. (D) Significant decrease in fast oscillation period with 100 μM TEA. * $P < 0.05$.

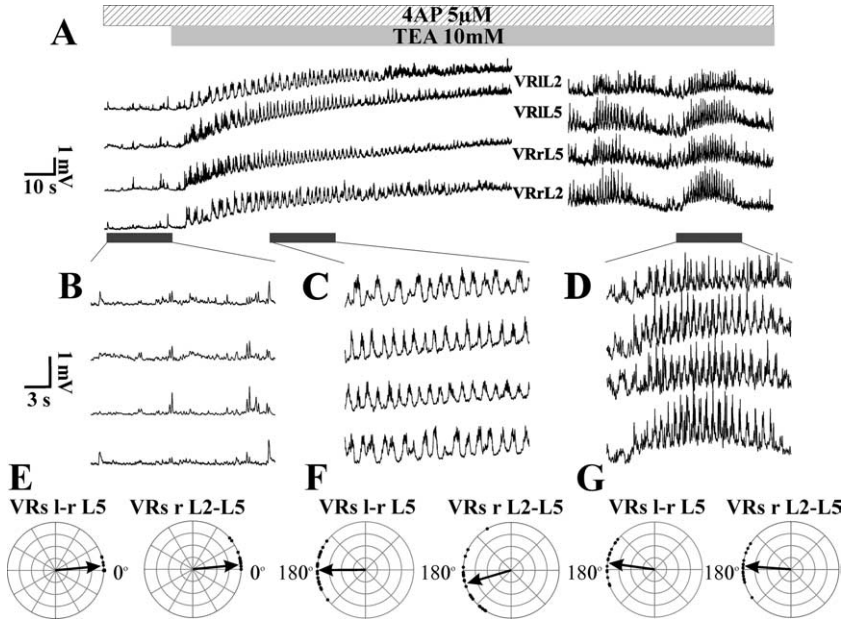

Fig. 7. Rhythmic activity produced by co-application of 4-AP and TEA. (A) 4-AP (5 μM) is applied (hatched bar) before TEA (10 mM) and generates irregular VR oscillations. Subsequent application of TEA (gray bar) induces VR depolarization and replaces irregular oscillations with alternating patterns. Break in traces (approximately 3 min) shows that late slow waves with alternating oscillations emerge. Scale bars apply to all traces. (B–D) Expanded time-base traces of VR activity during the three epochs indicated by horizontal bars in A. Scale bars in B apply to all records. (E–G) Polar plots for activity corresponding to 4-AP alone, plus TEA and late during combined application of TEA and 4-AP. Note that in 4-AP solution data points are around 0° (E), while they are clustered around 180° when TEA is present (F, G). Further details are in the legend to Fig. 1.

application (Fig. 7D). The polar plots of Fig. 7E show that initial synchronicity of the 4-AP discharges (l–r L5 $\Phi=5°\pm6$; r L2-5 $\Phi=14°\pm12$; $n=11$ cycles) was replaced by alternating VR signals early (Fig. 7F; $\Phi=180°\pm19$ and $197°\pm30$, respectively; $n=21$ cycles) and late (Fig. 7G; $\Phi=172°\pm28$ and $176°\pm24$, respectively; $n=19$ cycles) from the start of TEA administration. The mean frequency of alternating oscillations in the presence of 4-AP and TEA was 1.4 ± 0.5 s, while the CCF value was -0.64 ± 0.03 ($n=3$).

Localization of spinal networks responsible for the action of TEA

Although the electrical activity generated by TEA (10 mM) was prominently expressed as VR discharges, DRs also showed irregular electrical events with average period of 4.1 ± 2.3 s (CV$=0.43\pm0.09$; $n=5$), and 5.3 ± 1.0 s duration of single events. To find out the topography of the DR events, horizontal sections of the spinal cord were carried out as exemplified in Fig. 8A (Taccola and Nistri, 2005). In this case, VR bursting evoked by 10 mM TEA persisted although, on average, it was slower (3.3 ± 1.8 s; CV$=0.39\pm0.19$; $n=5$) without alternating VR patterns (CCF$=0.26\pm0.24$). DR discharges remained with 4.9 ± 1.2 s period (CV$=0.27\pm0.08$; $n=5$) and 2.6 ± 0.9 s duration, though with poor phase coordination between DRs (CCF$=0.44\pm0.40$; $n=5$). On the same preparations, after >30 min washout of TEA, application of 4-AP (5 μM; Fig. 8B) elicited larger and faster DR oscillations, while no events were present on VRs (see Taccola and Nistri, 2005). Fig. 8C summarizes the DR bursting properties evoked by 10 mM TEA with respect to the discharges induced by 4-AP on the same sectioned preparations. It is clear that the TEA-dependent oscillations were significantly slower, more irregular, smaller and of longer duration.

DISCUSSION

The principal finding of the present study was the novel demonstration that the broad spectrum potassium channel blocker TEA could activate spinal rhythmogenic networks to generate alternating electrical discharges from homosegmental and homolateral VRs. Hence, TEA had actions distinct from those of the other prototypical potassium channel blocker 4-AP, which could not directly produce alternating VR patterns.

Effects of TEA on spinal neurons

Previous studies have shown that, through the block of various potassium channel types (Rudy, 1988), TEA largely increases the excitability of spinal neurons (Barrett and Barret, 1976; Schwindt and Crill, 1980; Buss et al., 2003) and augments synaptic inputs to motoneurons (Clements et al., 1986). By inhibiting hyperpolarizing outward currents, TEA actually facilitates persistent, slow inward currents which determine repetitive firing (Barrett and Barret, 1976). On interneurons this action of TEA is clearly manifested as unmasking of plateau potentials with sustained depolarization and bimodal firing behavior due to voltage-activated Ca^{2+} conductances (Harada and Takahashi, 1983; Russo et al., 1998; Perrier and Hounsgaard, 1999; Powers and Binder, 2000). The effects of TEA are likely to involve multiple classes of potassium channels as

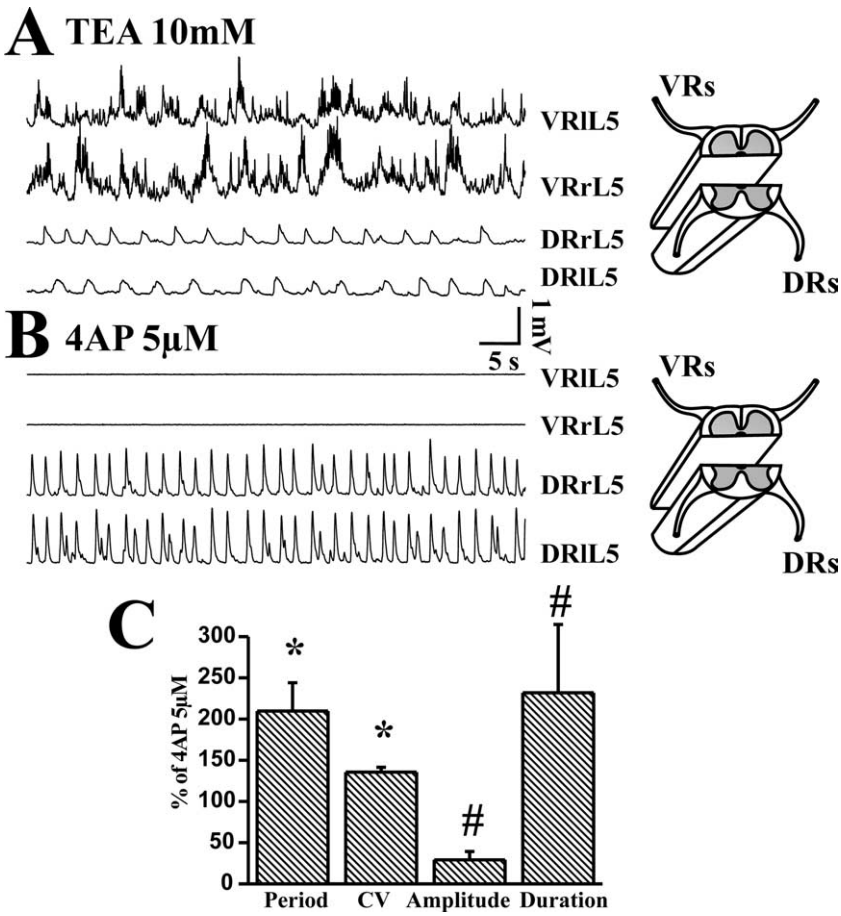

Fig. 8. Topography of oscillatory networks in the spinal cord. (A) TEA (10 mM) induces irregular oscillations on pair of L5 VRs and on the corresponding pair of DRs. The scheme on the right shows the section used to separate ventral from dorsal horn areas. (B) On the same preparation, after extensive washout of TEA, 4-AP (5 μM) does not produce any activity recorded from VRs, yet it elicits strong, synchronous oscillations from DRs. Scale bars apply to all records in A and B. (C) Histograms summarizing data for DR oscillations recorded after horizontal section and expressed as % of the actions produced by 5 μM 4-AP. Note that, unlike 4-AP oscillations, those induced by TEA are slower, more irregular, smaller and individually longer. Data are from $n=5$; * $P<0.01$; # $P<0.05$.

even careful titration of this drug concentration cannot ensure selectivity toward a particular potassium conductance (Rudy, 1988). Nevertheless, it is important to note that there was a dose-related change in spinal network excitability with consequent onset of synchronous VR discharges that were then converted into alternating motor patterns. This effect could not be attained with 4-AP (Taccola and Nistri, 2004, 2005).

It should be noted that the present results were obtained from neonatal spinal cord preparations. Because postnatally there is a steady increase in the expression and function of voltage-activated K^+ channels in spinal neurons to gradually reach adult levels (McLarnon, 1995), it is likely that the consequences of TEA-evoked block of K^+ channels in adult tissue are even more intense in terms of network excitability.

When the spinal cord is subjected to experimental injury, there is enhanced expression of certain K^+ channels (Kv1.1, Kv1.2 and Kv1.4) especially at the level of the white matter (Nashmi et al., 2000; Edwards et al., 2002; Karimi-Abdolrezaee et al., 2004). While Kv1.2 and Kv1.4 channels are relatively insensitive to TEA, Kv1.1 channels are readily blocked by low mM concentrations of this agent (Coetzee et al., 1999). It seems feasible that, in principle, high concentrations of TEA might be useful to inhibit such over-expressed Kv1.1 channels which may otherwise render spinal networks less susceptible to activation by excitatory inputs. Furthermore, other families of TEA-sensitive K^+ channels (in addition to the classical Kv2.1 believed to be responsible for the delayed rectifier of axons and neurons; Muennich and Fyffe, 2004) are suggested to be rhythmically active during vertebrate locomotor programs (Kuenzi and Dale, 1998; Grillner et al., 2001) and might contribute to the fictive locomotor activity evoked by high doses of TEA.

Synchronous rhythms evoked by small concentrations of TEA

Doses of TEA as low as 50 μM could already activate electrical discharges from VRs. While these events were synchronous at segmental and intersegmental level, they were also quite irregular. Slightly increasing the concentration of TEA merely decreased discharge periodicity

without improving its regularity. It is difficult to understand the mechanisms underlying these effects, because synchronicity should imply the operation of a broad spinal network capable of recruiting and constraining motoneurons into a simultaneous population discharge.

Despite increased network excitability, there was limited facilitation of fictive locomotor patterns induced, for instance, by DR stimuli or neurochemicals. Indeed, the most obvious change was a moderate increase in the number of alternating discharges caused by DR stimulus trains. Cazalets et al. (1999) also reported modest acceleration of alternating patterns evoked by NMDA and 5-HT, a phenomenon confirmed in the present study. In summary then, in terms of ability to facilitate the CPG activation by various inputs, TEA appeared to be less effective than 4-AP or low concentrations of the metabotropic glutamate agonist 3,5-dihydroxyphenylglycine (Taccola et al., 2004).

Alternating VR patterns elicited by large concentrations of TEA

Millimolar concentrations of TEA surprisingly generated rhythmic, alternating patterns with all the characteristics (period, phase, regularity etc.) of fictive locomotion. Such a phenomenon had a dose window as it appeared at 1 mM and disappeared at 20 mM when high frequency tonic discharges were produced.

The alternating rhythm evoked by TEA was associated with network (as recorded from VRs) and motoneuron (recorded intracellularly) depolarization of considerable amplitude. The observed depolarization was the sum of direct effects on the motoneuron membrane plus enhanced release by depolarizing transmitters within the local network. In fact, in the presence of blockers of glutamate, GABA and glycine receptors, the observed depolarization of motoneurons was halved, though still large (15–20 mV).

It was unexpected that a large depolarization of premotoneurons and motoneurons did not produce broad inactivation of voltage-gated channels and related arrest of network activity. Indeed, in most cases the initial depolarization evoked by TEA, at least in part, faded away, while alternating rhythmic patterns persisted. At this stage a novel type of spinal rhythmicity emerged with slow synchronous waves associated with cycles of locomotor like patterns and motoneuron firing with phase coupling necessary for fictive locomotion. The network origin of all VR responses evoked by TEA was suggested by their full disappearance in TTX solution.

Pharmacology of oscillations induced by large concentrations of TEA

The insensitivity of fast and slow oscillations to the gap-junction blocker carbenoxolone suggested they were not simply caused by motoneuron depolarizations spread through electrical junctions. Pharmacological tools enabled us to dissect out slow synchronous waves from fast alternating cycles. Blockers of ionotropic glutamate receptors (CNQX and APV) suppressed fast cycles as indeed expected for locomotor-like discharges (Beato et al., 1997), while leaving a background of smaller, slow irregular waves. The latter were largely amplified by the $GABA_A$ receptor antagonist bicuculline and the glycine antagonist strychnine. In the presence of ionotropic receptor blockers, the selective mGluR1 antagonist CPCCOEt fully suppressed these slow waves, indicating that they were mediated by mGluRs which, once activated, are known to generate oscillatory activity of premotoneurons and motoneurons in the rat spinal cord (Marchetti et al., 2003). However, CPCCOEt did not significantly change rhythmicity produced by TEA alone, suggesting that mGluRs contributed to rhythmicity in concert with other major players like ionotropic glutamate receptors. Only when the latter were inhibited, the action of mGluRs became detectable.

It is noteworthy that low-intensity activation of group I mGluRs can facilitate locomotor rhythms, while strong activation can actually suppress them (Taccola et al., 2004). There are, therefore, some analogies between the action of group I mGluRs and that of large concentrations of TEA: they both inhibit potassium conductances (Taccola et al., 2004), and can suppress locomotor-like patterns when administered during ongoing fictive locomotion. There are also important differences between them. For instance, activation of mGluRs alone cannot evoke fictive locomotion (Taccola et al., 2004). Furthermore, when mGluRs are blocked, TEA can still produce locomotor-like rhythms. Hence, the irregular slow waves emerging after block of ionotropic receptors in 10 mM TEA solution could not induce alternating (or even synchronous) motoneuron discharges. Rhythmic patterns seemed to be totally dependent on intact glutamatergic transmission at network level. When network synaptic transmission was blocked, motoneurons did exhibit membrane depolarization, very strong lengthening of their spike and irregular oscillations with potent spike discharges. All these effects were, however, incapable of being translated into a cohesive motor pattern, indicating that, even when potently excited by TEA, motoneuron discharges could not make up for impaired CPG networks.

Interaction between the effects of 4-AP and TEA

At the concentration of 5 μM 4-AP induces large electrical discharges synchronous on VRs and DRs (Taccola and Nistri, 2004, 2005). This appears to be the maximally effective dose as larger concentrations generate tonic, high frequency firing. When 10 mM TEA was applied during the synchronous rhythm by 4-AP, there was a large depolarization, indicating that there was no occlusion in the action of each potassium channel blocker. Alternating VR patterns were produced to replace the synchronous events due to 4-AP. In this sense, the switch off of the 4-AP pattern confirmed that the TEA-evoked rhythm had the characteristic of fictive locomotion because fictive locomotion turns off other spinal network patterns in rat spinal cord (Beato and Nistri, 1999; Marchetti and Nistri, 2001; Taccola and Nistri, 2004). Nevertheless, 4-AP could not speed up the locomotor-like rhythm. Thus, the TEA-induced activity was relatively stereotypic in its period which was not changed by using lower concentrations of TEA. Thus, by

changing the concentrations of TEA it was possible to determine how long the CPG remained active, but it was not feasible to modulate its output to motoneurons.

Localization of rhythmogenic networks activated by 10 mM TEA

DR discharges were observed in the presence of 10 mM TEA. When the dorsal and ventral areas of the spinal cord were sectioned, VR discharges persisted, albeit irregular and asynchronous, while DRs generated asynchronous oscillations. The DR activity evoked by TEA was very different from the one elicited by 4-AP because it was slower, irregular, longer, and of smaller amplitude. These characteristics suggest that the DR oscillators reputed to be activated by 4-AP were not the exclusive (or at least the main) target for the action of TEA. Furthermore, while isolated ventral horns lost rhythmic activity due to 4-AP, they retained it with 10 mM TEA. It seems likely that the action of TEA disclosed a broad ability of spinal networks to generate oscillations: topographically-sparse networks could generate oscillations, but their output lacked refinement like phase alternation and rhythm regularity. Next to raised neuronal excitability, network coupling was therefore an essential element to generate a motor program.

CONCLUSIONS

A high concentration of TEA per se brought about a spinal rhythm with all the properties typical of fictive locomotion. It is likely that this phenomenon was due to a wide-ranging block of multiple potassium conductances of spinal neurons including those belonging to the locomotor CPG. The TEA-evoked rhythm was less stable than and less susceptible to frequency modulation of fictive locomotion evoked by electrical stimuli or neurochemicals. These observations suggest that there were different pharmacological approaches to facilitate the operation of the locomotor CPG: on the one hand, a drug like 4-AP could favor the CPG operation, on the other hand, a substance like TEA per se could induce rhythmic, alternating VR discharges, but it actually often depressed the operation of the CPG when activated by other stimuli. These observations might provide the basis for future work designed at identifying more effective agents capable of activating the locomotor CPG via inhibition of strategically-restricted classes of potassium channels.

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RESEARCH NOTE

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Activation of group I metabotropic glutamate receptors depresses recurrent inhibition of motoneurons in the neonatal rat spinal cord in vitro

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Abstract This study examined whether activation of group I metabotropic glutamate receptors (mGluRs) could modulate synaptic inhibition of spinal motoneurons in the neonatal rat isolated spinal cord. Recurrent inhibitory postsynaptic potentials (IPSPs) generated by Renshaw cells were evoked via antidromic stimulation of motor axon collaterals and recorded intracellularly from lumbar motoneurons. The selective agonist of group I mGluRs DHPG (5 μmol L^{-1}) depressed the recurrent IPSP, an effect prevented by the selective antagonist AIDA (500 μmol L^{-1}). The depression by DHPG was use-independent and could be partly counteracted by increasing stimulus strength. Paired pulse depression observed at ≤ 50-ms intervals was blocked by DHPG in an AIDA-sensitive manner. These results suggest that, in the presence of DHPG, smaller recurrent IPSPs can contribute to the excitatory action of mGluR activation on spinal networks, including the generation of synchronous oscillations recorded from motoneurons.

Keywords Renshaw cell · Synaptic inhibition · DHPG · AIDA · Paired pulse depression

Introduction

Although detailed information on the structure, distribution and single-cell activity of metabotropic glutamate receptors (mGluRs) is currently available (Pin and Duvoisin 1995; Schoepp et al. 1999), their functional role within physiologically identified neuronal circuits remains incompletely understood.

The mammalian isolated spinal cord is useful for investigating the functional role of mGluRs (Ishida et al. 1993; Jane et al. 1994) that are widely distributed in spinal laminae (Berthele et al. 1999; Alvarez et al. 2000), suggesting they are important in the modulation of local network activity. Most mGluRs of the spinal cord belong to group I (Berthele et al. 1999; Alvarez et al. 2000) and are coupled to activation of phosphoinositol metabolism, membrane depolarization, and heightened excitability (Pin and Duvoisin 1995; Schoepp et al. 1999). In keeping with this view, our recent studies have shown that, in the rat spinal cord, group I mGluRs evoke motoneuron depolarization and electrical oscillations together with strong facilitation of spontaneous synaptic discharges (Marchetti et al. 2003). These phenomena are, however, associated with significant depression of excitatory synaptic transmission induced by electrical stimulation of dorsal root afferents—apparently because enhancement of glycinergic inhibition by group I mGluR activation leads to depression of excitatory glutamatergic responses (Marchetti et al. 2003). In this study we wished to explore further whether activation of group I receptors by the selective agonist DHPG could modify inhibitory transmission within a well-identified spinal pathway. For this purpose, we examined inhibitory postsynaptic potentials (IPSPs) mediated by Renshaw cells on motoneurons. Renshaw cells are a class of ventral horn interneurons, which, once excited by motor axon collaterals via cholinergic nicotinic receptors, inhibit motoneurons (Eccles et al. 1954). The IPSP of Renshaw cells is predominantly glycinergic (Werman et al. 1968; Marchetti et al. 2002) and functionally inhibitory even on neonatal motoneurons (Marchetti et al. 2002). Because group I mGluRs are largely present in the ventral horn (Berthele et al. 1999; Alvarez et al. 2000), the motoneuron IPSP, evoked by antidromic stimulation of a single ventral root (VR), is a convenient tool to explore interference by mGlu receptors with inhibitory synaptic transmission.

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Methods

Experiments were performed on lumbar spinal cord preparations isolated from neonatal Wistar rats (0–5 days old) under urethane anaesthesia (0.2 mL i.p. of a 10% *w*/*v* solution) as previously described (Taccola et al. 2004). These procedures were conducted in accordance with NIH guidelines, and Italian national laws DL 27/1/92 n. 116 following the European Community directives n. 86/609 and 93/88 (Italian Ministry of Health authorization D.M. 69/98-B). The spinal cord was superfused with Krebs solution of composition (mmol L^{-1}): NaCl, 113; KCl, 4.5; $MgCl_27H_2O$, 1; $CaCl_2$, 2; NaH_2PO_4, 1; $NaHCO_3$, 25; glucose, 11; gassed with 95% O_2–5% CO_2; pH 7.4 at room temperature. All agents were applied via the superfusing solution.

Intracellular current-clamp recording from functionally identified L3–L5 motoneurons used sharp electrodes filled with either 3 mol L^{-1} KCl (30–60 MΩ resistance), or 2 mol L^{-1} $KMeSO_4$ (60–120 MΩ resistance). Renshaw cell-mediated IPSPs were elicited by antidromic VR stimulation (stimulus intensity: 1–5 V; duration: 0.1 ms; 0.4 Hz unless otherwise stated) in accordance with standard procedures (Marchetti et al. 2002). QX-314·Cl (300 μmol L^{-1}; Marchetti et al. 2002) was added to the intracellular solution to block the somatic motoneuron spike and the slow inward rectifier, which would otherwise mask IPSPs. Synaptic responses were acquired after resetting (via intracellular current injection) the motoneuron membrane potential (V_m) to the value recorded before the test drug application, averaged (≥5) and quantified as means ± SD (*n* is the number of experiments). Statistical significance was assessed with Student's *t*-test, or ANOVA plus Tukey test. The accepted level of significance was $P = 0.05$. Substances were used at receptor subclass-selective concentrations (Schoepp et al. 1999; Marchetti et al. 2003). The following chemicals were purchased from Tocris: (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA, selective antagonist of type 1 of group I receptors; 500 μmol L^{-1}), (*RS*)-*a*-cyclopropyl-4-phosphonophenylglycine (CPPG, selective antagonist of group III receptors; 50 μmol L^{-1}), (*RS*)-3,5-dihydroxyphenylglycine (DHPG, selective agonist of group I receptors; 5 μmol L^{-1}), (2*S*)-α-ethylglutamic acid (EGLU, selective antagonist of group II receptors; 200 μmol L^{-1}), 2-methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP, selective antagonist of type 5 of group I receptors; 100 μmol L^{-1}).

Results

Effect of mGluRI activation on IPSPs

Figure 1Aa shows an example of multi-fibre recurrent IPSP recorded with a $KMeSO_4$ + QX-314-filled electrode from an L5 motoneuron ($V_m = -73$ mV) stimulated via its homolateral VR. As previously reported (Marchetti et al. 2003), 5 μmol L^{-1} DHPG evoked motoneuron depolarization with increased input resistance (not shown). After 3 min exposure to DHPG (5 μmol L^{-1}) and repolarization of the membrane potential to control level, the peak amplitude and the area of the IPSP were depressed to 64 and 55%, respectively (Fig. 1Ab), an effect reversible after 20 min washout (not shown). On a sample of nine motoneurons, the IPSP peak after 2–4 min application of DHPG became 84 ± 27% of control ($P < 0.05$) and the area was 57 ± 15% ($P < 0.001$; Fig. 1B) whereas the rise time and decay time were unchanged (93 ± 18 and 100 ± 19%, respectively; $P > 0.05$).

In control solution the size of the IPSPs readily grew with small increments in stimulus intensity (compare 1 and 2 V traces in Fig. 1D). In DHPG solution, however, a very large rise in stimulus strength (16 V) was necessary to match the IPSP amplitude initially observed in control solution (Fig. 1D).

Next, we explored the role of endogenous mGluR activity in shaping the IPSP. Figure 1Ac–f shows little change in IPSPs recorded in the presence of AIDA or MPEP. On average, IPSP area (87 ± 24%, $n = 9$; Figs. 1B and 2B), peak amplitude (94 ± 20%), rise (98 ± 9%) and decay (90 ± 10%) times were not significantly different in the presence of AIDA. Likewise, in the presence of MPEP, IPSP area (129 ± 41%, $n = 8$; Figs. 1B and 2B), peak amplitude (111 ± 24%), rise (114 ± 25%) and decay (93 ± 13%) time were not significantly different from control. Likewise, the IPSP area, measured in the presence of EGLU, was 140 ± 30% of control ($n = 5$; $P > 0.05$) with no significant change in peak amplitude (104 ± 6%, $n = 5$). Similarly, CPPG did not cause significant changes in area (116 ± 26%, $n = 3$), or peak (88 ± 25%) of the IPSP.

Because of the composite nature of the recurrent IPSPs with considerable amplitude fluctuation, to assess the sensitivity of the action of DHPG to antagonists we compared (on the same cells; Fig. 1C) changes in IPSP area in the presence of 5 μmol L^{-1} DHPG plus antagonist(s) with the response in the presence of the antagonist(s) alone. The IPSP depression induced by DHPG was prevented by AIDA (96 ± 15%, $n = 4$) or AIDA + MPEP (92 ± 9%, $n = 3$), yet persisted with MPEP + DHPG (47 ± 24%, $n = 4$; $P < 0.005$).

Stimulus-dependent short-term changes in Renshaw cell IPSPs

We tested if the inhibition of the IPSP by DHPG was use-dependent. Fig. 1E shows, for five motoneurons, a time profile plot of the normalized IPSP area during a train of ten stimuli at 0.33 Hz. In control solution the IPSP remained stable throughout the train (filled squares). Four minutes application of DHPG depressed the area of the IPSP that was also normalized in respect of the first response for sake of comparison (open circles in Fig. 1E). Even in this case, the depression remained stable throughout the stimulus train, indicating that,

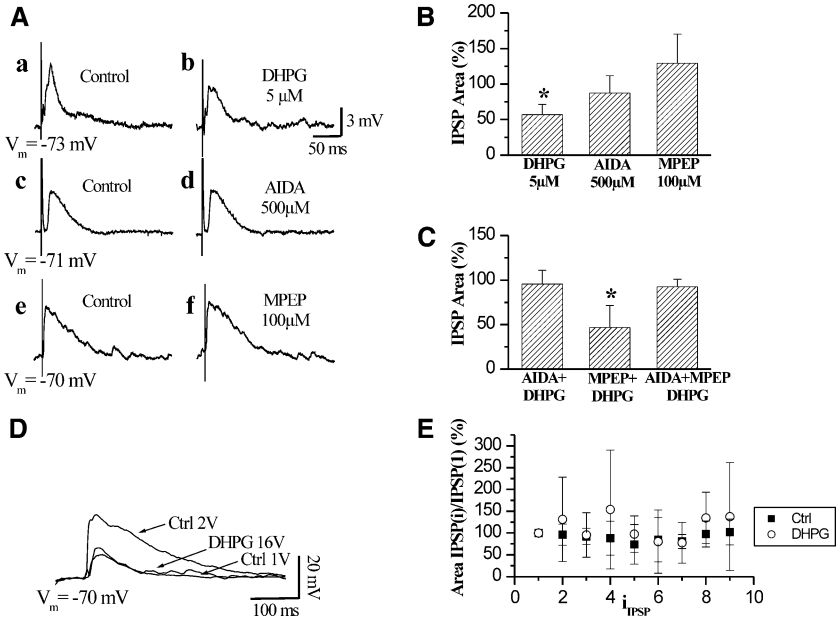

Fig. 1 **A–E** Effect of mGluR group I activation on recurrent IPSPs. **A** Recurrent IPSP (recorded with a $KMeSO_4$ + QX-314-filled electrode) at the same membrane potential value ($V_m = -73$ mV): response is depressed in the presence of DHPG (**a**, **b**), but not in the presence of AIDA (**c**, **d**; different cell from **a**, **b**; $V_m = -71$ mV) or MPEP (**e**, **f**, different cell from **a**, **c**; $V_m = -70$ mV). **B** Average IPSP area (percentage of control) during application of DHPG ($n = 9$), AIDA ($n = 9$), or MPEP ($n = 8$). $^*P < 0.001$. **C** Average IPSP area (percentage of value in the presence of antagonists applied for 10 min) during application of AIDA + DHPG ($n = 4$), MPEP + DHPG ($n = 3$), or AIDA + MPEP + DHPG ($n = 3$); $^*P < 0.005$. **D** Superimposed IPSPs elicited by stimuli at different intensities in control (ctrl, 1, 2 V) and in DHPG solution (16 V). **E** Time course of changes in IPSP area (normalized to area of first IPSP) in control (*filled symbols*) and in DHPG solution (*hollow symbols*): *IPSP(i)* indicates the number of IPSP in the sequence ($i = 1,...,9$); $n = 5$

despite more signal fluctuation, the synaptic block did not show use-dependent modification.

We next tested if DHPG might affect synaptic plasticity over a shorter time-frame. We changed the interstimulus interval in the ms range as shown in Fig. 2A for control (filled circles) or DHPG (open circles) solution. Short-term synaptic depression was quantified by taking the ratio (PPR) between the second response amplitude over the first. In the example of Fig. 2A, at ≤ 50 ms intervals, the second IPSP amplitude was reduced to $0.84 \pm 0.03\%$ in control solution (average first IPSP amplitude was 4.35 ± 1.04 mV; $n = 7$). Short-term synaptic depression was, however, absent in the presence of DHPG (Fig. 2A). This phenomenon is also illustrated by sample records in Fig. 2B (same cell as in A) for 30-ms stimulus interval and summarized in Fig. 2C, indicating significant short-term depression in control solution (left column) only. This effect of DHPG was sensitive to AIDA. In fact, while in DHPG solution PPR was close to 1 (1.05 ± 0.11; $n = 6$) and significantly different from control (0.88 ± 0.12; $P < 0.05$), the PPR value in the presence of AIDA (0.87 ± 0.07) or AIDA plus DHPG (0.89 ± 0.10; $n = 3$) was similar to that in control, indicating persistence of short term depression when the mGluR antagonist was applied.

Discussion

The main result of this study is the novel demonstration that activation of group I mGluRs depressed recurrent synaptic inhibition of lumbar motoneurons in the neonatal rat spinal cord in vitro. This effect was observed when activating the Renshaw cell pathway by stimulation of the corresponding VR.

Mechanisms of IPSP depression by DHPG

It was somewhat surprising that, after concluding that DHPG facilitated dorsal root-evoked glycinergic transmission in the spinal cord (Marchetti et al. 2003), we actually found DHPG to depress the IPSP of Renshaw cells that use glycine as their main transmitter (Werman et al. 1968; Marchetti et al. 2002). The recurrent IPSP is based on a disynaptic pathway whereby cholinergic nicotinic synapses (Eccles et al. 1954) activate Renshaw cells impinging upon motoneurons (Werman et al. 1968). Thus, the depressant action by DHPG might target either nicotinic transmission on Renshaw cells, or Renshaw cell firing-dependent inhibitory transmission, or both.

Even though DHPG reduces glutamate release on hippocampal cells (Fitzjohn et al. 2001; Faas et al. 2002), neurochemical studies have shown that DHPG actually facilitates release of endogenous glycine (de Novellis et al. 2002), thus making it unlikely that the observed reduction in Renshaw cell IPSP was because of depression of inhibitory transmitter release on spinal motoneurons. The release hypothesis cannot, however, be tested directly, because IPSPs exclusively generated by

Renshaw cells cannot be identified on a single motoneurone. Previous studies have not found block of glycine or GABA receptors by DHPG in spinal (Marchetti et al. 2003; Taccola et al. 2004) or brainstem (Sharifullina et al. 2004) networks, suggesting that depression of spinal recurrent IPSPs was not because of partial block of inhibitory receptors on motoneurons. In accordance with this view, the depressant effect of DHPG was not use-dependent as might have been expected if this agent acted as a non-selective channel blocker.

The alternative possibility is that DHPG blocked cholinergic transmission, thereby depriving Renshaw cells of their input drive. The DHPG-induced increase in motoneuron firing (Marchetti et al. 2003) should have actually enhanced acetylcholine release to Renshaw cells by analogy with data from rat brain synaptosomes (Marti et al. 2001). Although there is evidence for DHPG acting distinctly from nicotinic receptors (Arce et al. 2004), augmented release of acetylcholine may lead to persistent depolarization of Renshaw cells. If Renshaw cells (like other ventral horn interneurons) express group I mGluRs (Berthele et al. 1999; Alvarez et al. 2000), their direct depolarization by DHPG would add to that caused by enhanced release of acetylcholine. This condition might be expected to evoke some inactivation of voltage-dependent Na^+ channels of Renshaw cells with impairment in their ability to release inhibitory transmitter and consequent IPSP depression.

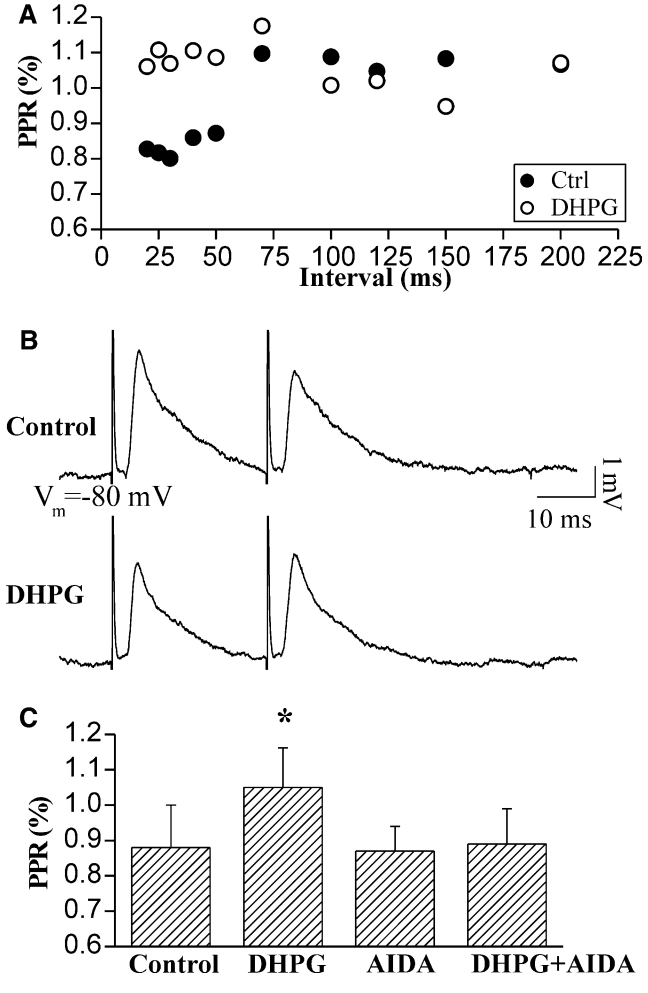

Fig. 2 **A–C** Short-term changes in recurrent IPSP in the presence of DHPG. **A** Plot of PPR versus inter-stimulus time in control solution or in the presence of DHPG. **B** Representative traces taken at 30-ms paired-pulse interval demonstrating loss of synaptic depression with DHPG application (same cell as in **A**). **C** average PPR at 30-ms paired-pulse interval in control ($n=6$), 5 µmol L^{-1} DHPG ($n=6$), 500 µmol L^{-1} AIDA ($n=3$), and AIDA plus DHPG solution ($n=3$); $^*P<0.05$ compared with control

Paired-pulse stimulation disclosed short-term depression of the IPSP, a phenomenon likely to reflect a transient fall in transmitter release, probably because of a rapid decrease in the available pool of synaptic vesicles after the first stimulus (Zucker and Regehr 2002). Interestingly, DHPG eliminated paired-pulse depression, perhaps because DHPG is known to increase intracellular free Ca^{2+} (Pin and Duvoisin 1995), a process, which might have expanded the vesicle pool to limit depression of the second IPSP. The ability by DHPG to minimize paired pulse depression was not, however, sufficient to counteract the fall in IPSP amplitude versus control in saline solution.

Although a persistent, large release of acetylcholine by DHPG might have facilitated nicotinic receptor desensitization and contributed to reduced inputs to Renshaw cells, this possibility could not explain the loss of paired-pulse depression in the presence of DHPG.

The mechanism responsible for the IPSP decrease in the presence of DHPG remains, however, conjectural because direct recording from Renshaw cells in the rat isolated spinal cord is very difficult, owing to their sparse location which makes rare their observation even in spinal slices (Dourado and Sargent 2002). Hence, this report has to rely on an indirect approach based on recording responses from motoneurons. Nevertheless, our results indicate that the IPSP depression was because of type 1 mGluRs in view of the antagonism by the selective blocker AIDA. It is noteworthy that, under the present conditions, there was no detectable activation of groups I–III receptors during antidromic stimulation, because none of the tested antagonists per se modulated the recurrent IPSP.

Functional implications for network activity

These data disclosing significant depression of recurrent IPSPs on lumbar motoneurons may help to account for one aspect of the general network activity triggered by mGluR activation in the rat spinal cord, namely the emergence of synchronous oscillations between motoneuron pools (Marchetti et al. 2003). It is widely accepted that glycinergic interneurons in the ventral horn contribute substantially to alternation of electrical oscillations between distinct groups of motoneurons (Butt et al. 2002). It is therefore feasible that impairment of Renshaw cell activity may lead to a synchronous

discharge pattern that does not, however, disrupt the fictive locomotor rhythm (Taccola et al. 2004).

Acknowledgements This work was supported by grants from MIUR (FIRB) and INFM.

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Electrophysiological effects of 4-aminopyridine on fictive locomotor activity of the rat spinal cord in vitro

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Summary

Recently the K^+ channel blocker 4-aminopyridine (4-AP) has been suggested to be useful to improve motor deficits due to spinal cord lesions. There is, however, little basic research support for this action of 4-AP. In this study we have used as a model the neonatal mammalian spinal cord in vitro that generates a rhythmic activity termed fictive locomotion (induced by bath-application of NMDA + 5-HT) with phasic electrical discharges alternating between flexor and extensor motor pools and between left and right motoneurons within the same segment. When 4-AP was added in the presence of sub-threshold concentrations of NMDA + 5-HT, there was facilitation of fictive locomotion which appeared with alternating patterns on all recorded ventral roots (VR). Furthermore, in the presence of 4-AP, weak dorsal root (DR) stimuli, previously insufficient to activate locomotor patterns, generated alternating discharges from various VRs. The present data show that 4-AP could strongly facilitate the locomotor program initiated by neurochemicals or electrical stimuli, indicating that the spinal locomotor network is a very sensitive target for the action of 4-AP.

Keywords: Central pattern generator; spinal cord lesion; rhythmic patterns; oscillations.

Introduction

Fampridine-SR, a new sustained release oral tablet form of 4-AP is currently under phase III clinic trial for its therapeutic efficacy in patients with Multiple Sclerosis (MS) and chronic spinal cord injury [2]. The rationale for this approach stems from the fact that low concentrations of 4-AP are considered to block transient, voltage activated, outward K^+ currents. The most striking effect seen with K^+ channel blockers is an enhancement of transmitter release at many central and peripheral synapses as a consequence of increased Ca^{++} influx into presynaptic terminals.

4-AP sensitive K^+ channels are also present in the internodal area of the axon membrane shielded under the myelin sheath. Traumatic injury causes apoptosis of oligodendrocytes with disruption of the myelin wrapping which then unmasks 4-AP sensitive K^+ channels located in juxtaparanodal and internodal regions. The activity of such previously-hidden K^+ channels results in axonal conduction failure at central and peripheral level [5]. Hence, block of voltage-dependent fast K^+ channels by 4-AP has two important effects that are thought to ameliorate the central conduction deficit experienced by patients following MS or traumatic cord injury: it prolongs the duration of the action current in focally demyelinated internodes and it enhances central and peripheral synaptic transmission.

We have considered the possibility that 4-AP might improve spinal cord function by modulating and/or reactivating the operation of the specialized spinal network devoted to generate rhythmic motor patterns responsible for locomotion. Such network is named Central Pattern Generator (CPG). The spinal CPG can generate in vivo, even in the absence of external stimuli, phasic electrical discharges alternating between flexor and extensor motor pools and between left and right motoneurons within the same segment.

A very similar pattern can be produced also by superfusing the isolated mammalian spinal cord with excitatory agents like NMDA and serotonin (5HT; see [1, 3]) or by repeated stimuli applied to one DR [4]. Since the main rhythmic burst in L2 is flexor-related and the main burst in L5 is extensor-related, it is common to call the rhythmic activity locomotor-like when the L2 and L5 bursts alternate on one side of the cord and when there is segmental left-right alternation.

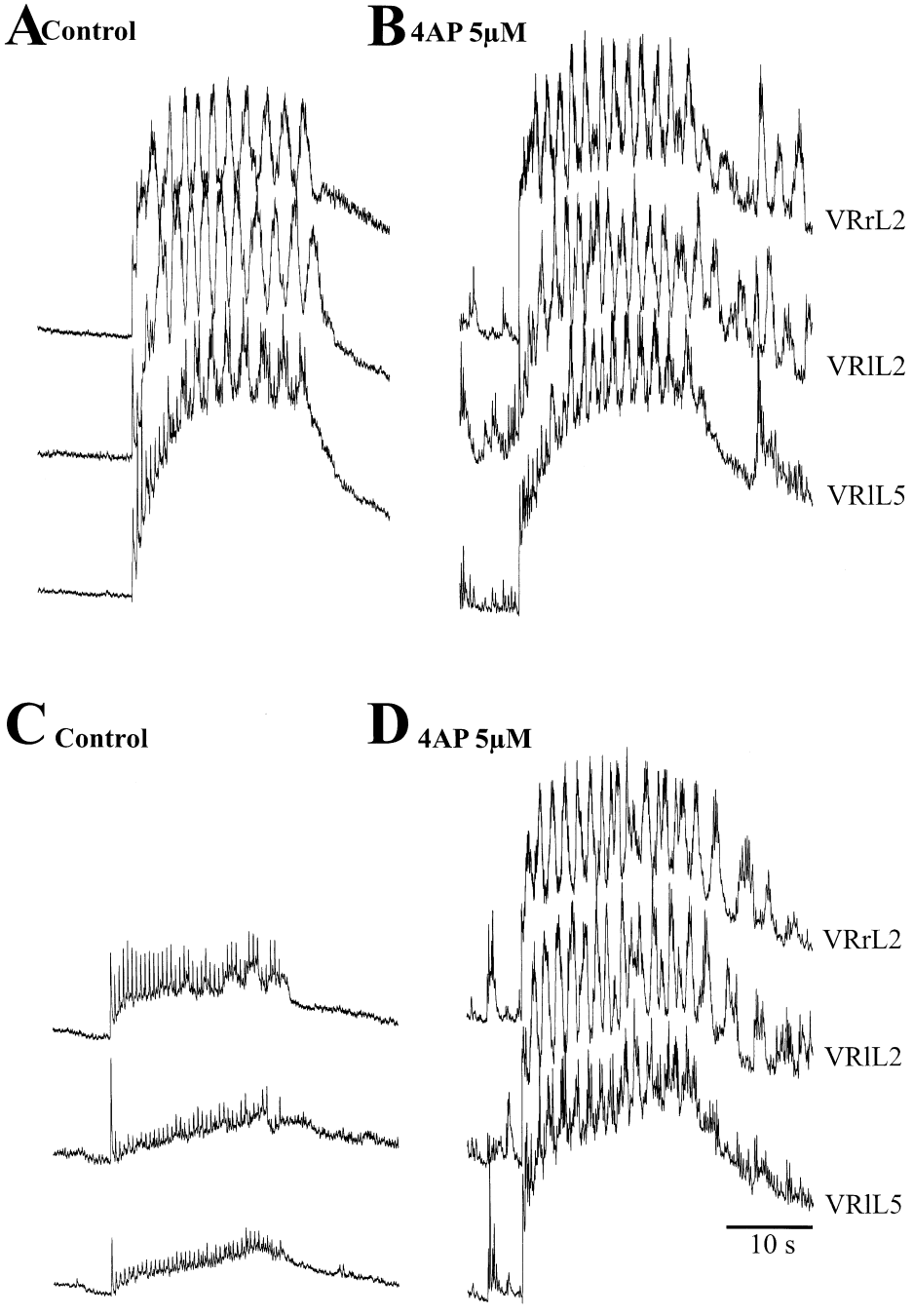

Fig. 1. 4-AP enhances alternating motor patterns during DR stimulation. Sample records are from three VRs (left, l, and right, r) whose segmental identification is abbreviated alongside the traces. All data are from the same preparation. (A) During a train of 40 strong pulses to a single DR, there is a slowly developing VR depolarization with superimposed alternating patterns. (B) When the test is repeated in the presence of 4-AP the number of alternating patterns is clearly increased. (C) The same preparation is stimulated with a train of weak DR pulses unable to induce cumulative depolarization or oscillations. (D) When the test is carried out in the presence of 4-AP, there is appearance of cumulative depolarization and alternating oscillations

Because of its well defined inputs via DR fibres and motor output via VR axons, and because of its long-term stability, the isolated spinal cord of the rat represents a very advantageous in vitro model to evaluate the pharmacological action of drugs, like 4-AP, proposed for the symptomatic treatment of spinal cord injured subjects. The present study sought to clarify if 4-AP could act on the spinal CPG.

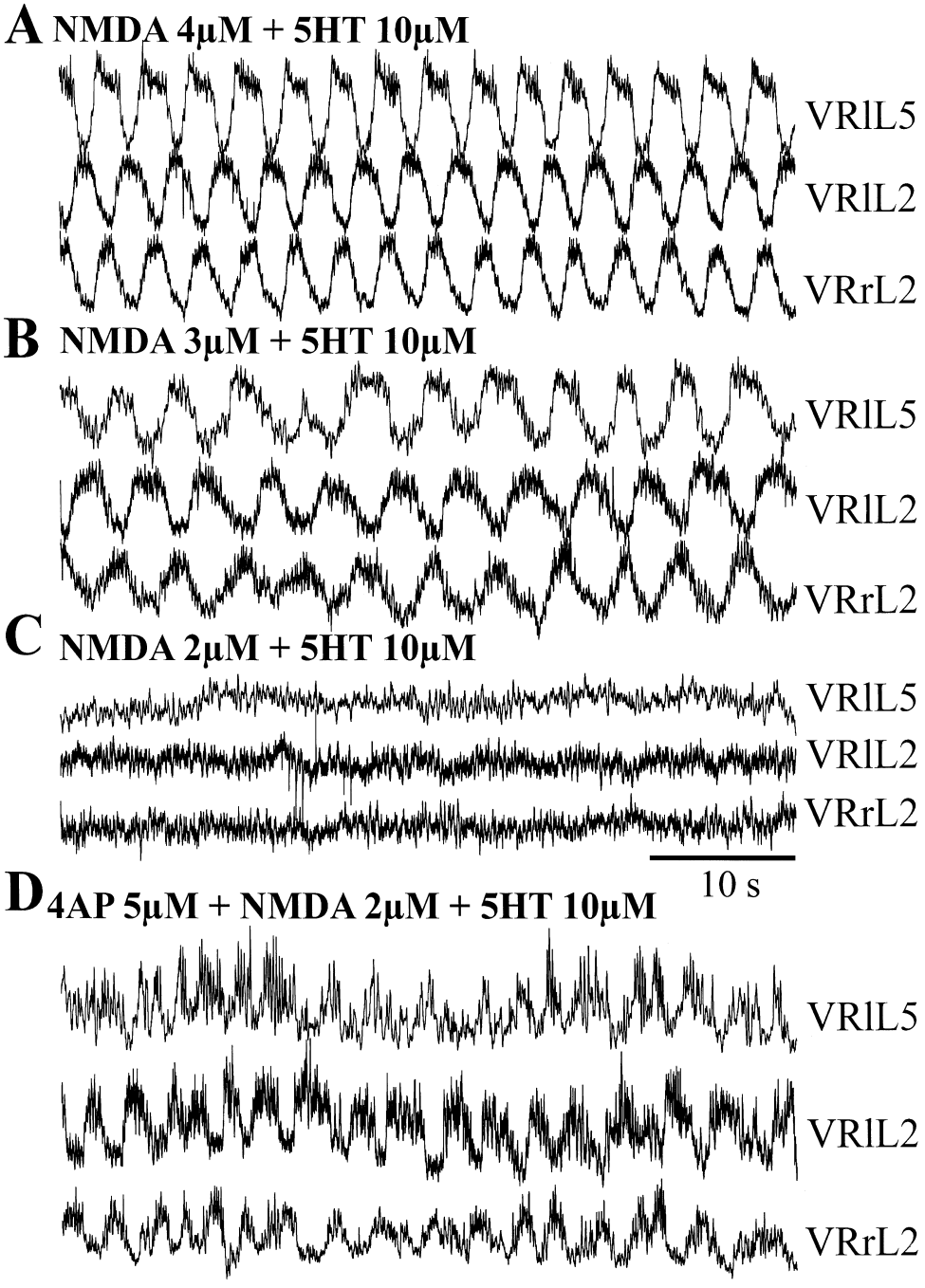

Fig. 2. Fictive locomotion is disclosed by 4-AP. Representative traces are all from the same preparation. (A) Stable fictive locomotion is recorded in the presence of 4 μM NMDA and 10 μM 5-HT. Note alternation between flexor and extensor motor pools. (B) Decreasing the NMDA concentration to 3 μM slows down the rhythm. (C) When the NMDA concentration is 2 μM, the rhythm is suppressed. (D) Administration of 5 μM 4-AP restores the rhythm despite the low concentration of NMDA

Methods

In accordance with NIH guidelines and the Italian act DL 27/1/92 n. 116 (implementing the European Community directives n. 86/609 and 93/88), experiments were performed on lumbar spinal cord preparations isolated from neonatal Wistar rats (0–5 days old) under urethane anaesthesia (0.2 ml i.p. of a 10% w/v solution). The experimental set-up was the same as described by Taccola *et al.* (2004) [6]. In brief, the neonatal rat spinal cord was superfused (7.5 ml min^{-1}) 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% O_2–5% CO_2; pH 7.4 at room tempera-

ture. All agents were bath-applied via the superfusing solution at the concentrations mentioned in the text. In view of the need to record fictive locomotion rhythms for a long time, the majority of experiments were based on DC-coupled recordings from lumbar VRs. DR electrical stimuli were employed to elicit either single VR responses (recorded from the ipsilateral VR of the same segment) or VR cumulative depolarization following a train of DR pulses.

Fictive locomotion was typically induced by continuously bath-applied NMDA plus 5-HT.

Results

A low concentration (5 μM) of 4-AP could not trigger the expression of fictive locomotion as this drug generated synchronous oscillations only (not shown). We therefore explored if 4-AP could facilitate the genesis of fictive locomotion in the presence of appropriate stimuli to the spinal locomotor CPG. Figure 1 A–B shows extracellularly recorded cumulative depolarization of three lumbar VRs following a train of 40 strong electrical pulses to a single DR. The cumulative depolarization which developed gradually during the pulse train presented superimposed alternating oscillations. In control condition such oscillations occurred between lL2 and lL5 and between rL2 and lL2, thus featuring the hallmarks of fictive locomotion. 4-AP (5 μM) increased the cumulative depolarization area by enhancing the number of oscillations without affecting their period. Note that during cumulative depolarization the synchronous oscillations induced by 4-AP disappeared to be replaced by the alternating ones.

The same experimental procedure was repeated by using a train of much weaker electrical pulses (see Fig. 1 C–D), which in control condition could evoke neither cumulative depolarization nor alternating oscillations. When this test was repeated in the presence of 4-AP (5 μM), the same stimulation pattern could now generate cumulative depolarization with alternating oscillations.

Figure 2 A shows a representative experiment of stable fictive locomotion induced by application of NMDA (4 μM) and 5HT (10 μM). This rhythm shows the characteristic double alternation between right and left side of the cord and between flexor and extensor motor pools. A small decrease in NMDA concentrations to 3 μM decelerated the rhythm which preserved its alternating patterns (Fig. 2 B). When NMDA was applied at 2 μM (Fig. 2 C), the rhythm disappeared. However, application of 5 μM 4-AP (in the presence of subthreshold concentration of NMDA and 5HT) could readily restored fictive locomotion (Fig. 2 D). When 4-AP was applied during a stable fictive locomotor pattern, it accelerated the rhythm (not shown).

Discussion

The present data show that 4-AP could strongly facilitate the locomotor program initiated by neurochemicals or electrical stimuli indicating that the operation of the central pattern generator responsible for locomotion could be significantly up-regulated by a very low dose of 4-AP. The present data therefore indicate a novel site of action for 4-AP in facilitating spinal motor programs in addition to its effects on axons and peripheral synapses. This effect of 4-AP was strictly dependent on the coincidence of 4-AP administration and stimulatory inputs to the CPG. For this reason 4-AP may have a possible therapeutic application to bring the CPG activity to threshold, although by itself it could not generate fictive locomotion.

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CHARACTERISTICS OF THE ELECTRICAL OSCILLATIONS EVOKED BY 4-AMINOPYRIDINE ON DORSAL ROOT FIBERS AND THEIR RELATION TO FICTIVE LOCOMOTOR PATTERNS IN THE RAT SPINAL CORD *IN VITRO*

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Abstract—4-Aminopyridine (4-AP) is suggested to improve symptomatology of spinal injury patients because it may facilitate neuromuscular transmission, spinal impulse flow and the operation of the locomotor central pattern generator (CPG). Since 4-AP can also induce repetitive discharges from dorsal root afferents, this phenomenon might interfere with sensory signals necessary to modulate CPG activity. Using electrophysiological recording from dorsal and ventral roots of the rat isolated spinal cord, we investigated 4-AP-evoked discharges and their relation with fictive locomotor patterns. On dorsal roots 4-AP (5–10 μM) induced sustained synchronous oscillations (3.3±0.8 s period) smaller than electrically evoked synaptic potentials, persistent after sectioning off the ventral region and preserved in an isolated dorsal quadrant, indicating their dorsal horn origin. 4-AP oscillations were blocked by tetrodotoxin, or 6-cyano-7-nitroquinoxaline-2,3-dione and D-amino-phosphonovalerate, or strychnine and bicuculline, suggesting they were network mediated via glutamatergic, glycinergic and GABAergic transmission. Isolated ventral horn areas could not generated 4-AP oscillations, although their intrinsic disinhibited bursting was accelerated by 4-AP. Thus, ventral horn areas contained 4-AP sensitive sites, yet lacked the network for 4-AP induced oscillations. Activation of fictive locomotion by either application of *N*-methyl-D-aspartate and serotonin or stimulus trains to a single dorsal root reversibly suppressed dorsal root oscillations induced by 4-AP. This suppression was due to depression of dorsal network activity rather than simple block of root discharges. Since dorsal root oscillations evoked by 4-AP were turned off when the fictive locomotor program was initiated, these discharges are unlikely to interfere with proprioceptive signals during locomotor training in spinal patients. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: motoneuron, presynaptic inhibition, burst, central pattern generator, spinal network.

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Abbreviations: ANOVA, analysis of variance; APV, D-amino-phosphonovalerate; CCF, correlation coefficient function; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CPG, central pattern generator; CV, coefficient of variation; DR, dorsal root; DR-DRP, dorsal root potential evoked by dorsal root stimulation; DR-VRP, ventral root potential evoked by dorsal root stimulation; EC_{50} value, concentration of drug producing 50% of the maximum effect; l, left; L, lumbar; NMDA, *N*-methyl-D-aspartate; r, right; Th, threshold; TTX, tetrodotoxin; VR, ventral root; 4-AP, 4-aminopyridine; 5-HT, serotonin.

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4-Aminopyridine (4-AP) is proposed to offer symptomatic treatment to spinal injury patients (Hansebout et al., 1993; Darlington, 2000). While the mechanism underlying such an effect is probably complex and dependent on the extent of spared spinal tissue, one contributing factor appears to be the facilitated operation of intrinsic spinal locomotor networks (collectively termed central pattern generator [CPG]) induced by low micromolar concentrations of 4-AP as demonstrated on the rat spinal cord *in vitro* (Taccola and Nistri, 2004).

Nevertheless, 4-AP is reported to exert an additional action on the mammalian spinal cord of either the cat *in vivo* (Dubuc and Rossignol, 1989a) or the hamster *in vitro* (Al-Zamil et al., 1988), namely, induction of repetitive discharges from dorsal roots (DRs). Although the processes responsible for this phenomenon are uncertain, this effect has been replicated also in rat spinal cord slices and suggested to represent a functional model of the abnormal discharges typically found during neuropathic pain (Ruscheweyh and Sandkuhler, 2003). If DR discharges can persist during the CPG activation brought about by 4-AP, one might suppose that they could interfere with the locomotor program on two accounts: (1) prevention by DR fiber backfiring of the modulatory tone from peripheral proprioceptive afferents to the CPG (reviewed by Barbeau et al., 1999); (2) limitation to the usefulness of 4-AP administration to spinal patients because it could induce neuropathic-like pain states.

In the present investigation we sought to clarify the interaction of 4-AP-elicited DR discharges with the locomotor CPG in the *in vitro* rat spinal cord model. Our goals were (1) investigating the interaction of DR oscillations induced by 4-AP with fictive locomotion; (2) establishing the spinal topography of the 4-AP induced oscillations; (3) identifying the minimal neuronal structure responsible for their generation; and (4) devising a wiring diagram to interpret the action of 4-AP in relation to locomotor networks.

EXPERIMENTAL PROCEDURES

In accordance with National Institutes of Health guidelines and the Italian act Decreto Legislativo 27/1/92 n. 116 (implementing the European Community directives n. 86/609 and 93/88), experiments were performed on lumbar (L) spinal cord preparations isolated from neonatal Wistar rats (0–5 days old) under urethane anesthesia (0.2 ml i.p. of a 10% w/v solution). All efforts were made to reduce the number of animals used and to minimize animal suffering. The experimental setup was the same as described by Taccola and Nistri (2004).

The spinal cord was superfused (6.5 ml min^{-1}) with Krebs solution of the following composition (in mM): NaCl, 113; KCl, 4.5; $MgCl_27H_2O$, 1; $CaCl_2$, 2; NaH_2PO_4, 1; $NaHCO_3$, 25; glucose, 11; gassed with 95% O_2–5% CO_2; pH 7.4 at room temperature (unless otherwise stated). All agents were bath-applied via the superfusing solution at the concentrations mentioned in the text. Lesions to ablate certain areas of the isolated spinal cord were carried out as described by Bracci et al. (1996). Various types of lesion were performed with a scalpel sectioning along the sagittal or horizontal plane of the spinal cord, or isolating a dorsal quadrant and its DR after carefully removing ventral horn and contralateral dorsal horn areas.

Recordings were obtained with glass suction microelectrodes (containing an Ag-AgCl pellet) filled with Krebs solution. For these extracellular data, records are often calibrated in terms of time only, as their amplitude is dependent on the access resistance of the suction electrodes. Fictive locomotion was typically induced by continuously bath-applied *N*-methyl-D-aspartate (NMDA; 4 or 5 μM) plus serotonin (5-HT; 10 μM; see Kiehn and Kjaerulff, 1998; Butt et al., 2002). To characterize fictive locomotor patterns, recordings were obtained from left (l) and right (r) L2 ventral roots (VRs) that mainly express flexor motor-pool signals to the hindlimbs, and from l and r L5 VRs (mainly expressing extensor motor-pool signals to the hindlimbs; Kiehn and Kjaerulff, 1998). VR discharges with alternation between the l and r VRs within the same segment, and between L2/L5 VRs on the same side were indicative of typical fictive locomotion (see Butt et al., 2002).

Miniature bipolar suction electrodes were used in order to deliver single or repetitive electrical stimuli to DRs to evoke either DR-DR potentials (DR-DRPs) or DR-VR potentials (DR-VRPs; see Kerkut and Bagust, 1995). Stimulus intensity was calculated in terms of threshold (Th), defined as the minimum intensity to elicit a detectable response in the homolateral VR.

Disinhibited bursting was produced by continuously bath-applied strychnine (1 μM) and bicuculline (10 μM) and remained unchanged for many hours. Oscillatory patterns recorded during this phenomenon were quantified from extracellular records.

All types of rhythmic activity were measured as described previously (Taccola and Nistri, 2004; Taccola et al., 2003, 2004). Rhythmic discharges were characterized on the basis of their period defined as the time between the onset of two cycles of oscillatory activity. When period values were averaged for a pool of preparations, data from each spinal cord were calculated as the mean of at least 20 cycles or bursts. The regularity of bursting was calculated in terms of coefficient of variation (CV; given by S.D. $mean^{-1}$) of the period. To ascertain if discharges were produced synchronously among various DRs or VRs, we produced cross-correlograms with pCLAMP 9 software (Molecular Devices, Union City, CA, USA) to estimate the correlation coefficient function (CCF), as traces from the selected data set were shifted along themselves incrementally (in "lag"; expressed in ms). When the CCF value was >0.5 at zero ms lag, events were considered to be synchronous (fully synchronous events occurred when $CCF=1$). Peristimulus histograms were calculated to assess quantitatively the reduction in DR oscillation occurrence following a train of DR stimuli. Bins (4 s long) of records prior and immediately after the train were analyzed for their number of events and plotted with pCLAMP 9 software. Data across experiments with DR train stimuli were averaged and their values expressed as oscillation frequency.

All data were expressed as mean±S.D., while "*n*" denoted the number of experiments. Before assessing statistical differences between groups, a normality test was performed to select the use of parametric or non-parametric tests. For comparison between two groups, the following tests were performed depending on data: Student's *t*-test (paired or unpaired) for normally distributed data, and Mann-Whitney test for non-normally distributed data. For multiple comparisons, ANOVA (analysis of variance) tests were used (ANOVA on ranks, in the case of non-parametric data), followed by a post hoc test (Tukey test). Accepted level of significance was $P\leq0.05$. To calculate the concentration of 4-AP producing 50% of the maximum effect (EC_{50} value), 4-AP concentrations were applied in a sequentially incrementing fashion. Individual EC_{50} values were first calculated for each preparation and then averaged for the sample size.

To verify the extent of coronal or sagittal hemisection or the remaining tissue in dorsal quadrants, specimens were fixed at the end of each experiment with paraformaldehyde (4% in Krebs solution). In order to cryoprotect the tissue, specimens were kept overnight in sucrose (30% in Krebs solution), embedded in Tissue-Tek O.C.T. compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands), frozen at −20 °C, and finally sectioned (25 μm) with a cryostat. The sections were mounted on gelatin-coated slides, dehydrated with ethanol and, after staining with Toluidine Blue (1% in distilled water), were examined with a Zeiss Axioskop 2 MOT microscope (5× or 10× magnification; Carl Zeiss AG, Oberkochen, Germany).

The following chemicals were purchased from Tocris (Bristol, UK): 4-AP, NMDA, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), D-amino-phosphonovalerate (APV), nifedipine, tetrodotoxin (TTX). 5-HT, bicuculline, and strychnine were from Sigma, Milan, Italy.

RESULTS

Rhythmic activities induced by 4-AP in the isolated spinal cord

Our previous work on the isolated spinal cord of the neonatal rat has shown that, in the absence of CPG activation, 5 μM 4-AP elicits repetitive discharges from VRs (Taccola and Nistri, 2004). We first examined how these were related to the DR discharges reported in former studies (Kerkut and Bagust, 1995; Ruscheweyh and Sandkuhler, 2003). As exemplified in Fig. 1A, application of 5 μM 4-AP evoked rhythmic discharges detected from DRs and VRs at different segmental level (L2 and L5). On average, the period of 4-AP induced oscillations on DRs was 3.3±0.8 s ($n=10$) with CV value of 0.36±0.11. The 4-AP EC_{50} value for inducing DR discharges was 3±2 μM with a dose Th at 0.1 μM concentration ($n=3$). Conversely, spontaneous DR and VR discharges in the absence of 4-AP were rarely detected (five of 40 spinal cords, namely, 12.5% occurrence).

Root recordings are unsuitable to provide absolute values of discharge amplitude which is mainly determined by the suction electrode access resistance. Hence, in order to evaluate the relative size of the 4-AP discharges, we compared them (on the same preparation) with the synaptic DR-DRP and DR-VRP elicited by a single weak (1.5–2×Th) stimulus applied to one L5 DR. The examples in Fig. 1A (shown at the start of each trace) indicate that DR-VRPs recorded from L2–L5 VRs and DR-DRPs recorded from L2–L5 DRs were all larger in amplitude than the corresponding 4-AP-induced discharges. On average, the peak amplitude of 4-AP-induced DR oscillations was 34±10% of DR-DRPs, while the one of 4-AP-evoked VR oscillations was 25±6% of DR-VRPs ($n=3$).

While 4-AP is known to enhance DR-VRPs significantly (Taccola and Nistri, 2004), in the present experiments it also increased the peak amplitude and area of the DR-DRP by 97±54% ($n=8$; $P<0.01$) and 115±53% ($n=8$; $P<0.01$), respectively. Furthermore, 4-AP-induced poten-

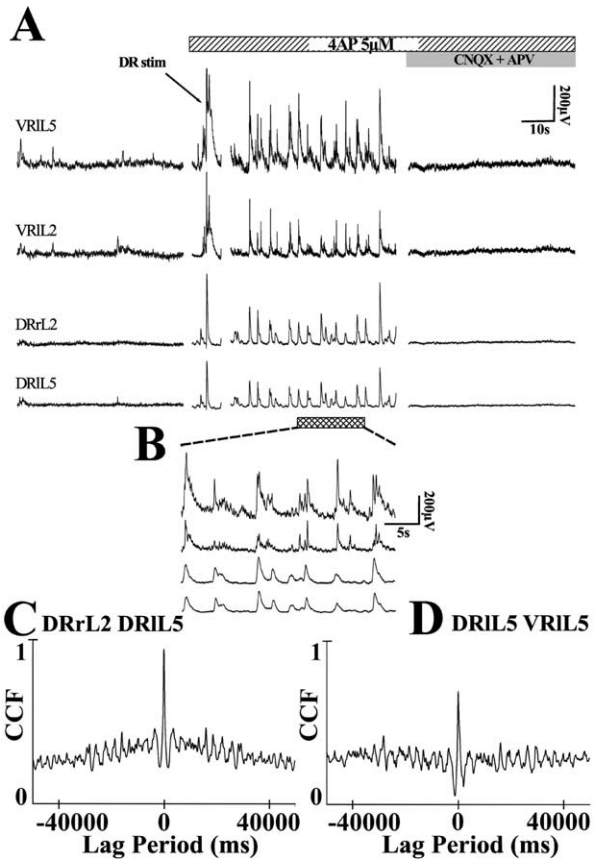

Fig. 1. Rhythmic oscillations induced by 4-AP in the rat isolated spinal cord. (A) l, simultaneous records of VR and DR activity before and during application of 5 μM 4-AP as indicated by dashed horizontal bar. Each trace initially shows control baseline activity, then, in the presence of 4-AP, reflex response induced by electrical stimulation of DR rL5 (DR stim), repetitive oscillations and, finally, complete suppression of all oscillatory activities following application of 10 μM CNQX and 50 μM APV (to block inotropic glutamatergic transmission). (B) Sample records of 4-AP-evoked oscillations recorded on a faster time base taken from records in (A) as shown by cross-hatched bar. (C) Cross-correlogram analysis of synchronous oscillatory coupling between DRrL2 and DRIL5 from the preparation shown in (A). Note sharp peak of CCF value in correspondence with zero lag period to indicate strong synchronicity between events recorded from such roots. (D) Similar cross-correlogram plot between DRIL5 and VRIL5 activity showing limited synchronicity between DR and VR events at the same segmental level.

tiation was not use-dependent because DR-DRPs generated by a train of pulses (40 stimuli, 2 Hz, 2–4×Th applied to a single DR) were uniformly enhanced: the first response in the train was increased (123±49%; $n=9$; $P<0.001$) as much as the last one (96±59%; $n=9$; $P<0.01$).

Application of CNQX (10 μM) plus APV (50 μM) to block ionotropic glutamatergic transmission completely suppressed the 4-AP evoked discharges on all four roots (Fig. 1, r; similar data were obtained on three other preparations) in accordance with previous studies (Kerkut and Bagust, 1995; Ruscheweyh and Sandkuhler, 2003). Application of 20 μM bicuculline to preparations generating robust 4-AP induced oscillations ($n=3$) readily suppressed DR and VR discharges, which were replaced by very slow waveforms (22±8 s period) with highly erratic occurrence (CV=0.74±0.11). The high Th Ca^{2+} channel antagonist nifedipine (20 μM) did not affect the DR and VR oscillations evoked by 4-AP ($n=4$).

Our former investigation reported that VR oscillations evoked by 4-AP are synchronous on VRs (Taccola and Nistri, 2004). Analogous, strong synchronicity (CCF=0.93) was observed between the two DRs even at different segmental level (see cross-correlogram plot in Fig. 1B). However, synchronicity between one DR and the corresponding VR within the same segment (L5) was poor (CCF=0.57; see plot in Fig. 1C) while VR-VR synchronicity had a large (0.90) CCF (not shown). On average, DR-DR discharge temporal coupling was very tight (CCF=0.85±0.17; $n=5$), whereas DR-VR coupling was consistently weaker (CCF=0.59±0.24; $n=13$; $P<0.02$).

Functional coupling of dorsal and ventral networks investigated with spinal sections

A sagittal section of the isolated spinal cord (see Fig. 2A) did not abolish the 4-AP rhythmicity recorded from ipsilateral DRs and VRs. Synchronicity between homolateral DRs was more limited (see cross-correlogram in Fig. 2E with CCF=0.72) than in the whole preparation (cf. Fig. 1C); on four preparations the average CCF value was 0.79±0.14. The cross-correlogram of Fig. 2F shows that for DR-VR coupling the CCF was 0.66 (average CCF=0.65±0.20; $n=5$). For hemisected preparations the 4-AP EC_{50} value for DR discharges was 1.4±0.4 μM with dose Th of 0.1 μM ($n=4$). With the standard dose of 5 μM 4-AP, the DR discharge period was 1.7±0.6 s with CV=0.37±0.10 ($n=9$). On the same preparations bathed in control saline, spontaneous discharges occurred in nine of 17 cases with 4.0±0.7 s period ($P<0.001$ vs 4-AP data) and CV=0.30±0.07 ($P<0.05$ vs 4-AP data).

A different pattern emerged following horizontal sections of the spinal cord as depicted in Fig. 2B. This procedure fully suppressed any VR discharge in the continuous presence of 4-AP (Fig. 2D; $n=7$), while it spared DR ones, indicating that the site of initiation of discharges resided within the dorsal portion of the spinal cord. This observation is consistent with earlier findings in the feline spinal cord in which VR discharges can be produced in part by DR reflex propagation to afferent terminals with consequent induction of excitatory postsynaptic potentials on motoneurons and their firing (Eccles, 1964).

In order to further characterize the properties of oscillations arising from dorsal horn regions, we performed multiple recording from four DRs in the horizontally sectioned preparations as shown in Fig. 3A. Almost complete synchronicity was observed between L2 and L5 DRs on the same side (Fig. 3B; CCF=0.99 for both ipsilateral pairs of DRs), while it was absent between lDRs and rDRs of the same segment (Fig. 3C; CCF=0.38 for l-rL5 and 0.35 for l-rL2). On average, synchronicity between DRs at the same segmental level was not present (CCF=0.36±0.22; $n=10$; $P<0.05$ versus data from isolated preparations),

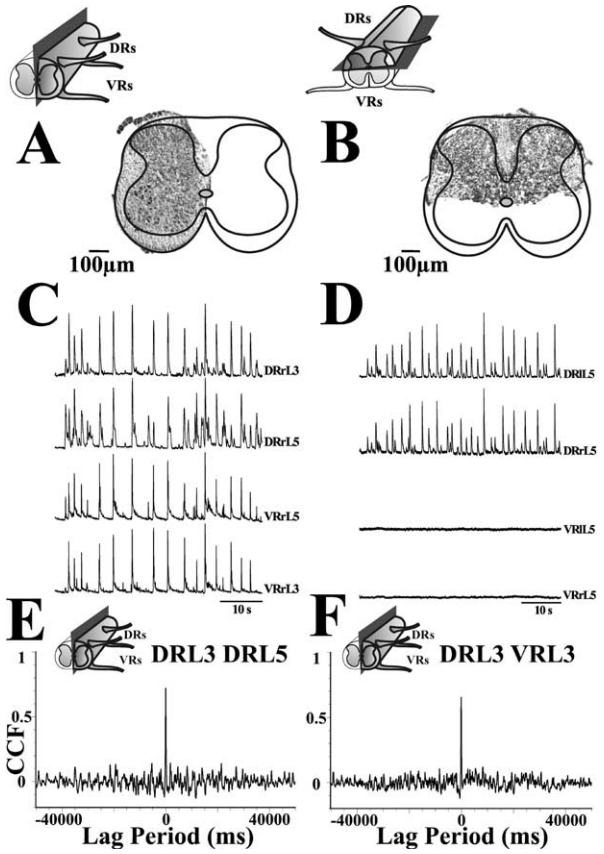

Fig. 2. Effect of spinal cord hemisection on 5 μM 4-AP-evoked oscillations. All data are obtained in the continuous presence of 4-AP. (A) Example of sagittal hemisection with inlaid histological section of the spinal cord. (B) Example of horizontal hemisection with inlaid histological section of the spinal cord. (C) Simultaneous DR and VR records from sagittally hemisected preparation. Note good preservation of oscillation synchronicity. (D) On the horizontally hemisected preparation DRs can still generate oscillatory activity while VRs become silent. (E) Cross-correlogram analysis of DRL3-DRL5 events shown in (C). High CCF value at zero lag indicates good degree of synchronicity. (F), Cross-correlogram analysis of DRL3-VRL3 events from the same sagittally hemisected preparation shown in (C). Note value of CCF suggesting good degree of synchronicity.

while it was strong between DRs on the same side (CCF=0.85±0.21; n=4; P<0.02).

On DRs of horizontally sectioned preparations, the 4-AP EC_{50} value for discharge period acceleration was 1.4±0.8 μM (n=5) with 0.1 μM Th. With 5 μM 4-AP the average discharge period was 2.4±0.7 s (n=5). Raising the concentration of 4-AP was associated with more regular rhythmicity as indicated by the decrement in period CV value which fell from 0.40±0.10 (0.1 μM 4-AP; n=6) to 0.24±0.06 (50 μM 4-AP; n=5). CNQX (10 μM) and APV (50 μM), or TTX (1 μM) fully abolished 4-AP evoked DR discharges of horizontally hemisected preparations (n=6).

In an attempt to identify the minimal spinal structure necessary to support 4-AP evoked DR discharges, responses were recorded from isolated spinal quadrants containing one dorsal horn area and its afferent DR. With this type of preparation discharges induced by 5 μM 4-AP were small (25±1 μV; n=7). To increase the signal-to-noise ratio we then applied 10 μM 4-AP that significantly (P=0.002) increased the mean response amplitude to 67±2 μV (n=7). Average data show that the quadrant discharge period (3.1±1.6 s; CV value=0.46±0.15; n=7) in the presence of 5 μM 4-AP was similar to the one of the horizontally sectioned preparations, and that it was significantly (P<0.002) accelerated (2.0±0.6 s; CV=0.49±015; n=7) when the 4-AP concentrations was 10 μM. These results thus show that a dorsal quadrant already contained the elementary structures necessary for 4-AP elicited DR bursting.

Suppression of 4-AP-induced patterns at 33 °C

Relatively small decreases in temperature are associated with significant rise in input resistance and membrane depolarization of spinal neurons *in vivo* with consequent increase in excitability (Hubbard et al., 1969). Since the present data were collected at room temperature, it seemed possible that this condition heightened neuronal excitability so as 4-AP-evoked discharges on DRs were unusually facilitated. This possibility was tested in experiments, like the one shown in Fig. 4A, on sagittally he-

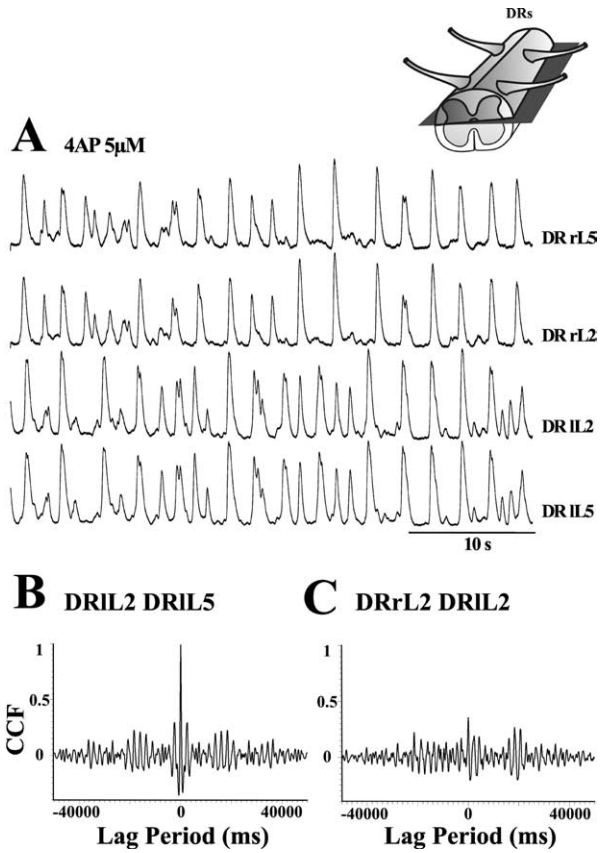

Fig. 3. Analysis of oscillation timing among DRs in a horizontally hemisected preparation. All data are obtained in the continuous presence of 5 μM 4-AP. (A) Simultaneous records of L2 and L5 DR oscillations. (B) Cross-correlogram analysis shows very high CCF value for lL2 and lL5 events indicating very strong synchronicity. (C) Analogous analysis for r and l L2 DRs shows lack of event synchronicity. All data are from the same preparation.

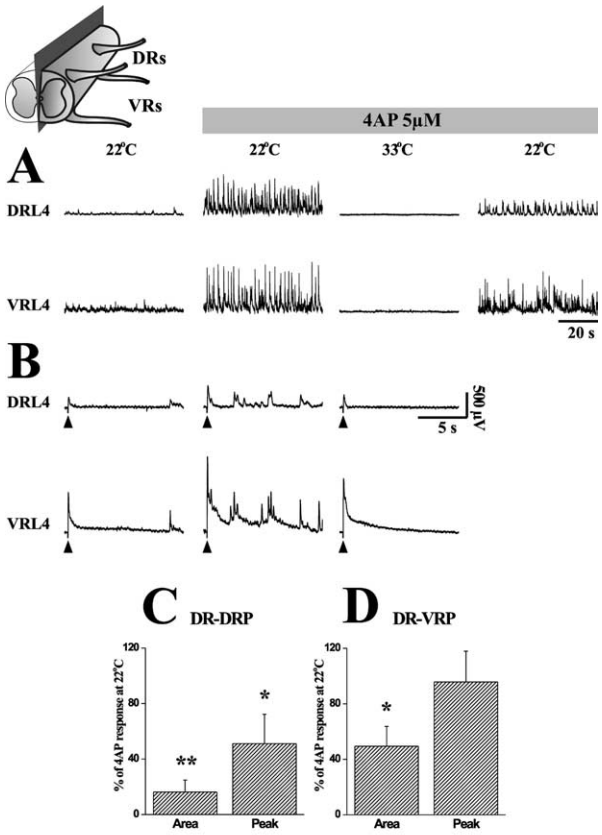

Fig. 4. Warmer recording temperature strongly reduces the action of 4-AP on hemisected preparations. (A) Simultaneous records of L4 DR and VR oscillations in the presence of 4-AP (shaded horizontal bar) at 22 °C. Raising the bath temperature to 33 °C completely suppresses oscillations that partially recover when the bath temperature is again lowered to 22 °C. (B) DR-DRP (top) and DR-VRP (bottom) induced by electrical stimulation (3×Th) of DRL5 (indicated by arrowheads) recorded at 22 °C in control solution (l), in the presence of 4-AP at 22 °C (middle) or at 33 °C (r). (C, D), Histograms of peak amplitude and area of DR-DRP or DR-VRP in the continuous presence of 4-AP at 33 °C. Data are all expressed as percentage of reflex responses in 4-AP solution measured from the same preparations at 22 °C. * $P<0.02$; ** $P<0.05$; $n=8$.

misected preparations ($n=8$) to improve bathing solution exchange. 4-AP was first applied to the preparation bathed with standard saline at 22 °C to induce typical repetitive patterns recorded from L4 DR and VR. The bathing solution was then warmed up (over about 10 min) with gradual reduction in root discharge amplitude which disappeared at 33 °C. Cooling again the same preparation partially restored 4-AP-evoked patterns (Fig. 4A). The result at higher temperature might have originated from either decreased excitability due to the warmer temperature or development of transient hypoxia due to the larger metabolic requirements of the tissue. To explore these possibilities, we next investigated reflex responses evoked by root stimulation at different temperature. In control solution at 33 °C, the DR-DRP peak and area fell to 22±3 and 3±1%, respectively ($n=4$) of those found at 22 °C ($P<0.005$). Likewise, peak and area of DR-VRP became 47±25 and 19±12%, respectively, of control at 22 °C ($P<0.005$). These results indicate that warming the preparation particularly impaired polysynaptic pathways underlying the late components of the reflex responses. When similar experiments were done in the presence of 4-AP (see example in Fig. 4B), the drug-dependent enhancement of the DR-DRP and DR-VRP observed at 22 °C (cf. Fig. 1A) was largely diminished at 33 °C. Pooled data from eight preparations in the presence of 4-AP (Fig. 4C, D) indicate that, at the warmer temperature, together with full loss of repeated patterns from DRs and VRs (Fig. 4A), the reflex area was decreased much more than the peak, which in the case of the DR-VRP was not even significantly impaired.

Interactions between 4-AP induced patterns and chemically-induced fictive locomotion

In Fig. 5A fictive locomotor discharges with typical oscillation alternation (top three traces) were evoked by NMDA (5 μM) plus 5-HT (10 μM). The bottom record of Fig. 5A

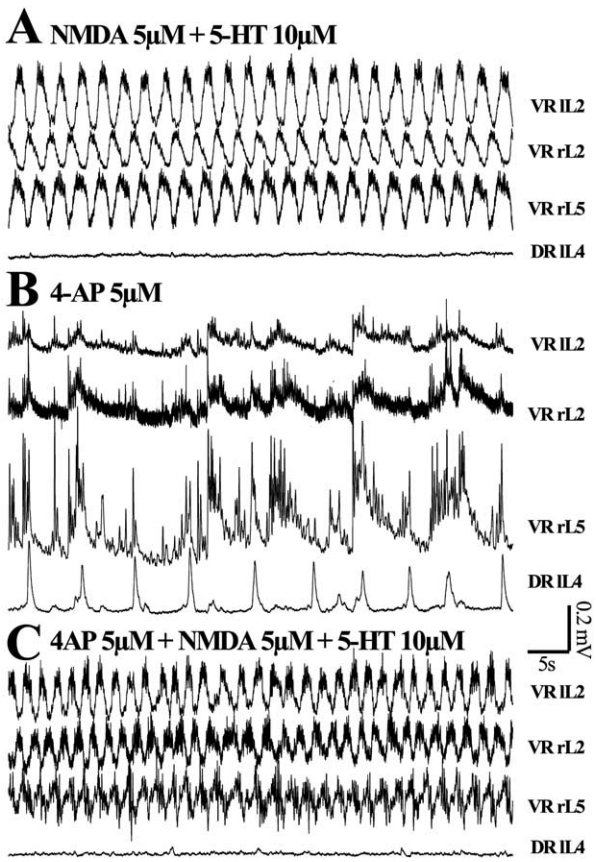

Fig. 5. Interaction between fictive locomotor patterns and the 5 μM 4-AP-induced oscillations recorded from the isolated spinal cord. (A) Typical fictive locomotor patterns evoked by co-application of 5 μM NMDA and 10 μM 5-HT with homo- and intersegmental alternation. Note minimal activity recorded from one DR. (B) After washout of NMDA and 5-HT and disappearance of fictive locomotion, application of 4-AP evokes irregular discharges on all roots. (C) Applying again NMDA and 5-HT to the same preparation in the continuous presence of 4-AP restores fictive locomotor patterns and strongly depresses DR discharges.

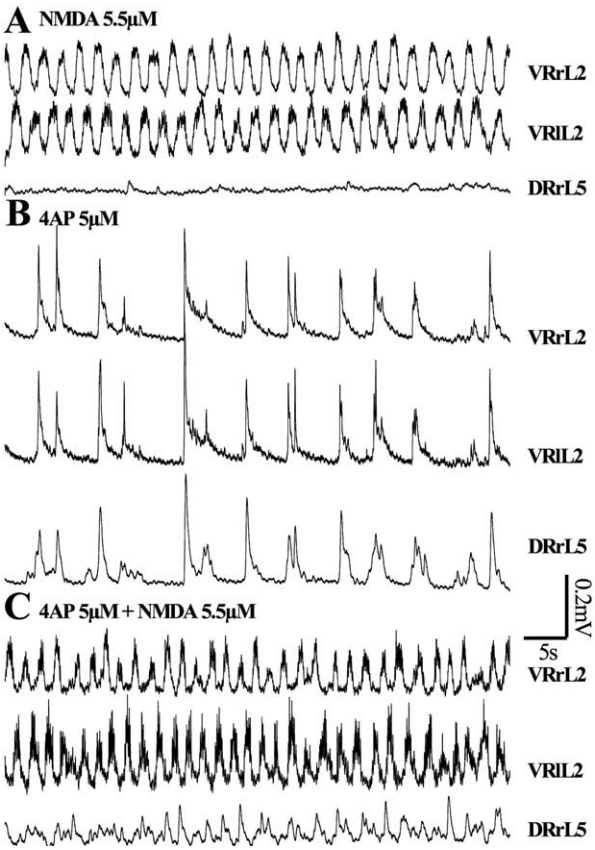

Fig. 6. Interaction between fictive locomotor patterns evoked by NMDA alone and the 5 μM 4-AP induced oscillations recorded from the isolated spinal cord. (A) Application of 5.5 μM NMDA generates stable fictive locomotor patterns with l/r alternation and minimal activity on L5 DR. (B) After washout of NMDA and return to rest, subsequent application of 4-AP induces irregular discharges on all roots. (C) Applying NMDA again restores fictive locomotor patterns and largely reduces the DR discharges. All data from the same preparation.

(same gain as in the other traces) shows that, on lL4 DR, electrical discharges were minimal, in accordance with the very small amplitude oscillations observed with intracellular recording from dorsal afferents of the *in vivo* cat spinal cord (Gossard et al., 1989). After washing out NMDA and 5-HT, application of 4-AP (5 μM) evoked synchronous patterns from VRs, and a large irregular discharge from the L4 DR (Fig. 5B). Subsequent addition of NMDA and 5-HT to the same preparation restored the fictive locomotor pattern and led to strong depression of DR discharges (Fig. 5C). Thus, in eight preparations in the presence of 4-AP, the mean amplitude of DR oscillations fell to 20±6% ($P<0.01$), while their mean period was decreased by 77±9% ($P<0.001$) during fictive locomotion.

Although 5-HT is an important agent to stabilize the locomotor pattern of the isolated rat spinal cord (Kiehn et al., 1999), it also has effects on primary afferent fibers (Levy, 1977) manifested as depression of DR excitability (Sillar and Simmers, 1994; Lopez-Garcia and King, 1996). The latter action might have caused the suppression of DR discharges evoked by 4-AP. To investigate this possibility, locomotor-like patterns were elicited by applying NMDA only as exemplified in Fig. 6A. After washout of NMDA, 4-AP evoked typical repetitive discharges (Fig. 6B) that were largely blocked by subsequent application of NMDA (Fig. 6C). On average, the reduction by NMDA of DR discharges in the presence of 4-AP was 49±18% and 60±9% ($n=6$; $P<0.02$) in terms of period and amplitude, respectively.

Finally, we tested the effect of NMDA or 5-HT on spinal quadrants (Fig. 7A) in the presence of 4-AP (10 μM). Fig. 7B shows that DR bursting induced by 4-AP was accelerated by NMDA that also decreased the pattern amplitude. The average period acceleration in the presence of 5 μM NMDA is reported in Fig. 7C, while the average discharge amplitude decreased by 58±9% ($n=6$; $P<0.001$). On spinal quadrants 10 μM 5-HT strongly inhibited 4-AP discharges (Fig. 7B) with lengthening of bursting period (Fig. 7D) and corresponding fall in discharge amplitude (44±11%; $n=5$; $P<0.05$). These results thus show opposite actions by NMDA and 5-HT on the 4-AP-evoked discharges of isolated dorsal quadrants. Application of 10–50 μM 4-AP to isolated DRs failed to induce electrical discharges ($n=6$).

Interaction between 4-AP-induced discharges and fictive locomotion elicited by DR stimuli

The observation that chemically induced fictive locomotion could inhibit the DR discharges evoked by 4-AP raised the

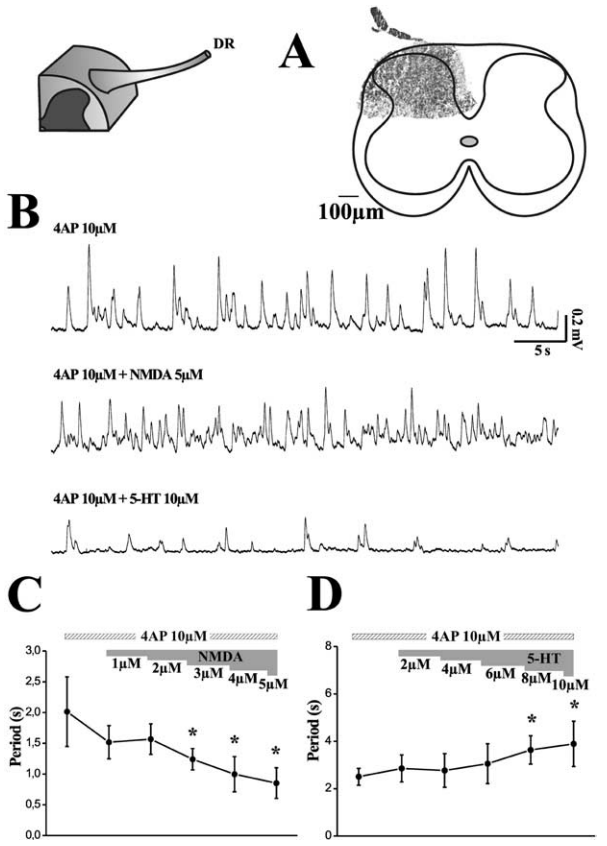

Fig. 7. Oscillatory activity of dorsal quadrants. (A) Schematic diagram of dorsal quadrant with inlaid histological section. (B) Continuous presence of 10 μM 4-AP generates irregular discharges (top) which are accelerated by 5 μM NMDA (middle) and depressed by 10 μM 5-HT (bottom). (C) NMDA dose-related decrease in event period. Asterisks indicate $P<0.001$ ($n=6$). (D) 5-HT dose-related increase in event period. Asterisks indicate $P<0.05$ ($n=5$).

issue whether similar alternating patterns generated by repeated DR stimulation (Marchetti et al., 2001) could also depress the DR discharges. This possibility was explored in experiments like the one depicted in Fig. 8 using an isolated spinal cord preparation bathed in 5 μM 4-AP solution. Under these circumstances the standard rhythm induced by 4-AP appeared on L2 and 5 VRs and one L4 DR. A train of 25 electrical pulses (2.5×Th; 10 Hz; see gray horizontal bar in Fig. 8A, top) was applied to the contralateral rL4 DR to generate an epoch of alternating patterns on VRs, outlasting the train duration and associated with depression of DR discharges (see also expanded traces in Fig. 8B). On 11 preparations the DR train led to an average reduction in DR discharge amplitude of 38±14% ($P<0.001$) measured over the time period up to the last VR alternating discharge. The peristimulus histograms of Fig. 8C quantify the depression in discharge frequency of DRs immediately following a train of DR stimuli: a significant ($P<0.001$) fall was transiently detected in coincidence with the bin just after the DR train, while discharge rate recovery occurred in the subsequent bins ($n=11$).

Contribution by GABAergic and glycinergic transmission to 4-AP-induced DR discharges

Application of strychnine (1 μM) and bicuculline (20 μM) abolished DR discharges induced by 4-AP (5 or 10 μM) in isolated spinal cord ($n=5$), horizontally hemisected ($n=7$) as well as DR quadrant ($n=7$) preparations. One experiment is shown in Fig. 9A in which, in the presence of strychnine and bicuculline, recording from one VR of the intact spinal cord displayed typical disinhibited bursting (Bracci et al., 1996), while the corresponding ipsilateral DR generated minimal responses. Subsequent application of 4-AP (5 μM) led to acceleration of VR disinhibited bursting without appearance of typical DR discharges (Fig. 9B). Average speeding up of VR rhythmicity by 4-AP was significant (Fig. 9C).

Even if strychnine and bicuculline prevented the expression of 4-AP-evoked discharges from DRs, it seemed interesting to investigate any contribution by dorsal horn areas to the disinhibited bursting recorded from VRs. For this purpose the ventral area of horizontally hemisected preparations was used to study disinhibited bursting (Bracci et al., 1996; see example in Fig. 9D) which was accelerated by 5 μM 4-AP (Fig. 9E) and made more regular because the period CV value fell from 0.42±0.24 to 0.07±0.02 in the presence of 4-AP ($n=4$; $P<0.05$). Average period acceleration is shown in Fig. 9F. On horizontally sectioned preparations bathed in standard control solution without strychnine and bicuculline, 4-AP (5 μM) could not generate oscillatory discharges from VRs ($n=7$; not shown).

DISCUSSION

The principal finding of the present study is the demonstration that the DR oscillations evoked by low micromolar concentrations of 4-AP and originating within dorsal horn areas did not interfere with the fictive locomotor program expressed by the rat isolated spinal cord. Onset of fictive locomotion actually inhibited 4-AP-evoked DR discharges,

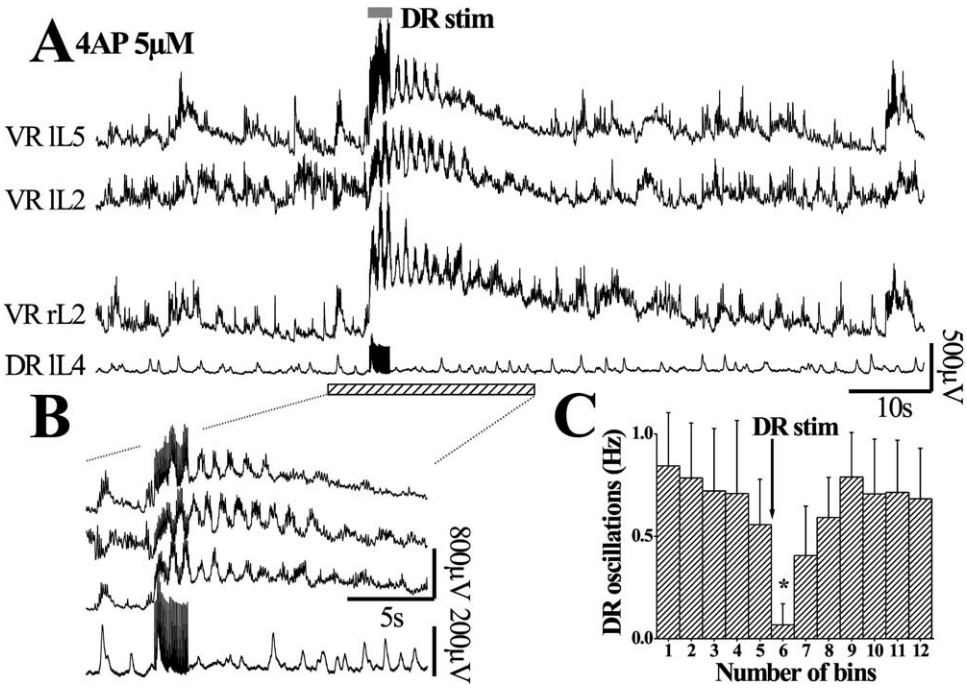

Fig. 8. Influence of DR train stimuli on 4-AP-induced DR oscillations. (A) Representative traces of discharges recorded from three VRs and one DR during continuous application of 5 μM 4-AP. The gray horizontal bar indicates application of a train of stimuli (10 Hz) to the contralateral rL4DR to induce VR cumulative depolarization with superimposed alternating patterns. Meanwhile, the lL4DR discharges are temporarily depressed. (B) Higher amplification of the record segments indicated with dashed bar in (A) to show depression of DR discharges in coincidence with VR patterns. (C) Peristimulus histograms show significant depression of DR discharge frequency immediately after the train of stimuli applied the contralateral DR. * $P<0.001$; $n=11$.

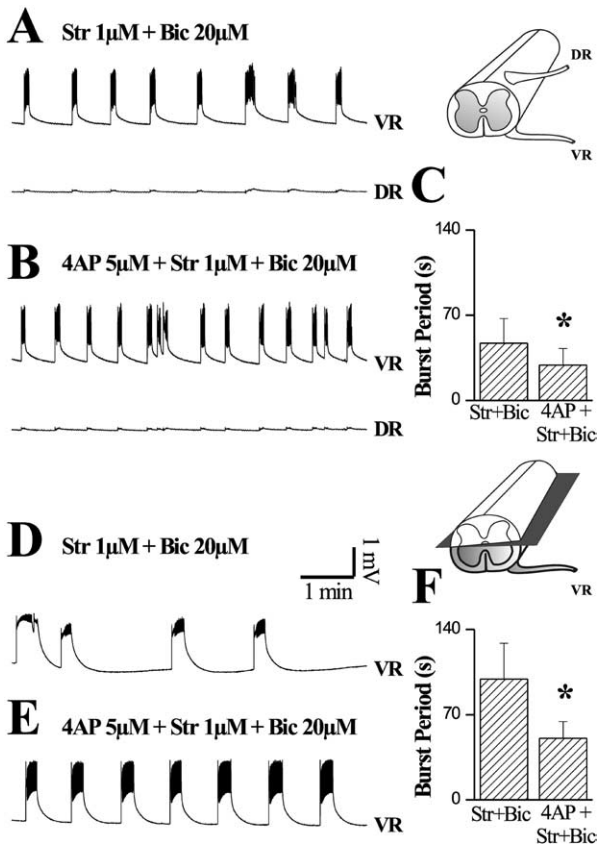

Fig. 9. Effect of 4-AP on disinhibited bursting evoked by strychnine and bicuculline. (A) Regular rL5 VR bursts are associated with small corresponding signals on contralateral L5 DR in an isolated spinal cord preparation (see inset). (B) Application of 5 μM 4-AP accelerates bursting. (C) Histograms to quantify the VR burst acceleration by 4-AP (5 μM); * $P<0.001$; $n=11$. (D, E) VR recording from a horizontally hemisected preparation (see inset) shows persistence of disinhibited bursting which is accelerated by 4-AP (5 μM). (F) Histograms to quantify the VR burst acceleration by 4-AP (5 μM) in horizontally hemisected preparations; * $P<0.01$; $n=5$.

indicating that the locomotor CPG believed to reside in ventral horn areas (Kjaerulff and Kiehn, 1996; Kremer and Lev-Tov, 1997; Kiehn and Butt, 2003) was a powerful gain-setter of these oscillations arising from overactivity within dorsal networks.

Cellular processes underlying DR discharges

The excitability of DR fiber terminals is controlled by various mechanisms which collectively contribute to the process of presynaptic inhibition of peripheral inputs manifested as depressed presynaptic release of the excitatory transmitter glutamate (Rudomin and Schmidt, 1999). One major contributor is activation of GABA receptors, while raised extracellular K^+ and activation of presynaptic ionotropic glutamate receptors may also be involved in reducing fiber excitability (Lee et al., 2002; Bardoni et al., 2004). Although it is debatable what cellular processes are ultimately responsible for presynaptic inhibition (Stuart and Redman, 1992), it seems likely that substantial depolarization of presynaptic terminals of primary afferents can inhibit transmitter release via inactivation of their sodium and/or calcium channels. Under control conditions the isolated spinal cord can even spontaneously generate small, random DR discharges (Ryan et al., 1984; Kerkut and Bagust, 1995), indicating that the local circuitry underlying DR discharges is entirely contained within the spinal cord and exhibits a degree of ongoing activity. Much slower discharges are also detected from DRs in the presence of bicuculline and attributed to fluctuation in extracellular K^+ (Kremer and Lev-Tov, 1998), a phenomenon confirmed in the present study. In standard saline solution spontaneous DR and VR discharges rarely occurred (12%) in the whole spinal cord preparations and had slower periodicity than the ones evoked by 4-AP.

Although the present study is ill suited to unveil the molecular mechanisms underlying the electrical oscillations induced by 4-AP on dorsal afferents, it accords with previous investigations (Ruscheweyh and Sandkuhler, 2003), indicating that blockers of glutamate, glycine and $GABA_A$ receptors suppressed DR oscillations evoked by 4-AP. It is therefore apparent that DR oscillations elicited by 4-AP required a dorsal horn network comprising glutamatergic, glycinergic and GABAergic transmission, and that they were not merely caused by transient changes in extracellular K^+ (Kremer and Lev-Tov, 1998). For sake of simplicity we shall term this structure the "4-AP oscillatory network" and we shall assume it capable of exciting DR fibers strongly and in a sustained manner via enhanced excitability of network elements (Ruscheweyh and Sandkuhler, 2003). Our interpretation is compatible with observations suggesting the presence (within the dorsal horn) of a network capable of generating fast oscillations after focal depolarization (Asghar et al., 2005). Russo and Hounsgaard (1996) found that in some dorsal horn cells of the turtle spinal cord the interaction between plateau potentials and a slow membrane depolarization can produce damped oscillations in which L-type calcium channels play a major role. In the present experiments the L-type calcium channel blocker nifedipine failed to affect 4-AP oscillations of DRs, despite the fact that the same concentration of nifedipine inhibits oscillations elicited by metabotropic glutamate receptor activation (Taccola et al., 2003). It seems therefore likely that the 4-AP-induced discharges did not rely on L-type calcium channel activity.

Temperature dependence of 4-AP-induced discharges

The use of ambient temperature for electrophysiological recording is a standard tool to optimize long survival of rodent spinal cord preparations *in vitro* (Deshpande and Warnick, 1988; Kerkut and Bagust, 1995). However, lowering the temperature of the *in vivo* feline spinal cord increases neuron input resistance and produces membrane depolarization with associated rise in excitability (Hubbard et al., 1969). In theory then, the rat spinal cord at ambient temperature may mimic this condition so as to strongly facilitate 4-AP-evoked oscillations: this might then represent an experimental circumstance very different from the one found *in vivo*. Indeed, increasing recording

temperature of the rat spinal cord from 22° to 33 °C fully suppressed 4-AP oscillations (with partial recovery returning to ambient temperature). This phenomenon was accompanied by a large reduction in the area of electrically evoked reflexes. Nevertheless, in the absence of 4-AP, the reduction in reflexes at the warmer temperature was actually stronger. The simplest interpretation of these data is therefore that 4-AP retained its ability to potentiate electrically evoked synaptic transmission (and network dependent discharges) even at higher temperature. However, at 33 °C *in vitro*, this effect was counterbalanced by the functional loss of a large fraction of polysynaptic circuits necessary to support the onset of oscillatory activity probably because of hypoxic mismatch between increased metabolic demand and relative lack of oxygen supply in a preparation where O_2 is delivered by diffusion only (Wilson et al., 2003). It is noteworthy that, even in the feline spinal cord *in vivo*, 4-AP elicits oscillations recorded from DRs and VRs (Dubuc and Rossignol, 1989a,b), suggesting that *in vitro* rat spinal cord preparations used at ambient temperature to retain their functional network activity are suitable models to study the action of 4-AP.

Spinal topography of 4-AP-evoked oscillations

Although, in the presence of 4-AP, oscillations could be recorded from DRs and VRs, their timing displayed certain topological characteristics. In fact, against strong intrasegmental synchrony between a pair of DRs or a pair of VRs, there was modest synchronicity between DRs and the corresponding VRs within the same segment. Furthermore, the isolated ventral region per se could not express 4-AP-induced oscillations, even if 4-AP accelerated the disinhibited rhythm in the same ventral region. The latter result, thus, demonstrates ventrally located, 4-AP-sensitive sites unable to trigger oscillations. Evidently, the main origin of the 4-AP rhythmicity was in the dorsal horn areas where the 4-AP oscillatory network was purported to be, and was relayed with some delay (or even filtered out) to motoneurons because of the limited synchronicity between DRs and VRs. This notion also suggests that mere presence of 4-AP-sensitive sites is insufficient to generate oscillations and that oscillations are a topographical (presumably network-dependent) property rather than just the expression of generalized heightened excitability.

The persistence of oscillations in the dorsal horn areas of horizontally sectioned preparations indeed confirmed that such discharges originated dorsally as supported by their presence even in isolated dorsal quadrants. Their strong homolateral intersegmental phase coupling suggests that projection interneurons might have been responsible for synchronicity on the same side. Vice versa, the lessened coupling between l and r DR discharges indicated that ventral regions contributed to regularize DR rhythmicity (as actually observed in the isolated spinal cord) perhaps because coupling between l and r DRs required an intact pathway coursing via a region ventral to the central canal.

Interactions between the 4-AP oscillatory network and the locomotor CPG

Previous experiments have shown how activation of fictive locomotion by NMDA plus 5-HT led to suppression of the synchronous rhythm evoked by 4-AP on VRs (Taccola and Nistri, 2004). The present study shows that fictive locomotion suppressed synchronous DR oscillations as well. This finding could be due to two main causes: (1) functional suppression by CPG activity of the 4-AP oscillatory network; (2) direct inhibition by NMDA and 5-HT of primary afferent oscillations. Although the second hypothesis is particularly relevant to 5-HT which has been proposed to be involved in presynaptic inhibition (Thompson and Wall, 1996), it has been recently suggested that NMDA receptors on primary afferents can also down-regulate fiber excitability (Bardoni et al., 2004).

To examine these possibilities, experiments were carried out on isolated spinal cord preparations in the presence of NMDA alone, demonstrating that for suppression of 4-AP-evoked DR oscillations it was not necessary to have the combined application of NMDA and 5-HT. NMDA per se maintained such an effect whenever fictive locomotion was generated. Tests on isolated DR quadrants (which had obviously lost the ventrally located CPG) demonstrated that 5-HT could still slow down the 4-AP oscillations, whereas NMDA actually accelerated them. Hence, while 5-HT had probably a direct depressant action on DR excitability, NMDA did not. Any block of 4-AP oscillations by NMDA thus implied functional activation of the CPG as actually supported by the experiments when the CPG activity was evoked by pulse trains applied to a single DR. The simplest interpretation is therefore that the operation of the CPG switched off the 4-AP oscillatory network. Note that, in the rat spinal cord, standard fictive locomotor patterns were not detectable from DRs, a result which accords with the report that antidromic DR firing during fictive locomotion is observed only when extracellular Cl^- is halved to boost GABAergic signaling (Vinay et al., 1999).

A scheme to account for rhythmic oscillations in the rat spinal cord

Fig. 10 is a simplified block diagram to illustrate how the 4-AP oscillatory network and the locomotor CPG might interact. It should be emphasized that this is an idealized scheme drawn on the basis of functional data reported in the present experiments and requiring future validation on the basis of histological results. Its value is therefore heuristic rather than anatomically descriptive.

Since distinct spinal lumbar segments expressed analogous oscillatory activity, this finding is compatible with the notion that oscillatory networks are arranged in a modular fashion (Cheng et al., 1998). 4-AP primarily activated dorsal horn regions (Ruscheweyh and Sandkuhler, 2003) which sent strong synaptic inputs to DRs. In the absence of 4-AP the likelihood to detect spontaneous oscillations from DRs was low probably because these responses were perhaps inhibited by contralateral networks, as suggested by their higher probability of occurrence in hemisected

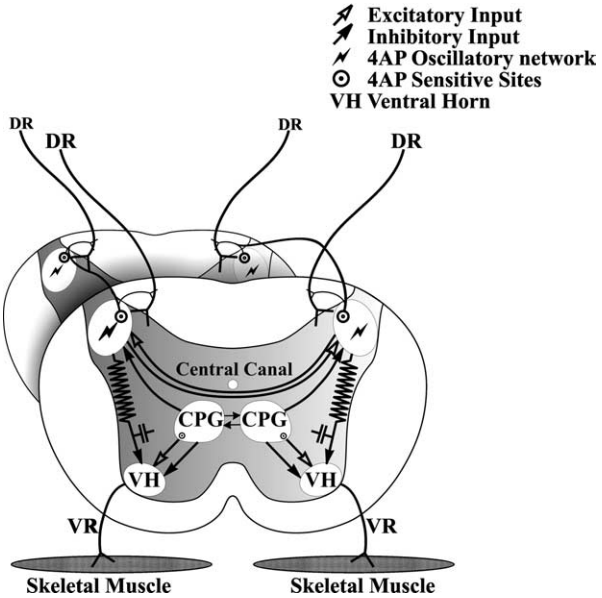

Fig. 10. Idealized wiring diagram of oscillatory circuits in the rat spinal cord. The locomotor CPG is conventionally represented as two ventrally located components (with cross-inhibitory links) projecting to ventral horn areas excitatory and inhibitory signals for agonist and antagonist muscles of the same limb. The locomotor CPG also projects inhibitory inputs to the dorsal network responsible for 4-AP-evoked DR oscillations so as fictive locomotor patterns can depress DR oscillatory activity. When the CPG is inactive, dorsal networks are activated by 4-AP and synchronized via commissural pathways coursing below the central canal. Signals from such networks are then transmitted to afferent DR fibers to express DR oscillations. The 4-AP sensitive dorsal networks also activate ventral horn neurons with some delay (represented by a resistor and a capacitor). Strong coupling of 4-AP dependent DR oscillations on the same side is supposed to be due to heterosegmental ipsilateral connections among dorsal horn networks. The extent of distribution of 4-AP sensitive sites within the CPG or dorsal horn areas is indicated with ⊙ of different size.

preparations. In the presence of 4-AP discharges from such dorsal horn regions were phase coupled among DRs on the same side and, through ventrally coursing projections, with analogous dorsal horn areas on the other side. Inputs from the 4-AP oscillatory network to ventral horn areas including motoneurons were probably phase shifted and filtered via polysynaptic pathways rather than large modulation of intrinsic conductances of ventral horn neurons. In fact, the strong regularity of the VR disinhibited bursting in the presence of 4-AP after removal of the dorsal areas suggests that the intrinsic bursting properties of these regions were not directly altered by 4-AP. Furthermore, low concentrations of 4-AP did not change motoneuron spikes or axon conduction (Taccola and Nistri, 2004).

The operation of the CPG was expressed as alternating patterns recorded from VRs together with concurrent depression of the 4-AP evoked DR oscillations, presumably via an inhibitory input to the 4-AP oscillatory network. Previous studies have shown how the locomotor CPG produces a particularly strong program that can switch off other forms of spinal rhythmicity (Whelan et al., 2000). In the present scheme the block by CGP operation of the synchronous VR oscillations due to 4-AP was reputed to be indirect, via suppression of the dorsally located 4-AP oscillatory network.

Functional implications

The current investigation cast some light to the complex issue regarding the usefulness of 4-AP administration to spinal injury patients and the potential intervening risks. Clearly, any strong discharges of afferent axons by 4-AP might have been expected to induce pain as well as to disrupt the proprioceptive signals modulating CGP output, thus blocking any attempt to restore locomotion. This scenario, at least from the merely experimental angle of the *in vitro* preparation of the rat spinal cord, did not materialize. DR oscillations were suppressed as soon as the operation of the CPG started. Nevertheless, it is difficult to predict what the clinical consequences of DR oscillations by 4-AP in the absence of CPG operation might be. Pain is reported to be a relatively rare side effect of 4-AP treatment (Grijalva et al., 2003). Perhaps DR oscillations, the amplitude of which was observed to be relatively small with respect to synaptically evoked responses, could actually depress nociceptive inputs in a manner akin to presynaptic inhibition. Clinical electrophysiological studies in patients will be necessary to clarify this issue.

In summary, the present study identified the topography of 4-AP-evoked oscillatory activity within the rat spinal cord and demonstrated that repeated DR oscillations did not interfere with the locomotor network operation which actually suppressed the DR discharges.

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LOW MICROMOLAR CONCENTRATIONS OF 4-AMINOPYRIDINE FACILITATE FICTIVE LOCOMOTION EXPRESSED BY THE RAT SPINAL CORD *IN VITRO*

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Abstract—Upregulating the operation of spinal locomotor networks is one mechanism to restore, at least partially, lesion-impaired locomotion. We investigated if the K^+ channel blocker 4-aminopyridine (4-AP) could facilitate spinal locomotor networks in addition to its well-known effect on motor nerve conduction. Fictive locomotor patterns were recorded from ventral roots (VRs) of the isolated spinal cord of the neonatal rat. 4-AP (0.1–50 μM) produced synchronous VR oscillations which did not develop into fictive locomotion. These oscillations had network origin, required intact glutamatergic transmission and were probably amplified via electrotonic coupling because of their depression by the selective gap junction blocker carbenoxolone. 4-AP (5 μM) slightly increased input resistance of lumbar motoneurons without affecting their action or resting potentials. Dorsal root (DR) evoked synaptic responses were enhanced (217±65%) by 5 μM 4-AP without changes in axon conduction. 4-AP (5 μM) accelerated fictive locomotion induced by *N*-methyl-D-aspartate (NMDA) and serotonin (5-HT) without altering cycle amplitude and facilitated the onset of fictive locomotion in the presence of sub-threshold concentrations of NMDA and 5-HT. Furthermore, in the presence of 4-AP, weak DR stimuli, previously insufficient to activate locomotor patterns, generated alternating VR discharges. Thus, although 4-AP per se could not directly activate the locomotor network of the spinal cord, it could strongly facilitate the locomotor program initiated by neurochemicals or electrical stimuli. These data suggest that the reported improvement by 4-AP in locomotor activity of spinal-injury patients may include activation of locomotor networks when low concentrations of this drug are administered in coincidence with appropriate stimuli.

Key words: central pattern generator, motoneuron, spinal cord lesion, rhythmic patterns, oscillations, cumulative depolarization.

The K^+ channel blocker 4-aminopyridine (4-AP; Fampridine®) is currently undergoing phase III clinical trials for the symptomatic treatment of incomplete chronic spinal cord injuries (see http://www.acorda.com). Despite the large amount of clinical and animal studies demonstrating the benefits of 4-AP in restoring part of lost function (Blight and Gruner, 1987; Hansebout et al., 1993), up to date the mechanism and site of action of the 4-AP effects remain unclear. This is an important issue because understanding the action of 4-AP on spinal neurons may help to devise more effective strategies to mitigate the devastating outcome suffered by chronic spinal-injured patients.

Although early reports have indicated that 4-AP facilitates transmitter release from central (Tokunaga et al., 1979) and peripheral (Molgo et al., 1977) synapses, the current notion is that 4-AP may help to re-establish axon conduction in those fibers spared by the first traumatic event, but devoid of their myelin sheath as a consequence of secondary damage triggered by initial impact (Nashmi and Fehlings, 2001). However, the high concentration of 4-AP necessary to improve axon conduction in the white matter (Fehlings and Nashmi, 1996) can induce seizures when applied to the whole spinal cord (Haghighi et al., 1998) and is much larger than the one detected in human serum (Segal et al., 2000) after administration of Fampridine®, the sustained-release, clinically effective form of 4-AP (Darlington, 2000).

The aim of the present study was to investigate if low concentrations of 4-AP, comparable to those attained *in vivo*, might activate the central pattern generator (CPG) of locomotion or affect the excitability of motoneurons directly. For this purpose we used, as an experimental model, the neonatal rat isolated spinal cord (Kerkut and Bagust, 1995) which is an advantageous tool to investigate spinal network properties expressed as well-defined motor output from ventral roots (VRs).

In the rat spinal cord *in vitro*, the spinal locomotor CPG can be activated by applying excitatory agents, such as *N*-methyl-D-aspartate (NMDA) and serotonin (5-HT; Kiehn and Kjaerulff, 1998; Butt et al., 2002), or by delivering a high frequency train of electrical stimuli to a dorsal root (DR; Marchetti et al., 2001). Our present data suggest that the beneficial effects of 4-AP on partially paraplegic people may be, at least in part, due to a direct increase in excitability of the CPG. This action appeared at rather low 4-AP concentrations, indicating that the spinal CPG represents a very sensitive target for this substance.

EXPERIMENTAL PROCEDURES

In accordance with NIH guidelines and the Italian act DL 27/1/92 n. 116 (implementing the European Community directives n. 86/609 and 93/88), experiments were performed on lumbar spinal cord preparations isolated from neonatal Wistar rats (0–5 days old) under urethane anesthesia (0.2 ml i.p. of a 10% w/v solution).

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Abbreviations: APV, D-amino-phosphonovalerate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CPG, central pattern generator; CV, coefficient of variation; DR, dorsal root; EPSP, excitatory postsynaptic potential; l, left; MN, motoneuron; NMDA, *N*-methyl-D-aspartate; r, right; S.D., standard deviation; Th, threshold; TTX, tetrodotoxin; VRs, ventral roots; 4-AP, 4-aminopyridine; 5-HT, serotonin; Φ, mean phase.

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All efforts were made to reduce the number of animals used and to minimize animal suffering. The experimental setup was the same as described by Marchetti et al. (2003), while full details about procedures have been previously published (Bracci et al., 1996a,b; Beato and Nistri, 1999). In brief, the neonatal rat spinal cord was superfused (7.5 ml min^{-1}) 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% O_2–5% CO_2; pH 7.4 at room temperature. All agents were bath-applied via the superfusing solution at the concentrations mentioned in the text. In view of the need to record fictive locomotion rhythms for a long time, the majority of experiments were based on DC-coupled recordings from lumbar VRs. For these extracellular data, records are calibrated in terms of time only, as their amplitude is dependent on the access resistance of the suction electrodes.

Intracellular recordings (from L3–L5 motoneurons) were obtained using sharp electrodes filled with 3 M KCl (30–60 MΩ resistance). The input resistance of motoneurons was measured by delivering hyperpolarizing current steps (0.1–0.9 nA, 30–50 ms) through the intracellular electrode and constructing I/V curves which were linear within the voltage range considered. Measurements of wind-up and cumulative depolarization (Barbieri and Nistri, 2001) always relied on simultaneous motoneuron and VR recordings. DR electrical stimuli employed to elicit either single VR responses (recorded from the ipsilateral VR of the same segment) or cumulative depolarization, had intensity (1–10 V range; 0.1 ms duration) expressed as multiples of threshold (Th) necessary to evoke a detectable synaptic response from the recorded motoneuron (see Marchetti et al., 2001). DR induced responses were averages of at least three events. VR stimulation (1–5 V; 0.1 ms) was used to elicit antidromic spikes.

Fictive locomotion was typically induced by continuously bath-applied NMDA (4 or 5 μM) plus 5-HT (10 μM; see Kiehn and Kjaerulff, 1998; Butt et al., 2002). Rhythmic discharged were characterized on the basis of their period defined as the time between the onset of two cycles of oscillatory activity. When period values were averaged for a pool of preparations, data from each spinal cord were calculated as the mean of at least 30 cycles or bursts. The regularity of bursting was calculated in terms of coefficient of variation (CV; given by standard deviation [S.D.] $mean^{-1}$) of the period. To calculate axon conduction velocity, isolated DRs were electrically stimulated at one end while the compound action potential was recorded at the other end and the length of the root measured with a calibrated caliper under the microscope. Data were quantified as means±S.D.; statistical significance was assessed with the Student's *t*-test, or ANOVA plus Tukey test for non-parametric data. The Rayleigh test was used for statistical significance of phase coupling (Drew and Doucet, 1991) where *R* is the concentration of phase values around the mean phase (see Marchetti et al., 2001). The accepted level of significance was always $P=0.05$.

The following chemicals were purchased from Tocris (Bristol, UK): 4-AP, NMDA, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), D-amino-phosphonovalerate (APV), tetrodotoxin (TTX). 5-HT and carbenoxolone were from Sigma, Milan, Italy.

RESULTS

The database of the present study comprises 17 intracellularly recorded motoneurons with −77±5 mV resting potential and 37±13 MΩ input resistance. VR responses were collected from 71 spinal cord preparations.

Effects of 4-AP on spinal motoneurons and network properties

Application of 4-AP (5 μM) to control spinal cord preparations did not induce significant depolarization of the motoneuron membrane (0.7±1.2 mV; $n=9$) and only a small increase in input resistance (109±10%; $P<0.05$). The Th for the antidromic spike generation by motoneurons was unchanged (105±15% versus control), like the spike amplitude (96±16%) and halfwidth (103±16%). Despite lack of substantial changes in motoneuron properties, sustained application of 4-AP elicited a discharge pattern recorded extracellularly and intracellularly (Fig. 1A) characterized by rhythmic depolarizing waveforms with superimposed spikes. This phenomenon was apparent after 20±5 min from the start of application, persisted as long as 4-AP was bath-applied (1–2 h) and, despite extensive washout (≥1 h), it could not be completely eliminated. As exemplified in Fig. 1A, discharges were more complex and longer lasting when recorded from the VR than from a single motoneuron. The rhythmic discharges were readily blocked (within 169±42 s from the start of application;

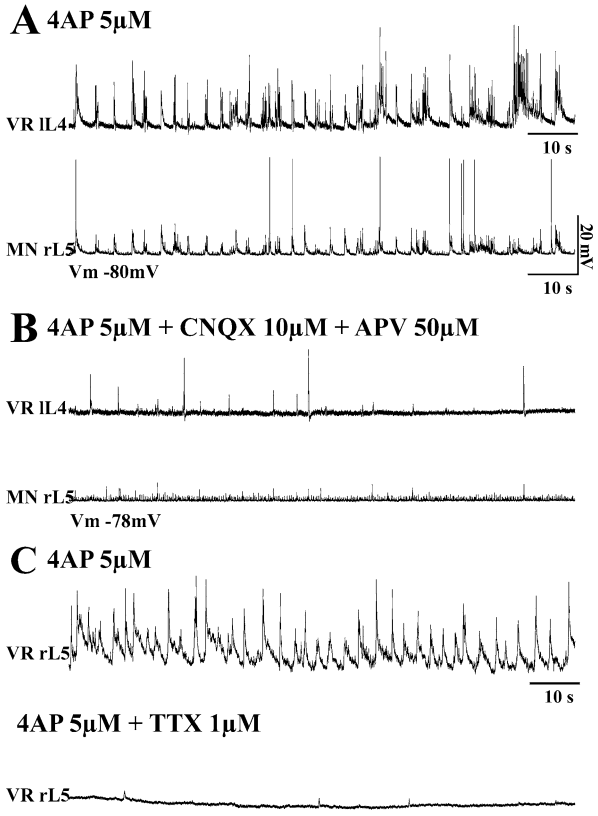

Fig. 1. Spinal network oscillations induced by 4-AP. (A) Simultaneous records from left lumbar VR 4 (lL4; top) and single right lumbar 5 motoneuron (rL5 MN) obtained after 20 min continuous application of 5 μM 4-AP. Note irregular discharges appearing synchronously on VR and contralateral motoneuron. Vm indicates resting membrane potential of motoneuron. Fast deflections are truncated action potentials. (B) On the same preparation depicted in (A), strong suppression of oscillations by CNQX (10 μM) and APV (50 μM). (C) 4-AP-induced (5 μM) oscillations recorded from rL5 VR (different preparation from A, B) are abolished by TTX (1 μM).

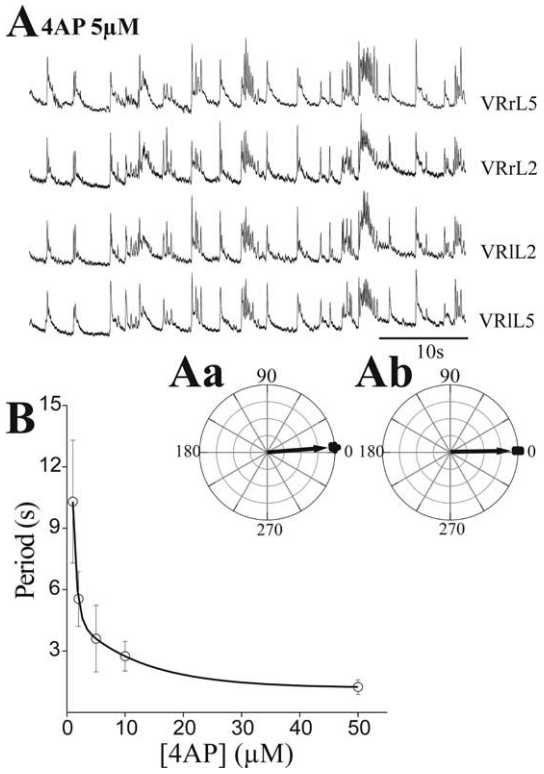

Fig. 2. Strong synchrony of 4-AP induced oscillations. (A) Simultaneous recording from four VRs (left and right L2, and left and right L5) show strong synchronous oscillations after 20 min exposure to 4-AP (5 μM). Note also irregular occurrence and shape of individual discharges. (Aa) and (Ab) show polar plots of phase discharge coupling for right L2 and right L5 VRs (Aa) or for left and right L2 VRs (Ab). Data points are grouped around 0° indicating full phase coincidence. The length of the vector (arrows) represents the strength of coupling between phases calculated for 20 cycles indicated by filled circles (due to the close similarity of values, data points largely overlap). (B) Dose response graph for the oscillation period value produced by concentrations of 4-AP ranging from 1 to 50 μM; $n=6$ preparations.

$n=7$) by CNQX (10 μM) and APV (50 μM) as exemplified in Fig. 1B for the same preparation shown in Fig. 1A. These observations suggested that the rhythm had a network origin, a notion confirmed by its suppression (within 246±48 s from the start of application; $n=4$) by TTX (1 μM; see example in Fig. 1C with extracellular recording).

Characteristics of rhythmic discharges induced by 4-AP

The network origin of such a pattern prompted exploring its main properties in terms of VR synchronicity and periodicity. Bursts were identified as sudden depolarizations exceeding the S.D. of the baseline noise by at least 10-fold. Fig. 2 shows that rhythmicity induced by 4-AP was synchronous on four lumbar VRs on either side of the spinal cord and was confirmed with the polar plots depicted in Aa for coupling between right L2 and right L5 VRs (mean phase [Φ]=4.1±1.3°; $n=20$ cycles), and in Ab for coupling between left and right L2 VRs (Φ=1.5±0.9°; $n=20$ cycles). Significant coupling for such responses was demonstrated with the Rayleigh test ($P<0.05$ for either pair). The period of discharges was dependent on the concentration of 4-AP as indicated by the plot in Fig. 2B, in which for six preparations concentrations of 4-AP in the range 1–50 μM were applied at 30 min intervals in a cumulative fashion. Concentrations higher than 50 μM produced chaotic bursting and were not further used. We also explored the Th concentration of 4-AP required to elicit patterned discharges. Doses of 0.1–0.5 μM evoked occasional cycles of rhythmic activity but no stable pattern. These results indicate that 4-AP was a potent agent to trigger spinal rhythmicity at concentrations between 1 and 50 μM. The average period induced by 5 μM 4-AP was 3.9±1.1 s ($n=32$). Despite its synchronicity, the pattern was rather irregular as quantified by its relatively large value of period CV (0.43±0.11).

While the drive for 4-AP evoked bursting was crucially dependent on glutamatergic ionotropic transmission (see Fig. 1B), its synchronicity suggested that additional mechanisms were necessary to recruit and synergize the discharge of neurons throughout spinal segments. In view of the histological and functional reports of gap junctions among rat spinal neurons (Fulton et al., 1980; Walton and Navarrete, 1991; Kiehn and Tresch, 2002), we tested the possibility that gap junctions were involved in synchronization of 4-AP evoked discharges. For this purpose, once bursting induced by 5 μM 4-AP was fully developed, we used a standard protocol relying on 100 μM carbenoxolone, a typical gap junction blocker applied for at least 50 min to obtain its effect (Kiehn and Tresch, 2002).

As shown in Fig. 3A, B, carbenoxolone strongly decelerated the rhythm elicited by 4-AP (5 μM) with reduced background discharges between bursts. Mean data ($n=11$) are quantified in Fig. 3C in which a significant ($P<0.001$) lengthening of period values was produced by carbenoxolone while the period CV value (0.43±0.07) was unchanged.

Synaptic activity and impulse flow in the presence of 4-AP

In analogy with previous studies of cat motoneurons *in vivo* (Jack et al., 1981; Jankowska et al., 1982), the area of averaged excitatory postsynaptic potentials (EPSPs; intracellularly-recorded from motoneurons following single pulses of 1–3×Th intensity delivered to one DR) was significantly enhanced in the presence of 5 μM 4-AP (217±65% versus control; $n=8$; $P<0.001$). Since high concentrations (mM range) of 4-AP are reported to affect axon conduction velocity (Fehlings and Nashmi, 1996), we wondered whether the EPSP enhancement observed in the present study could be due to facilitation of incoming volleys. For this purpose we measured axon conduction velocity in isolated DRs using stimuli in the 2–15 V range (1–5×Th) and we analyzed the earliest peak amplitude which in control conditions gave a conduction velocity of 0.61±0.15 m s^{-1}. This parameter was unmodified by 5 μM 4-AP (97±3%; $n=4$). Nevertheless, it is possible that fibers within the spinal cord had differential sensitivity to 4-AP. As a first approximation to this issue, we calculated the Th taken as the minimal stimulus necessary to evoke EPSPs (recorded intracellularly from motoneurons) because weak

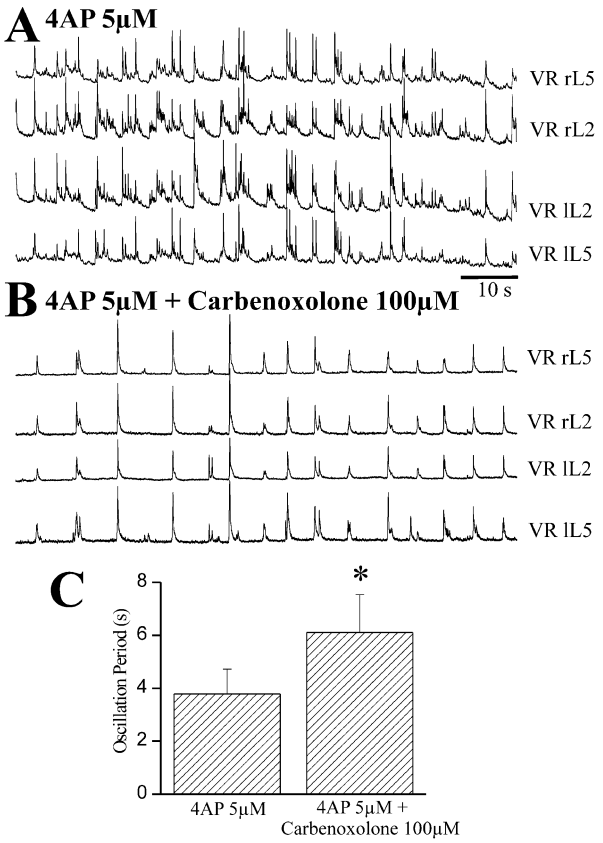

Fig. 3. 4-AP-evoked oscillations are largely depressed by carbenoxolone. (A) Simultaneous records from four VRs (identified by their abbreviation alongside each trace) 20 min from the start of 5 μM 4-AP application. (B) Carbenoxolone (100 μM; 50 min) strongly reduces oscillatory discharges. (C) Histograms showing significant (* $P<0.001$) prolongation of oscillation period values by carbenoxolone ($n=11$ preparations).

stimuli around Th are known to activate monosynaptic Ia inputs to such cells (Jack et al., 1981). In the presence of 5 μM 4-AP, EPSP Th was 104±11% of control value ($n=9$). These data collectively indicated that enhancement by 4-AP of motoneuronal EPSPs was an intrinsic phenomenon not merely due to facilitated conduction and/or generation of afferent inputs.

Alternating patterns induced by DR stimuli in the presence of 4-AP

Following a train of DR stimuli, spinal interneurons and motoneurons generate an incrementing depolarization (cumulative depolarization) associated with alternating rhythmic discharges due to activation of the CPG for locomotion (Whelan et al., 2000; Marchetti et al., 2001). In the present study we tested if this phenomenon was modulated by 4-AP because it would be an important manifestation of the ability of this drug to stimulate locomotor programs of the spinal cord. Fig. 4A, B shows an example of cumulative depolarization plus superimposed discharges recorded from right L2 and L5 VRs of the same spinal cord preparation. The rhythmic discharges, emerging when the cumulative depolarization was approaching plateau, were alternating between these roots as quantified by the polar plot (inset to Fig. 4A) in which the discharge phase values are grouped around a mean Φ of 155±27° ($n=13$ oscillations; $P<0.05$ with Rayleigh test for coupling strength between L2 and L5 oscillations). When similar measurements were repeated in the presence of 5 μM 4-AP (Fig. 4B), there was a larger number of oscillations with Φ value of 149±33° ($n=15$ oscillations; see polar plot inset to Fig. 4B; $P<0.05$), demonstrating again strong coupling between these VR signals. Thus, during cumulative depolarization the synchronous discharges typically evoked by 4-AP (Fig. 1) were replaced by an alternating, locomotor-like rhythm. Fig. 4C shows average data from 11 preparations in which 5 μM 4-AP significantly augmented the number of oscillations which was reflected in a significant increase in the cumulative depolarization area. Nevertheless, the period of such oscillations remained unchanged as much as the cumulative depolarization amplitude. It was interesting to observe that the gap junction blocker carbenoxolone had an effect opposite to the facilitatory one induced by 4-AP. In fact, carbenoxolone per se significantly depressed the peak amplitude (69±11%; $P<0.03$) and area (68±14%; $P<0.01$) of cumulative depolarization elicited by submaximal (2×Th) stimuli applied to a DR.

While 4-AP could increase the locomotor-like output of CPG activated by repeated stimuli, it was important to find out if 4-AP could actually activate the CPG operation. To this end repeated, weak DR stimuli were applied to generate a gradually decrementing depolarization (habituation; see for example Baranauskas and Nistri, 1996) as depicted in Fig. 4D for L2 and L5 VRs. Twenty minutes after application of 5 μM 4-AP the same stimulus train generated cumulative depolarization with alternating oscillations (Fig. 4E). Fig. 4F shows a polar plot of these oscillations which had Φ value of 203±40° ($n=20$ oscillations; $P<0.05$) in support of strong event coupling. Similar results were observed in five preparations indicating that 4-AP could trigger the generation of a locomotor rhythm when the afferent inputs were subTh for it. Smaller concentrations of 4-AP (0.5–1 μM) could not generate alternating patterns during repeated DR stimuli of intensity (1.5–2×Th) below Th for cumulative depolarization ($n=4$). Conversely, the largest concentration of 4-AP able to express alternating patterns during trains of weak (1.5–2×Th) DR stimuli was 20 μM ($n=3$).

Effects of 4-AP on chemically induced fictive locomotion

The potential by 4-AP to activate and facilitate the locomotor CPG was further explored by inducing fictive locomotion with the standard method of bath application of NMDA (4–5 μM) and 5HT (10 μM). Fig. 5A shows typical patterns induced by NMDA plus 5HT recorded from four VRs. Discharge alternation was present between left and right VRs of both segments (L2 and L5), and also between L2 and L5 of the same side as it represents alternations between flexor (L2) and extensor (L5) motor pool signals to the same hindlimb. In the presence of 5 μM 4-AP, the pattern

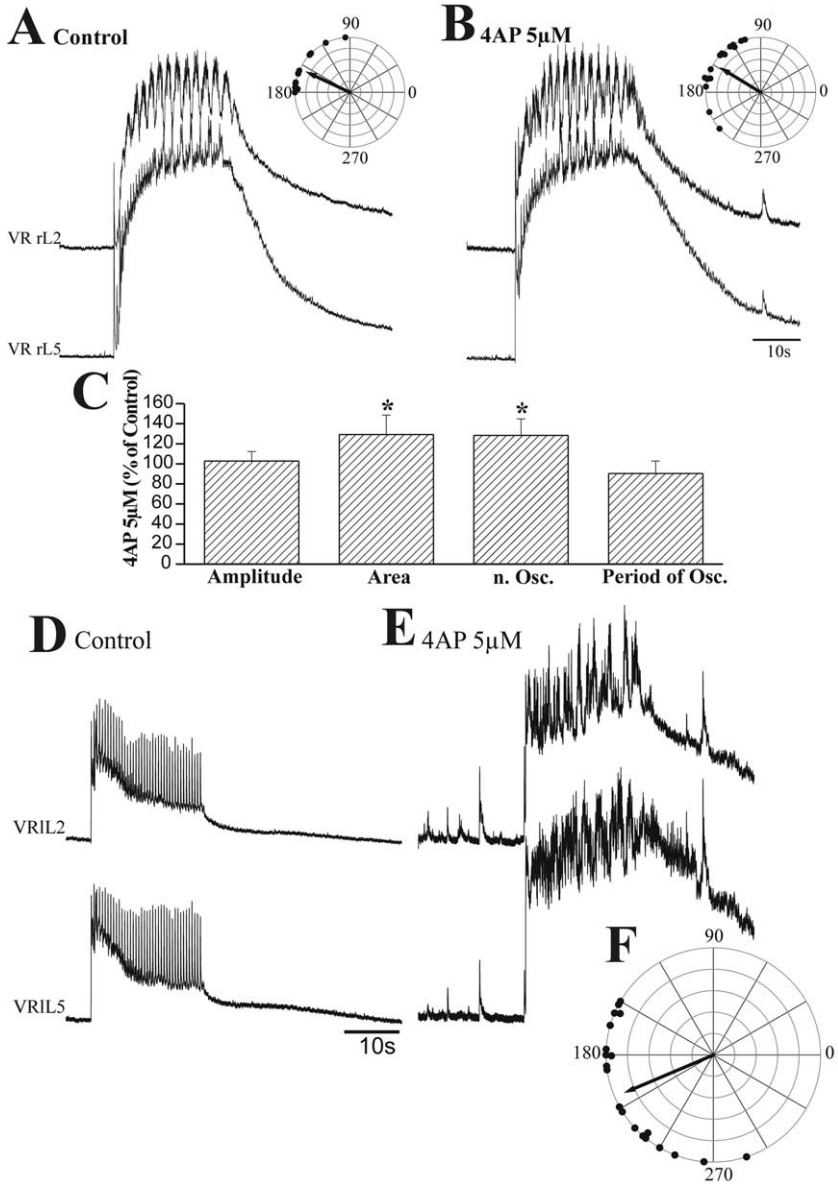

Fig. 4. 4-AP can enhance or disclose alternating patterns during repeated DR stimuli. (A) In control conditions a train of 40 stimuli (2 Hz; 3×Th) applied to right L5 DR generates cumulative depolarization with associated alternating pattern recorded from right L2 and right L5 VRs. The inset shows a polar plot for these oscillations which are predominantly grouped near 180° (155±27°; $n=13$) indicating their phase alternation. Further details are in the legend to Fig. 2. (B) On the same preparation, enhancement of alternating oscillations in the presence of 5 μM 4-AP (20 min). Inset depicts corresponding polar plot with data points close to 180° (149±33°; $n=15$). (C) Histograms for percent changes in average data relative to cumulative depolarization amplitude, area, number of oscillations and their period value in the presence of 5 μM 4-AP. * $P<0.02$ (number of oscillations) * $P<0.001$ (area); $n=12$ Note that only the cumulative depolarization area and number of oscillations were significantly changed. (D) Simultaneous records from left L2 and L5 VRs during stimulation with a train of 40 pulses (2 Hz; 1.5×Th) applied to left L5 DR. Note decrementing baseline during the train and lack of alternating patterns. (E) In the presence of 4-AP (5 μM) the same stimulation protocol evokes much larger VR response with cumulative depolarization and alternating oscillations. (F) Polar plot for oscillations which are on average close to 180° (203±40°; $n=20$) indicating their phase alternation.

period was accelerated although signal alternation was well preserved among all VRs (Fig. 5B) while synchronous discharges typical of 4-AP were absent. On average, period was reduced by 4-AP to 82±8% (control was 2.6±0.7 s; $n=8$; $P<0.005$) while the discharge amplitude was unchanged (100±29%).

The facilitatory action by 4-AP on the fictive locomotor rhythm was manifested even when the concentration of NMDA and 5-HT was subTh for fictive locomotion. This experiment is illustrated in the example of Fig. 6. First, NMDA (5 μM) plus 5-HT (10 μM) were coapplied to generate a stable alternating rhythm (Fig. 6A). After full washout for 20 min, the same agents were applied again at a lower concentration (3 and 5 μM, respectively) which was ineffective in generating a pattern, although it evoked high-frequency firing from VRs (Fig. 6B). After washout of

NMDA and 5-HT, 4-AP (5 μM) was applied for 20 min to induce a stable synchronous rhythm (not shown) which was readily transformed into alternating locomotor-like discharges by subsequent application of sub-Th NMDA and 5-HT (Fig. 6C). Note that under such conditions the rhythm period was 3.41±1.69 s, a value not significantly different from the one of alternating fictive locomotion (2.99±0.82 s) in the absence of 4-AP. Inspection of raw data in Fig. 6C seemed to suggest that during the entire length of each oscillatory cycle there was multiple spike activity. This observation was confirmed by analyzing the average cycle area which was 76±21% of control (n=6; P<0.02). This finding is compatible with the rise in network excitability evoked by 4-AP (see Figs. 1, 2).

Facilitation by 4-AP of fictive locomotion was observed on six preparations in which either 4-AP (5 μM) was added in the continuous presence of sub-Th concentrations of NMDA (0.5–5 μM) and 5-HT (1–5 μM), or sub-Th concentrations of NMDA and 5-HT were administered in the presence of a synchronous rhythm evoked by 4-AP. Note that the 5 μM concentration of 4-AP was "Th" for inducing alternating patterns in the presence of sub-Th doses of NMDA plus 5-HT. In fact, lower concentrations of 4-AP

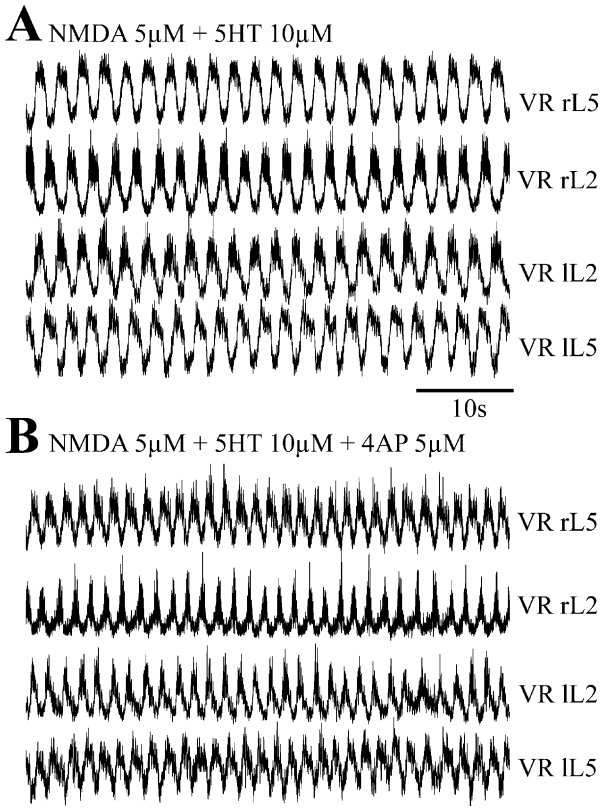

Fig. 5. Acceleration of fictive locomotor patterns by 4-AP. (A) Control fictive locomotor pattern induced by co-applied NMDA (5 μM) and 5HT (10 μM). Note that oscillations display typical alternation at homosegmental level (left and right L2; left and right L5) and between flexor (L2) and extensor (L5) motor pools on the same side. (B) Records from the same preparation following application of 5 μM 4-AP (20 min). Note faster rhythm with well-preserved phase alternation homolaterally and homosegmentally.

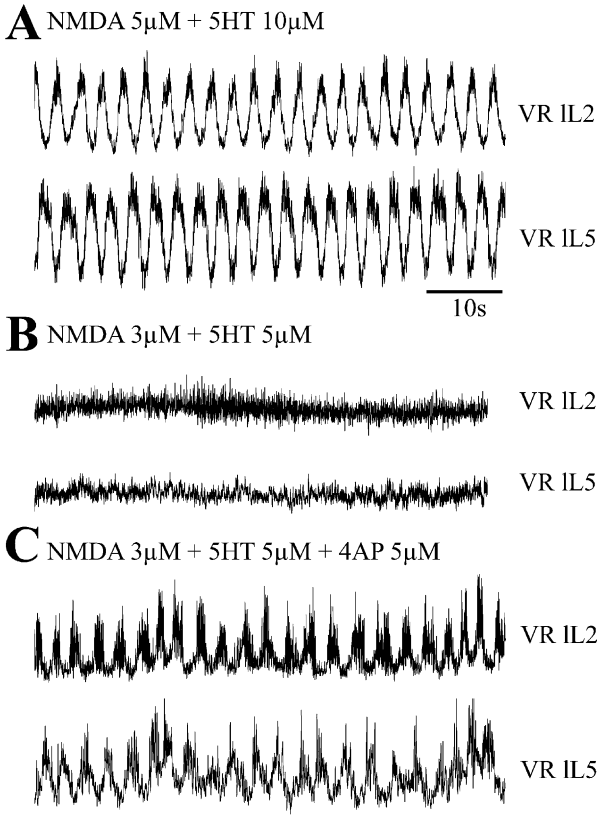

Fig. 6. Facilitation by 4-AP of fictive locomotor patterns. (A) Homolaterally alternating pattern between left L2 and L5 VRs in the presence of NMDA (5 μM) and 5HT (10 μM). (B) After washout, a lower dose of NMDA and 5HT (3 and 5 μM, respectively) is applied without generating fictive locomotor pattern, although high frequency firing is detectable from VRs. (C) After washout 5 μM 4-AP is applied for 20 min to induce synchronous VR oscillations (not shown) and subsequent addition of the sub-Th dose of NMDA and 5HT, in the continuous presence of 4-AP, can now induce an alternating pattern.

(0.5–1 μM) failed to activate fictive locomotion as VR records displayed irregular discharges only (n=3).

DISCUSSION

The principal finding of the present study is that low μM doses of 4-AP together with appropriate paradigms of concurrent CPG stimulation could activate and facilitate the function the locomotor CPG in the rat spinal cord. This result indicates that the mechanisms responsible for the action by 4-AP on spinal injury patients should include a central action of 4-AP, in addition to its well documented effect on conduction of nerve fibers (reviewed by Nashmi and Fehlings, 2001). In the absence of even subliminal activation of the CPG, 4-AP induced only a synchronous pattern mediated by a wide spinal network dependent on glutamatergic transmission and reinforced via gap junctions.

Strong potency of 4-AP action on the spinal cord

One interesting aspect of the present study is the demonstration that the distinct changes in spinal network excit-

ability could be produced by low micromolar concentrations of 4-AP substantially below those normally used for electrophysiological experiments (reviewed by Coetzee et al., 1999). The effects of 4-AP included onset of synchronous oscillations and larger amplitude of EPSPs without comparatively large alterations in membrane potential, input resistance and spike properties of motoneurons. These observations indicate that electrical synchronicity elicited by 4-AP had a network origin which commanded a patterned output from motoneurons, by themselves little sensitive to such low concentrations of this drug. Since CNQX plus APV completely blocked this oscillatory pattern, it is clear that intact glutamatergic transmission at network level was essential to express this activity. The high CV value of 4-AP-induced rhythmicity demonstrates its lack of regular discharges and suggests a multifocal origin of this activity with shifting foci of local excitability rises. Previous studies have shown that 4-AP increases the amplitude of excitatory and inhibitory potentials recorded from spinal motoneurons *in vivo* (Jankowska et al., 1977) because it reduces the probability of release failure at single boutons (Jack et al., 1981). This effect is likely responsible for the generation of spinal network discharges reported in the present study. Interestingly, enhancement of synaptic transmission requires $>$10 min to develop fully even after i.v. injection of this drug (Jack et al., 1981) in analogy with the present observations on the latency of action of 4-AP bath-applied to the isolated spinal cord.

Synchronicity of 4-AP elicited pattern

It is important to note that the 4-AP evoked discharges were synchronous on all four VRs recorded in the present study. This finding clearly indicates that such electrical signals, even if generated by motor pools, were incompatible with locomotion. There is no evidence that synchronicity was due to block of cross-inhibitory pathways. In fact, the synchronous rhythm emerging spontaneously from block of synaptic inhibition in the spinal cord (Bracci et al., 1996a) is much slower and characterized by long bursts with silent intervals. Furthermore, inhibitory synaptic processes are typically enhanced by 4-AP (Jankowska et al., 1977), a mechanism essential for observing any favorable action by this drug in promoting the generation of locomotor-like patterns.

Nevertheless, the strong synchronicity of 4-AP oscillations implied a very effective coupling process which, despite the probabilistic nature of the synaptic inputs arising from the network, ensured simultaneous firing of motoneurons. One potential mechanism underlying this property appeared to be electrotonic coupling among spinal neurons since the gap junction blocker carbenoxolone substantially attenuated discharges. This result is analogous to the effect of carbenoxolone on 4-AP induced discharges in the rat hippocampus (Ross et al., 2000) and cerebral cortex (Szente et al., 2002). Timofeev and Steriade (2004) have proposed that transient fluctuations in the extracellular space during bursting can increase the efficiency of electrical coupling among neurons, a general phenomenon which may be applicable to rat spinal networks as well. Nevertheless, the persistence of discharges, albeit on a smaller scale, in the presence of carbenoxolone indicated that additional factors, like activation of certain spinal circuits hierarchical to premotoneurons and motoneurons and involved in reflex synchronization (Kerkut and Bagust, 1995), were responsible for simultaneous pattern occurrence.

Cellular targets for 4-AP action

The very strong sensitivity of spinal networks to 4-AP restricts the range of voltage activated K^+ channels potentially implicated in the action of this drug. Among them Kv1.4 and Kv3.1 channels (see database in http://iuphar-db.org) largely expressed in the ventral and dorsal horn gray matter (Deuchars et al., 2001; Brooke et al., 2002; Edwards et al., 2002) seem to fulfill this role because of their exquisite sensitivity to 4-AP (Grissmer et al., 1994; Coetzee et al., 1999). Both sets of channel present rapid voltage-dependent activation and inactivation kinetics (Coetzee et al., 1999). It is noteworthy that Kv1.4 inactivation is only partial and that recovery from full inactivation of Kv3.1 occurs quite slowly (2–4 s). These properties may render such channels suitable to pace the typical rhythm of fictive locomotion.

Effects of 4-AP on axonal conduction

Lesions of the myelin sheath uncover paranodal K^+ channels which, once activated by depolarization, contribute to failure of axonal action potential propagation (Bostock et al., 1981; Kocsis, 1985). Thus, one important strategy to improve axonal conduction is pharmacological block of such channels (Bostock et al., 1981). In the case of lesions affecting spinal axons, this goal can be achieved with 4-AP (Hayes et al., 1993; Waxman, 1993; Nashmi and Fehlings, 2001) although administered at concentrations considerably larger (Fehlings and Nashmi, 1996) than those found to be effective on spinal networks in the present study. Spinal axons might contribute to generation of spinal rhythms in analogy with their important role in hippocampal fast oscillations via axonal gap junctions (Schmitz et al., 2001) whose efficiency may be dynamically modulated by several mechanisms (LeBeau et al., 2003) including K^+ channels sensitive to 4-AP. Since we observed no significant change in axon conduction velocity of DR fibers or in the voltage Th to evoke EPSPs or VR antidromic spikes, it seems likely that spinal networks rather than projecting axons were the most sensitive target for the action of 4-AP.

Fictive locomotor patterns in the presence of 4-AP

Fictive locomotor patterns require alternation of regular discharges between flexor and extensor motor pools (Kiehn and Kjaerulff, 1998; Marchetti et al., 2001; Butt et al., 2002), a condition thus very different from the synchronous discharges induced by 4-AP alone. Nevertheless, it seems likely that certain K^+ conductances have a role in generating the spinal locomotor pattern in the lamprey (Hess and El Manira, 2001), amphibian embryo (Kuenzi and Dale, 1998), and rat (Cazalets et al., 1999). It is,

however, noteworthy that all those studies have reported block of alternating patterns by high doses of 4-AP. We also confirmed that relatively large concentrations of 4-AP produced highly irregular discharges incompatible with locomotor cycles (Cazalets et al., 1999). Nevertheless, the novel feature of the present work was to demonstrate that low concentrations of 4-AP (targeted to block certain populations of K^+ channels rather than broad spectrum K^+ conductance inhibition) did facilitate locomotor-like oscillations with clear alternation among appropriate motor pools and simultaneous disappearance of the synchronous patterns typical of 4-AP alone. In particular, the number of oscillatory patterns induced by repeated DR stimuli was increased by 5 μM 4-AP. When DR stimuli were insufficient to evoke cumulative depolarization, their association with 4-AP could then generate typical alternating patterns.

Unlike 4-AP, the gap junction blocker carbenoxolone had opposite, depressant effects on cumulative depolarization elicited by submaximal DR stimuli. While carbenoxolone may exert additional effects on brain neurons like activation of mineralocorticoid receptors with consequent increase in network excitability (Ross et al., 2000), had this effect occurred in the spinal cord, it would have been expected to enhance rather than depress cumulative depolarisation, contrary to the present observations. Since carbenoxolone does not affect either AMPA or NMDA receptors (Oleskevich et al., 1993) or excitability and firing properties of spinal motoneurons (Kiehn and Tresch, 2002), our results suggest that gap junction coupling might have facilitated VR responses due to depolarization of non-synaptically activated motoneurons.

When fictive locomotion was induced by the standard protocol of NMDA plus 5-HT application, 4-AP increased the frequency of rhythm in a persistent manner without significant changes in discharge amplitude. When the concentration of NMDA and 5-HT was sub-Th for fictive locomotion, 4-AP triggered this phenomenon which was characterized by cycles containing multiple spike activity. The functional outcome of this enhanced firing does not imply disrupted locomotor activity *in vivo* because a former study of spinalized cats has demonstrated that i.v. injection of 4-AP could bring locomotor activity to Th in the presence of L-DOPA administration (Zangger, 1981). Although such *in vivo* experiments could not elucidate the site or mode of action of 4-AP, they nevertheless demonstrate that locomotor facilitation observed in the rat spinal cord *in vitro* is also present in the cat *in vivo*.

Collectively all these observations suggest that 4-AP per se could not generate fictive locomotion, yet it facilitated it as long as the drug was administered in conjunction with appropriate stimuli (even below Th) for the locomotor CPG. In a way, this notion is compatible with the possibility that 4-AP application enhanced the excitability of the CPG so as subsequent signals of different origin could fully activate it.

Network mechanisms underlying the action of 4-AP

4-AP can generate seizure-like activity in various brain areas as exemplified by recording from cortical and hippocampal neurons (Avoli et al., 1988; Watts and Jefferys, 1993; Lopantsev and Avoli, 1998). In the spinal cord, the network underlying discharges evoked by 4-AP appears to be incompatible with locomotion because it lacked motor alternation. However, the 4-AP sensitive network could interact with the locomotor CPG. In fact, it could activate fictive locomotion in association with certain stimuli, indicating that the density (and/or location) of the 4-AP channels within the CPG was not a major factor to suppress spontaneous, ongoing operation of the CPG. 4-AP could therefore enhance the ability of CPG neurons to act as "coincidence detectors" when suitable pulses reached them. Conversely, once the CPG network fully expressed locomotor-like patterns, it switched off the activity of the 4-AP network to avoid overlapping patterns. This property of CPG neurons to turn off other spinal patterns is a well-known phenomenon observed with other spinal patterns irrelevant to locomotion (Whelan et al., 2000; Taccola et al., 2004).

Functional implications of the action by 4-AP

The present study provides some implications that may be useful to improve the clinical efficacy of 4-AP on spinal patients. First, the concentration of 4-AP should be low, carefully titrated and kept below the one which would disrupt motor patterns. This result closely accords with clinical studies that have shown the therapeutic plasma level of 4-AP in man to be about 0.3–0.6 μM (Segal et al., 2000), a value not far from those found to be effective in the present experimental conditions. Our experimental observations suggest that a four-fold increase in the Th dose of 4-AP was the maximum permissible to preserve alternating patterns. A recent review on 4-AP concludes that "there is a narrow therapeutic range" for this drug and that overdose is associated with dystonic movements or even generalized convulsions (Darlington, 2000). These findings are, therefore, fully compatible with the present study. Second, administration of 4-AP per se appears to be insufficient to trigger motor patterns. Thus, 4-AP should be given together with appropriate sensory stimuli to maximize the chance of successful clinical response. Interestingly, clinical studies have proposed 4-AP to be beneficial only in patients with incomplete spinal lesions (Hayes et al., 1993; Segal et al., 1999). Presumably in such cases, lesion-spared spinal networks might be sufficient to produce synergy of action with 4-AP. Third, more selective blockers of certain populations of K^+ channels might in future be proven of even greater benefit to improve spinal deficits in voluntary motor activity.

In summary, the present study carried out on the rat *in vitro* spinal cord provides novel data suggesting that the ameliorating action of 4-AP on spinal injury patients may be generated at the level of locomotor networks in the spinal cord which are highly sensitive to the action of this drug.

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RESEARCH ARTICLES

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Role of group II and III metabotropic glutamate receptors in rhythmic patterns of the neonatal rat spinal cord in vitro

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Abstract Electrophysiological recordings were used to explore the role of group II and III metabotropic glutamate receptors (mGluRs) in oscillatory patterns generated by the neonatal rat spinal cord in vitro. Neither the group II agonist DCG-IV (and the selective antagonist EGLU), nor the group III agonist L-AP4 (and its selective antagonist CPPG) had any effect on lumbar motoneuron membrane potential or input resistance. This observation suggests that motoneurons expressed no functional group II and III mGluRs and received no network-based, tonic influence mediated by them. DCG-IV or L-AP4 strongly depressed synaptic responses evoked by single dorsal root (DR) stimuli, an effect counteracted by their respective antagonist. EGLU or CPPG per se had no effect on synaptic responses, indicating no mGluR autoreceptor-dependent control of transmitter release. L-AP4 largely depressed cumulative depolarization, windup and associated oscillations, whereas synaptic depression induced by DCG-IV waned with repeated stimuli. L-AP4 slowed down fictive locomotor patterns and arrested disinhibited bursting, which could, however, be promptly restored by DR electrical stimulation. DCG-IV had no significant effect on fictive locomotion, but it blocked disinhibited bursting. EGLU facilitated bursting, suggesting that burst termination was partly controlled by group II mGluRs. All these effects were reversible on washout. It is concluded that activation of group II and III mGluRs differentially modulated rhythmic patterns recorded from motoneurons via network-dependent actions, which probably included decrease in the release of neurotransmitters at key circuit points.

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Introduction

The neonatal mammalian spinal cord in vitro preparation is very useful to investigate neuronal network properties because of its well-defined inputs via dorsal root (DR) fibres and motor output via ventral root (VR) axons (Kerkut and Bagust 1995). Spinal networks can generate, even in the absence of external stimuli, a repertoire of rhythmic activities (reviewed by Kiehn and Kjaerulff 1998; Butt et al. 2002). One of them is termed fictive locomotion (induced by bath-application of excitatory agents) with phasic electrical discharges alternating between flexor and extensor motor pools and between left and right motoneurons within the same segment (Kiehn and Kjaerulff 1998; Butt et al. 2002). This is a network-dependent program that requires glutamatergic transmission (Cazalets et al. 1992; Beato et al. 1997) and utilizes glycine and γ-amino butyric acid (GABA) to generate rhythm alternation (Cowley and Schmidt 1995; Kremer and Lev-Tov 1997). Fictive locomotor patterns can also be evoked by repeated stimuli applied to one DR (Whelan et al. 2000; Marchetti et al. 2001a), a protocol that elicits cumulative depolarization of interneurons and motoneurons (Thompson et al. 1990, 1994; Sivilotti et al. 1993; Baranauskas et al. 1995; Baranauskas and Nistri 1996), which is thought to be a model of sensitization of nociceptive pathways (Baranauskas and Nistri 1998; Herrero et al. 2000). It is possible that spinal oscillatory discharges are the electrophysiological expression of the aversive locomotor response following intense nociceptive inputs.

Blocking spinal GABA- and glycine-mediated synaptic inhibition elicits spontaneous slow bursting (termed disinhibited bursting; Bracci et al. 1996a, 1996b), and demonstrates the inherent ability of spinal networks to generate rhythmic patterns. The disinhibited rhythm is initiated via periodic release of glutamate by intrinsically

active interneurons (Tscherter et al. 2001; Darbon et al. 2002). Although this pattern is incompatible with locomotion because of its slow frequency and lack of alternation, disinhibited bursting is similar to the natural rhythm observed in late embryonic life (Nakayama et al. 2002), and is believed to be generated by the same pattern generator of fictive locomotion (Beato and Nistri 1999). Thus, disinhibited bursting represents a useful model to study network mechanisms for burst onset and termination.

While such studies have demonstrated the key role of glutamate as excitatory neurotransmitter acting via ionotropic receptors, much less is known about the role of metabotropic glutamate receptors (mGluRs), which mediate a variety of effects on central neurons (Pin and Duvoisin 1995; Schoepp et al. 1999). There are three main mGluR groups, termed I, II and III, all coupled to different G proteins. Activation of class I receptors leads to intracellular inositol triphosphate (IP_3) formation and Ca^{2+} increase, while class II or III mGluR activation inhibits adenylyl cyclase with associated depression of Ca^{2+} and K^+ conductances (Pin and Duvoisin 1995; Schoepp et al. 1999).

Recent studies of the rat spinal cord in vitro have demonstrated that activation of mGluRs by a broad spectrum agonist inhibits fictive locomotion and speeds up disinhibited bursting (Taccola et al. 2003). These observations suggest a pleiotropic action of mGluRs on spinal rhythmicity and point to further investigations to dissect out the relative contribution by various mGluR groups. Group I receptors modulate synaptic responses and trigger electrical oscillations in the rat spinal cord (Marchetti et al. 2003) and can generate fictive swimming patterns in the lamprey spinal cord (El Manira et al. 2002). Comparatively less is known, however, about the network role of group II and III receptors, which are mainly found in the most superficial dorsal layers of the dorsal horn and are weakly expressed in laminae VIII and IX of the ventral horn (Berthele et al. 1999; Aronica et al. 2001; Azkue et al. 2001). Previous work has indicated that the depressant action of group II or III mGluRs on synaptic transmission is exerted at the presynaptic level via inhibition of transmitter release (Pinco and Lev-Tov 1993; Cao et al. 1995, 1997; Dong and Feldman 1999). The present study addressed how pharmacological agonists and antagonists of group II and III mGluRs could affect motoneuron excitability and network-mediated discharges during fictive locomotion, cumulative depolarization and disinhibited bursting.

Methods

Procedures involving animals were conducted in accordance with Italian Law DL 27/1/92 n. 116, following the European Community directives n. 86/609 and 93/88 (Italian Ministry of Health authorization for the local animal care facility in Trieste: D.M. 69/98-B). Full details about the experimental methods have been published previously (Bracci et al. 1996a, 1996b; Beato and Nistri 1999; Rozzo et al. 2002). In brief, the neonatal rat spinal cord was superfused (7.5 ml/min) 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% O_2/5% CO_2; pH 7.4 at room temperature.

In view of the need to record for a long time fictive locomotion and disinhibited rhythms, the majority of experiments were conducted with DC-coupled lumbar ventral root (VR) recordings via tightly fitting suction electrodes. Intracellular recordings (from L3 to L5 motoneurons) were obtained using sharp electrodes filled with either 3 M KCl (30–60 MΩ resistance), or 2 M $KMeSO_4$ (60–120 MΩ resistance) with no significant difference in resting membrane potential. Bridge balancing was continuously checked and capacitative transients minimized by negative capacity compensation. Input resistance was calculated from the amplitude of hyperpolarizing electronic potentials or from the slope of linear current/voltage (I/V) relations. Experiments with the cumulative depolarization protocol (Barbieri and Nistri 2001) always relied on simultaneous VR and intracellular motoneuron recording. Dorsal root electrical stimuli, delivered via miniature bipolar suction electrodes, were employed to elicit single VR responses (recorded from the ipsilateral VR of the same segment) or cumulative depolarization. Stimulus intensity (1–10 V range, 0.1 ms duration) was calculated in terms of threshold (Th), which was defined as the minimum intensity to elicit a detectable reflex response in the ipsilateral VR (see Marchetti et al. 2001a).

Cumulative depolarization amplitude and area, plus wind-up (expressed as number of spikes in the train) were measured as previously reported (Barbieri and Nistri 2001). Oscillatory patterns recorded during cumulative depolarization were quantified from extracellular records. In each test DR-evoked reflexes were quantified by averaging at least three responses and measuring their area. Fictive locomotion was typically induced by continuously bath-applied *N*-methyl-D-aspartate (NMDA, 4 or 5 μM) plus serotonin (5-HT, 10 μM; see Kiehn and Kjaerulff 1998; Butt et al. 2002). Disinhibited bursting was generated by continuously bath-applied strychnine (1 μM) and bicuculline (20 μM; Bracci et al. 1996a, 1996b). Rhythmic discharged were characterized on the basis of their period (T) defined as the time between the onset of two cycles of oscillatory activity for fictive locomotor-like rhythm or of two bursts of activity for disinhibited rhythm. When period values were averaged for a pool of preparations, data from each spinal cord were calculated as the mean of at least 20 cycles or bursts. The stereotypic structure of each disinhibited burst (Bracci et al. 1996a, 1996b) comprises, after a rapid upstroke, a depolarized plateau (whose duration, T_1, was measured) followed by a series of intraburst oscillations (whose periodicity was also measured).

mGluR agonists were applied for 10–30 min: once their effect had stabilized, data were collected and quantified as means ±SD with *n* representing the number of motoneurons for intracellular records or number of spinal cord preparations for extracellular records. Note that in these protocols, owing to the long-lasting nature of experiments on disinhibited bursting, data were only collected with extracellular recording. mGluR antagonists were applied for a minimum of 10 min prior to the application of their agonist and maintained throughout. Representative traces shown in Figs. 1, 2, 3, 4, and 5 were taken under steady state conditions for drug effects. Statistical significance was assessed with the Student's *t*-test, or analysis of variance (ANOVA) plus Tukey's test.

The following mGluR agents were purchased from Tocris (Bristol, UK): the selective group II agonist (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV), the selective group III agonist L-(+)-2-amino-4-phosphonobutyric acid (L-AP4), the selective group II antagonist (2*S*)-α-ethylglutamic acid (EGLU), and the selective group III antagonist (*RS*)-α-cyclopropyl-4-phosphonophenylglycine (CPPG). Full details concerning their pharmacological properties may be found in Schoepp et al. (1999). Stock solutions of DCG-IV and L-AP4 were prepared in distilled water, whereas stock solutions of EGLU and CPPG were prepared in 1 *N* NaOH and diluted 1,000- to 10,000-fold in Krebs solution. Equivalent amounts of NaOH added to the buffered Krebs solution had no effect on spinal preparations. Agonists were washed out for >10 min to obtain recovery. On each preparation, whenever antagonists were tested,

they were applied after having established that selective agonists had first evoked a reversible effect. After antagonist equilibration time of at least 10 min in the tissue, the agonist was applied again in the continuing presence of antagonist.

Results

The database of the present study comprises 45 isolated spinal cords for VR recording and 16 motoneurons with an average resting potential (V_m) of −74±5 mV and 30 ±16 MΩ input resistance.

Action of group II- and III mGluR agents on the spinal cord

DCG-IV (0.2–2 μM) or L-AP4 (1–50 μM), selective agonists for class II and III mGluRs, respectively, did not per se elicit any measurable effect on VR baseline (data not shown). Intracellular recordings confirmed that neither DCG-IV (0.4 μM, *n*=4) nor L-AP4 (1 μM, *n*=4) changed motoneuron membrane potential. The input resistance was not changed by 0.4 μM DCG-IV (27±12 MΩ versus 25 ±11 MΩ in control, *n*=4), or by 1 μM L-AP4 (18±10 MΩ versus 19±10 MΩ in control, *n*=4). Likewise, input resistance was not altered by the group II selective antagonist EGLU (200 μM; 16±3 MΩ versus 18±6 MΩ in control, *n*=4), or by the group III selective antagonist CPPG (50 μM; 26±15 MΩ versus 27±16 MΩ in control, *n*=4). Lack of any direct action by agonists or antagonists on motoneuron membrane properties prompted us to investigate how activation of class II and III mGluRs might affect synaptic transmission.

Effects of group II or III mGluR agents on DR-evoked single responses of motoneurons

Single DR stimuli applied at suprathreshold intensity are known to generate complex polysynaptic responses of motoneurons that can last several seconds (see review by Kerkut and Bagust 1995). Figure 1Aa,Ab shows the depressant effect of DCG-IV (0.4 μM) on the potential intracellularly recorded from one rL5 motoneuron following rL5 DR stimulation (3× Th intensity). Since the initial peak of synaptic response was unchanged, the depression was quantified in terms of area, and amounted to 40±10%

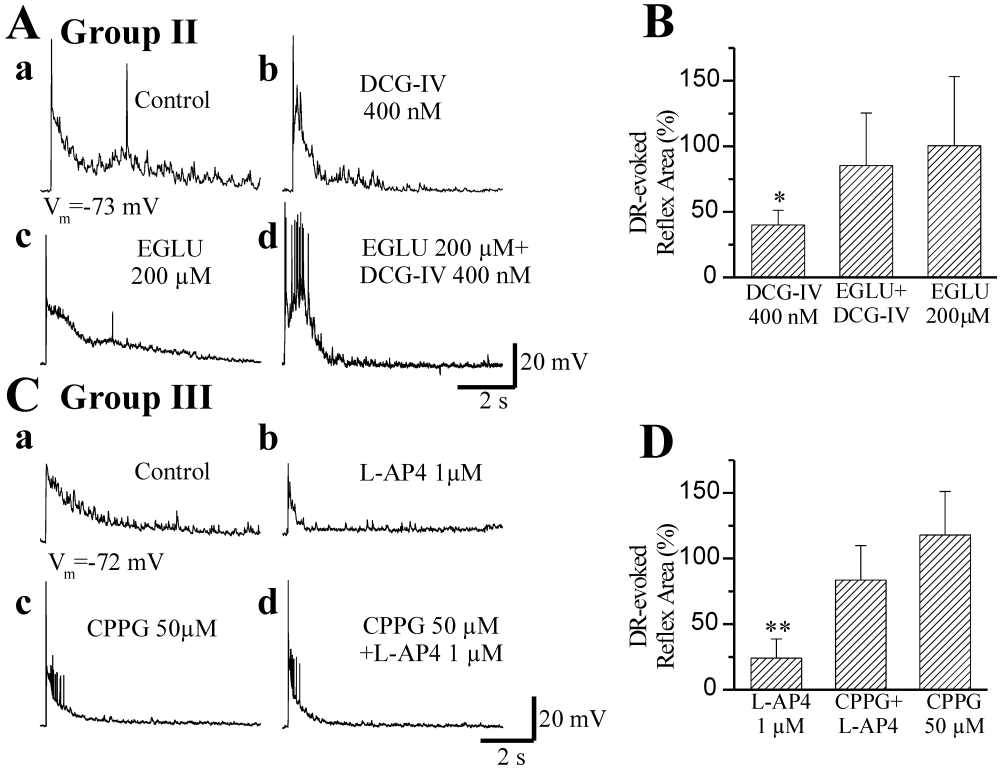

Fig. 1A–D Effect of activating or blocking group II or III metabotropic glutamate receptors (mGluRs) on dorsal root (DR)-evoked synaptic responses. All traces are intracellular records from single motoneurons. Data sampling rate clips fast peak responses. **A** Representative records of the action of group II mGluR agonist DCG-IV (0.4 μM) and its selective antagonist EGLU (200 μM) on DR-evoked responses recorded at the same membrane potential (V_m −73 mV, DR stimulus intensity 3× threshold): **Aa** control; **Ab** DCG-IV depresses synaptic response area (not the early peak); **Ac** EGLU does not significantly alter DR evoked response; **Ad** EGLU prevents DCG-IV induced depression. **B** Average of pooled data of DR-evoked reflex area for group II agonist and antagonist (*p<0.05, *n*=4–10). **C** Representative records of the action of group III mGluR agonist L-AP4 and its selective antagonist CPPG on DR evoked responses (V_m −72 mV, DR stimulus intensity 3× threshold): **Ca** control; **Cb** L-AP4 (1 μM) depresses synaptic response; **Cc** the group III antagonist CPPG (50 μM) does not significantly alter this response; **Cd** CPPG prevents L-AP4 induced depression. **D** Average of pooled data of DR-evoked reflex area for group III agonist and antagonist (**p<0.02, *n*=4–7)

(Fig. 1B). This decrease was not dependent on either stimulus intensity or the DCG-IV concentration in the 0.2–2 μM range. Whereas the group II antagonist EGLU (200 μM) per se did not alter the DR reflex response (Fig. 1Ac,B), it prevented the DCG-IV-induced depression (Fig. 1Ad).

A strong depressant effect on the DR-evoked response was also elicited by L-AP4, as exemplified in Fig. 1Ca,Cb where this agent caused a large (77%) decrease in synaptic response area of an rL4 motoneuron (3× Th DR stimulus). On average, L-AP4 (1 μM) depressed the response to 24 ±14% of control (Fig. 1D). The group III antagonist CPPG (50 μM), while leaving the control response unaltered (Fig. 1Cc), prevented the L-AP4-dependent depression (Fig. 1D).

The depressant action by DCG-IV or L-AP4 appeared within 3–5 min of the start of application and was maintained throughout their application period (10–30 min).

Cumulative depolarization changes in relation to group II and III mGluR activity

The VR records of Fig. 2A show that DCG-IV (2 μM) reduced cumulative depolarization. Intracellular records obtained from a single motoneuron before and during DCG-IV application are shown in Fig. 2C. The average reductions in area and amplitude were significant, yet relatively small (Fig. 2E), and did not lead to any change in the number of oscillations (99±39% of control, n=3) or

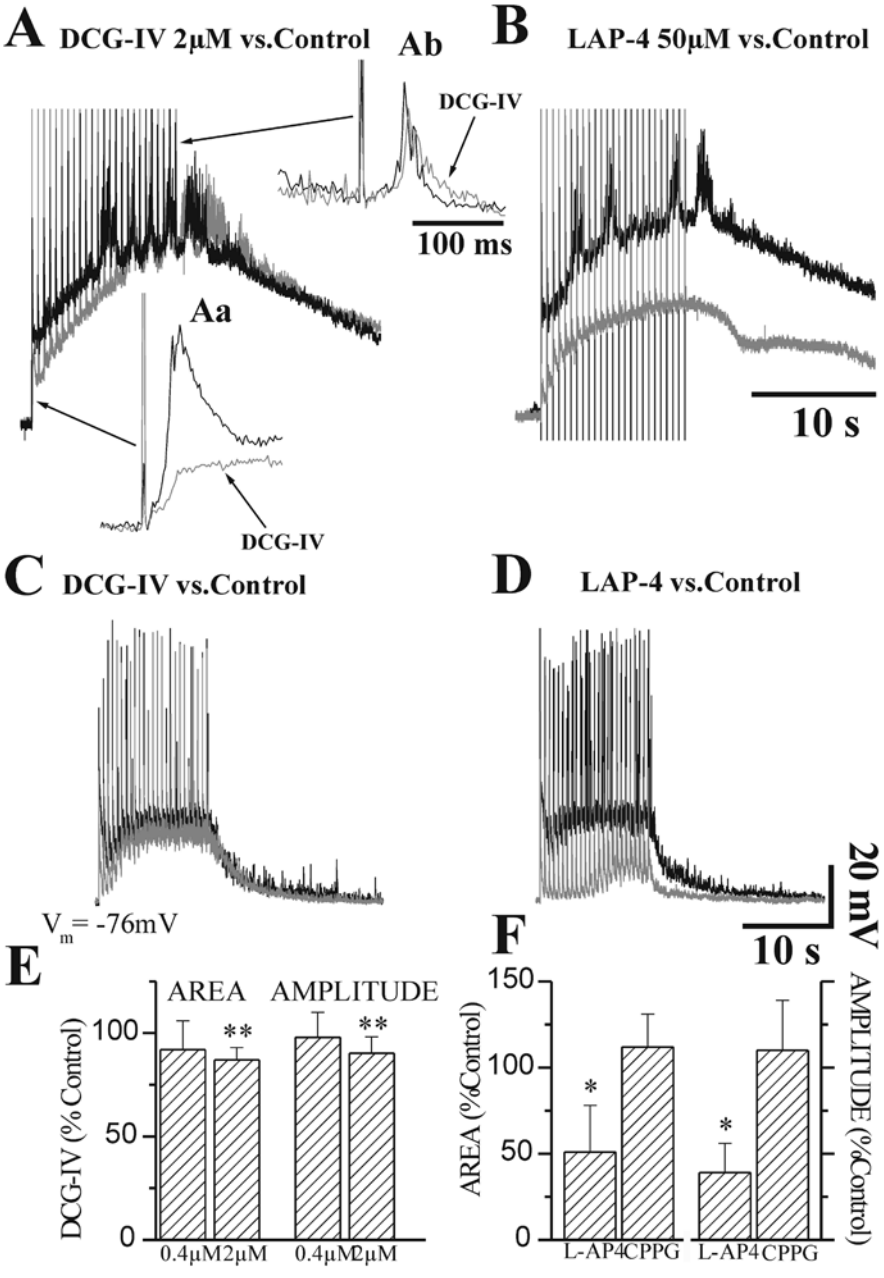

Fig. 2A–F Effect of group II or III mGluR agonists on cumulative depolarization induced by trains of dorsal root (DR) stimuli. **A** Ventral root (VR) response to a train of DR stimuli (25 stimuli, 2 Hz) in control (*top trace*) and in the presence of DCG-IV (2 μM, *bottom trace*). Note oscillatory patterns developing near the peak of cumulative depolarization in either trace. The first and the last evoked responses in the train are superimposed to show the differential action by DCG-IV (see insets **Aa** and **Ab**). **B** VR responses to analogous trains of DR stimuli in control (*top trace*) and in the presence of L-AP4 (50 μM, *bottom trace*). Note strong reduction in cumulative depolarization and loss of rhythmic patterns. **C, D** Intracellular records from the same motoneuron to demonstrate reduction in cumulative depolarization in the presence of DCG-IV (**C**) or L-AP4 (**D**). *Shaded traces* are taken during mGluR agonist application and superimposed on control traces. **E, F** Histograms indicating average values for cumulative depolarization area and amplitude in the presence of DCG-IV (**E**) or L-AP4 (**F**) vs. control (%). In **E** effects of two concentrations of DCG-IV are shown, while in **F** effects of 1 and 50 μM L-AP4 are pooled together as they were not dose related. The mGluR III antagonist CPPG per se is ineffective on cumulative depolarization (**F**) (**p<0.05, *p<0.01)

of spikes during the train (140±40%, n=5). This result was somewhat unexpected because DCG-IV depressed DR-evoked single responses recorded from motoneurons. To clarify this issue, we then compared the average amplitude of the first response in the stimulus train with the last one, as exemplified in the two insets to Fig. 2A. While the first response was consistently decreased (47±8%, p<0.001, n=5) by 2 μM DCG-IV, the last one was not significantly different from its corresponding control (88±20%, n=5). A lower concentration (0.4 μM) of DCG-IV, that had already reduced single synaptic responses (see Fig. 1), was also ineffective on the amplitude of cumulative depolarization (Fig. 2E). EGLU (200 μM) did not affect responses to trains of DR stimuli (2–7× Th) as cumulative depolarization area was 115±27% and amplitude 120±50% of control (n=25).

L-AP4 (1–50 μM) largely depressed responses to trains of DR stimulation by significantly reducing cumulative depolarization area and amplitude, as exemplified by extracellular (Fig. 2B) and intracellular (Fig. 2D) records, and by abolishing oscillations (Fig. 2B). On average, oscillations were 17±12% (p<0.01, n=4) of control. On the other hand, the group III antagonist CPPG (50 μM) did not significantly alter responses to trains of DR stimuli (area 112±19%, cumulative depolarization amplitude 110±29%, n=11), while it antagonized the depressant effect of L-AP4 (area 98±14%, cumulative depolarization amplitude 85 ±19%, n=9).

Effect of mGluRs on chemically induced fictive locomotion

The effect of L-AP4 on fictive locomotion is shown in Fig. 3A,B and summarized in Fig. 3C. The group III agonist (1 μM) significantly slowed down rhythmicity (133±12% of control, n=4), an effect blocked by the selective antagonist CPPG (50 μM). Conversely, DCG-IV (0.4 μM) had no significant effect on fictive locomotion. Neither CPPG nor EGLU (200 μM) changed fictive locomotor patterns. None of these mGluR compounds affected oscillation amplitude of fictive locomotor cycles.

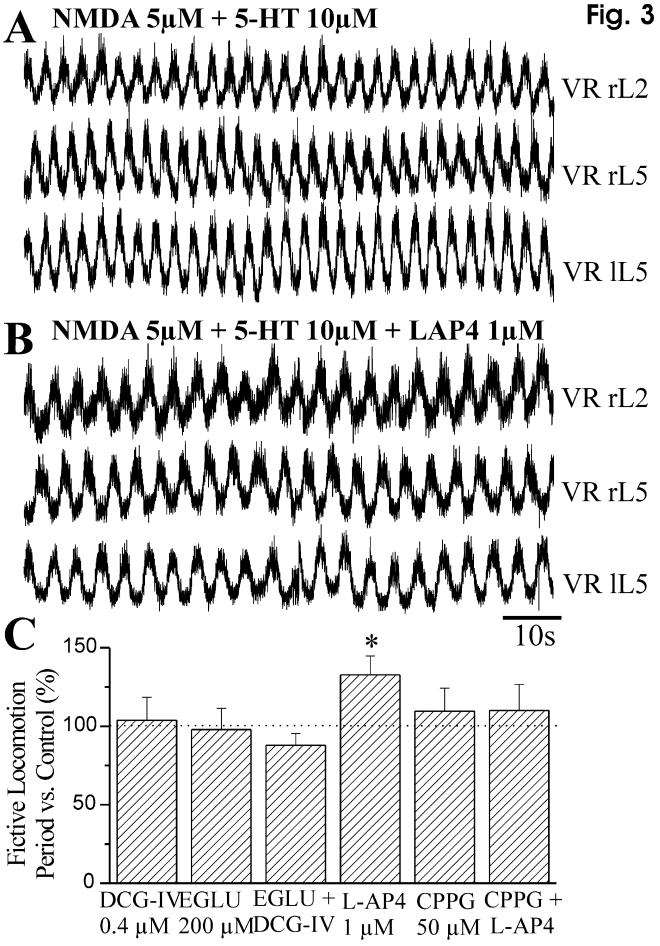

Fig. 3A–C Fictive locomotor patterns in the presence of group II or III agonists and antagonists. **A, B** Representative examples of alternating rhythmic discharges induced by bath-applied NMDA (5 μM) and 5-HT (10 μM) and recorded from three ventral roots (VRs; their abbreviations are alongside the records). Note typical patterns displaying phase alternation between left and right VRs at the same segmental level (L5) and between L2 and L5 VRs of the same right side. In the presence of L-AP4 (1 μM) there is slowing down of the rhythm (**B**). **C** Histogram of the effects of group II or III mGluR agents on period of fictive locomotion. Data for L-AP4, CPPG and EGLU are from four experiments while for DCG-IV n=10 (*p<0.05 for L-AP4 data only)

Group II and III mGluRs modulated disinhibited rhythm

DCG-IV (0.4 μM) slowed down the disinhibited rhythm (induced by bath-applied 1 μM strychnine and 20 μM bicuculline) without changing VR baseline level (Fig. 4A). This phenomenon was observed in 14 of 20 preparations. Taking average data from 14 preparations, burst period values rose to 200±40% of control, regardless of DCG-IV concentration (0.1–1 μM, n=14) but without significant change in burst duration (Fig. 4C, D). In the six remaining preparations (data not shown), the disinhibited rhythm was completely abolished by DCG-IV (0.1–0.4 μM), an effect reversible after 10–15 min washout. However, after washout, a further application of DCG-IV (0.4 μM) in the presence of EGLU (200 μM; Fig. 4A,C,D) had no effect on rhythmicity. Furthermore, EGLU (200 μM) alone could accelerate the rhythm (Fig. 4C), as exemplified in Fig. 4B, and it decreased average burst duration (Fig. 4D) without changing intraburst oscillation period or T_I (Fig. 4E), or VR baseline.

L-AP4 (1 μM) completely abolished the disinhibited rhythm in 9 of 10 preparations (see example in Fig. 5Aa, Ab), and slowed it down in the remaining case without changing VR baseline. The latency for the onset of burst suppression by L-AP4 was dose-related (2–3 min for 20 μM L-AP4, and 5–10 min for 1 μM L-AP4) and the effect of L-AP4 was always reversible after 10–15 min washout. It is noteworthy that, even when the rhythm was abolished, bursts could still be evoked by DR stimulation (2× Th), as shown in Fig. 5C; in this example, regular

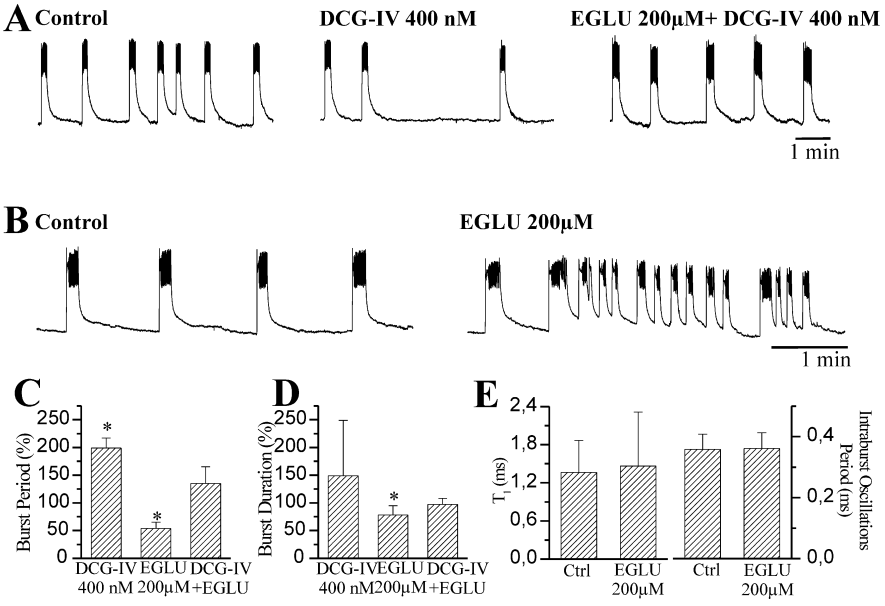

Fig. 4A–E Group II mGluRs modulate disinhibited rhythm. **A** Representative example of disinhibited bursts recorded from a lumbar ventral root (VR) in control condition and in the presence of DCG-IV (0.4 µM), which slows down rhythm without change in burst duration. The selective antagonist EGLU (200 µM) prevents the DCG-IV induced effect. **B** Application of EGLU (200 µM) during disinhibited rhythm accelerates bursting (different preparation from **A**). **C, D** Mean data for burst period and burst duration in the presence of DCG-IV (n=14), EGLU (n=7) or their combination (n=7); *p<0.05. **E** Average duration of depolarized plateau (T_1, see Methods section) and intraburst oscillation period in control conditions or in the presence of EGLU (n=7)

rhythmicity was abolished by 5 µM L-AP4 within 2 min from start of application. After 4 min from the last burst, a stimulus delivered to a DR evoked one burst (indicated by the filled arrowhead in Fig. 5C; artefact of stimulation not shown due to slow signal sampling frequency); a further DR stimulus again induced bursting 1.5 min later.

The suppressant action of L-AP4 was prevented by previous application of CPPG (Fig. 5Ac). On average, the inhibition generated by 1–20 µM L-AP4 was antagonized by 50 µM CPPG (Fig. 5Ac,D,E). However, CPPG (50–200 µM) could not prevent the rhythm suppression by 50 µM L-AP4 (n=4; data not shown). CPPG (50–200 µM) per se produced no significant change in disinhibited bursting (see pooled data in Fig. 5D,E,F), despite occasional rhythm acceleration (Fig. 5Bb).

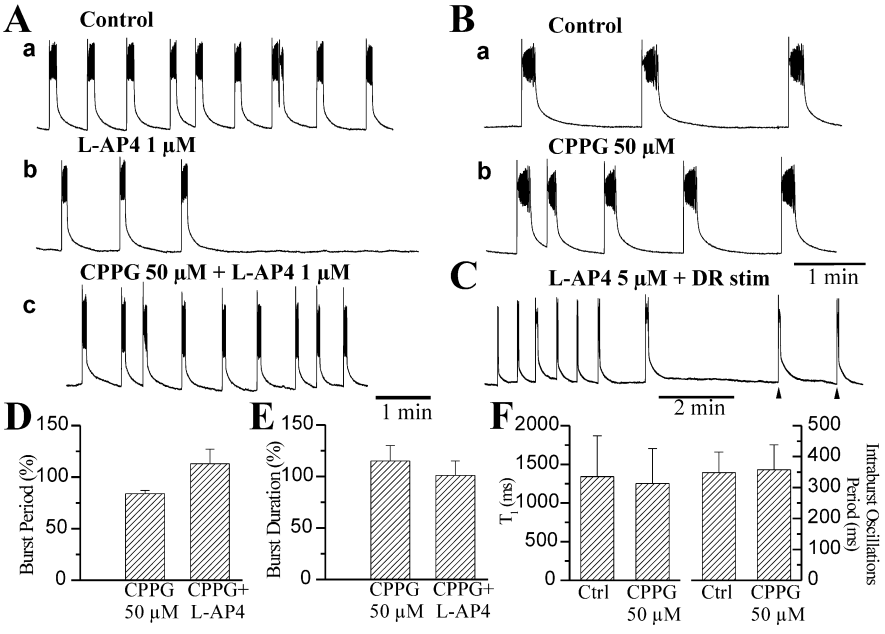

Fig. 5A–F Group III mGluRs modulate disinhibited rhythm. **Aa–Ac** L-AP4 (1 µM) abolishes rhythm within 5 min from start of application, an effect prevented by the selective antagonist CPPG (50 µM). **Ba, Bb** Application of CPPG (50 µM) during disinhibited rhythm does not alter bursting (different preparation from **A**). **C** Dorsal root stimulation (*arrowheads*) triggers bursts during the inhibition induced by L-AP4. **D, E** Histograms for average burst period and duration in the presence of CPPG (50 µM, n=4) or CPPG plus L-AP4 (1–5 µM, n=6). Data do not differ from control. **F** Average duration of depolarized plateau (T_1) and intraburst oscillation period in control conditions or in the presence of CPPG (50 µM, n=4)

Discussion

The principal finding of the present report is that activation of group II and III mGluRs differentially modulated the patterned activity generated by spinal networks following repeated DR stimuli or application of neurochemicals that induced fictive locomotion or disinhibited rhythms. It is noteworthy that the present data were obtained from neonatal rat spinal cords in which the expression of various mGluR groups is particularly high (Berthele et al. 1999). Since mGluR expression changes with tissue maturation (Berthele et al. 1999) it is unclear how subtype selective mGluR agents may influence synaptic transmission and network excitability in the adult. Nevertheless, the abundant expression of such receptors in neonatal tissue makes this preparation an excellent choice to exploit our understanding of the functional role of such receptors.

mGluRs and cumulative depolarization

In support of immunocytochemical studies demonstrating that group II and III mGluRs are scarcely found in the ventral horn of the mammalian spinal cord (Berthele et al. 1999; Aronica et al. 2001; Azkue et al. 2001), we confirmed that neither DCG-IV nor L-AP4 had any effect on membrane potential or input resistance of motoneurons. Interestingly, no change in membrane potential or input resistance of motoneurons was also observed with the antagonists EGLU and CPPG, suggesting that, under the present experimental conditions, there was insufficient ambient glutamate to tonically activate group II or III receptors on network cells impinging upon motoneurons. Thus, background neuronal excitability within the rat spinal cord was not dependent on persistent activation of group II receptors by extracellular glutamate as has been reported for the rat cerebral cortex (Bandrowski et al. 2003). However, particular conditions like sustained inhibition of glutamate uptake might raise glutamate levels sufficiently high to activate group II and III mGluRs (Bird et al. 2001).

In the present study, the ability by DCG-IV and L-AP4 to depress DR-evoked reflexes was consistent with a presynaptic reduction in transmitter release (Pinco and Lev-Tov 1993; Cao et al. 1995, 1997; Dong and Feldman 1999) and with the location of their mGluRs in the dorsal horn area to regulate afferent input flow (Berthele et al. 1999; Aronica et al. 2001; Azkue et al. 2001).

Intracellular recording from motoneurons indicated that DCG-IV depressed DR-evoked single synaptic potentials as has been previously observed with extracellular recording (Ishida et al. 1993). This effect was manifested as a decrease in the synaptic response area rather than in the early response peak, suggesting that polysynaptic networks were the prime target for mGluR II activation. It is interesting that the degree of reduction in synaptic responses could not be graded despite using DCG-IV concentrations ranging from 0.4 to 2 μM. This observation is compatible with expression of mGluR II activity being limited to certain synaptic pathways, which made constrained contribution to the overall size of the synaptic response recorded from motoneurons. Thus, for full suppression of synaptic activity it was necessary to activate group III rather than group II mGluRs.

Since the depressant action of DCG-IV was blocked by the selective group II antagonist EGLU, it was apparently mediated by group II mGluRs. Nevertheless, former reports on the action of DCG-IV on rat superficial dorsal horn neurons provide a complicated picture with inhibition or even facilitation of excitatory transmitter release (Gerber et al. 2000), or no effect (Chen et al. 2000). In the present study, DCG-IV attenuated cumulative depolarization without significantly changing spike windup or associated rhythmic oscillations. The overall reduction in cumulative depolarization was, however, of limited magnitude because the initial depressant action of DCG-IV on synaptic responses could not be maintained when synaptic inputs from DRs were repeatedly stimulated. This use-dependent waning of the inhibitory action by the group II agonist was unlikely to be due to its receptor desensitization and/or decay in the associated intracellular second messenger systems because the first synaptic response to subsequent stimulus trains remained depressed and because depression of disinhibited bursting persisted as long as DCG-IV was applied, indicating continuous drug effectiveness. Although DCG-IV inhibits GABAergic transmission in the rat cerebellum and can generate excitation through disinhibition (Vetter et al. 1999), this phenomenon is unlikely to have occurred in the spinal cord because DCG-IV did not disrupt fictive locomotor patterns, which are exquisitely dependent on efficient synaptic inhibition (Cowley and Schmidt 1995; Kremer and Lev-Tov 1997). It is also unlikely that any potential NMDA-like activity of high doses of DCG-IV (Breakwell et al. 1997; Uyama et al. 1997) could have limited the reduction in depolarization because even low concentrations of NMDA actually depress cumulative depolarization via network depolarization (Rozzo et al. 2002). In summary, it seems probable that repeated DR stimuli generated compensatory network changes to overcome the early depression of transmitter release by DCG-IV. While the identity of such mechanisms remains to be established by future investigations, it is feasible that one of them might be gradual build-up of extracellular K^+ with consequent facilitation of transmitter release (Marchetti et al. 2001b).

Unlike the action by DCG-IV, the effect of L-AP4 on cumulative depolarization was consistent with its ability to block strongly and reversibly synaptic transmission evoked by DR stimulation. It is noteworthy that, perhaps because of its waning inhibitory properties on synaptic transmission, DCG-IV is not an effective spinal analgesic drug when administered to rats in vivo (Fisher and Coderre 1996) whereas L-AP4 shows analgesic properties in certain rat (Fisher et al. 2002; Mills et al. 2002) or mouse (Onaka et al. 1996) chronic pain models.

mGluRs and fictive locomotion

Group II and III antagonists did not change fictive locomotion, indicating that, while this process requires endogenous glutamate (Cazalets et al. 1992; Beato et al. 1997), it did not crucially depend on activation of mGluRs. Notwithstanding the report of modest NMDA-like action by DCG-IV at concentrations higher than those used in the present study (Uyama et al. 1997), this agent did not significantly change locomotor rhythmic discharges.

It is, however, interesting that fictive locomotion was only moderately slowed down by 1 μM L-AP4, which strongly depressed synaptic transmission and cumulative depolarization. On the lamprey spinal cord, it was also surprising to find a similar discrepancy between the small decrease in locomotor rhythm frequency by L-AP4 and the strong depression of synaptic transmission (El Manira et al. 2002). It is clear that the inhibition caused by L-AP4 is not mediated via GABA or glycine (Gerber et al. 2000). Whelan et al. (2000) have suggested that fictive locomotion is the strongest functional pattern expressed by spinal networks because it has the highest threshold for onset but, once switched on, it has the ability to turn off the activity of most other networks. Our results are compatible with this view since fictive locomotion does not crucially require the activity of those spinal regions, which are particularly rich in group III mGluRs (Azkue et al. 2001). Our data also indicate that it might be possible, with application of a group III agonist, to reduce cumulative depolarization evoked by strong peripheral inputs without concomitant suppression of fictive locomotor patterns. This notion can help to explain why L-AP4 slowed but did not abolish fictive locomotor patterns, yet fully suppressed analogous oscillations evoked by repeated DR stimuli: in the first instance, oscillations were due to a central pattern generator chemically activated by NMDA and 5-HT, being weakly endowed with group III receptors, and downstream of the dorsal horn neurons processing afferent DR inputs. In the second case, the high density of activated group III mGluRs on dorsal horn neurons might have prevented adequate excitatory inputs reaching the central pattern generator.

Group II and III mGluRs role in disinhibited bursting

Disinhibited bursts are very large depolarizations due to synchronous activation of spinal interneurons and motoneurons (Bracci et al. 1996a, 1996b). Current evidence supports the view that fictive locomotion and disinhibited bursting may be generated by analogous spinal networks (Beato and Nistri 1999). However, while the central pattern generator of fictive locomotion is reputed to be located around the central canal (Butt et al. 2002), disinhibited bursting is triggered by spatio-temporal coincidence of random discharges by spontaneously active neurons often located in the ventral horn area (Tscherter et al. 2001; Darbon et al. 2002). While it is clear that neither pattern is dependent on the dorsal horn area where group II and III mGluRs are primarily found (Berthele et al. 1999; Aronica et al. 2001; Azkue et al. 2001), disinhibited bursting, more than fictive locomotion, critically depended on mGluRs for two reasons: (1) each rhythmic cycle is a relatively long excitatory phase (about 7 s; Bracci et al. 1996a), during which glutamate release might be sufficient for activation of a widespread family of mGluRs throughout the spinal cord to terminate a burst episode; (2) even a modest decrease in glutamate release caused by switching on a limited cluster of group II and III receptors with exogenously applied agonists might impair the ability of recurrent excitatory collaterals to recruit spinal neurons for burst onset and maintenance (Tscherter et al. 2001; Darbon et al. 2002).

In accordance with these views, the present study observed that activation of group II or III mGluRs consistently inhibited bursting, a phenomenon antagonized by their respective class-selective blockers. Bursts became slower or even disappeared without significant change in their duration. Even when burst inhibition was complete like in the case of L-AP4 application, electrical stimulation of one DR promptly evoked a burst, indicating that there was a large fall in network excitability, perhaps due to a reduced number of spontaneously active neurons (Tscherter et al. 2001; Darbon et al. 2002) unable to recruit other network elements to trigger bursts. Strong afferent inputs by the stimulated DR fibres then provided an adequate signal to boost excitability.

While the group II antagonist EGLU could facilitate bursting, the group III antagonist CPPG did not, suggesting that there was very limited activation of group III mGluRs by endogenously released glutamate in this experimental model. Conversely, group II receptors seemed to play a significant role in controlling burst duration and periodicity.

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Modulation of rhythmic patterns and cumulative depolarization by group I metabotropic glutamate receptors in the neonatal rat spinal cord *in vitro*

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Abstract

The role of group I metabotropic glutamate receptors (mGluRs), and their subtypes 1 or 5, in rhythmic patterns generated by the neonatal rat spinal cord was investigated. Fictive locomotor patterns induced by *N*-methyl-D-aspartate + serotonin were slowed down by the subtype 1 antagonists (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA) or 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt) and unaffected by the subtype 5 antagonist 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP). The group I agonist (*RS*)-3,5-dihydroxyphenylglycine (DHPG) depolarized ventral roots and disrupted fictive locomotion, an effect blocked by AIDA (or CPCCOEt) and reversed by increasing the *N*-methyl-D-aspartate concentration. Cumulative depolarization induced by low frequency trains of dorsal root stimuli was attenuated by DHPG and unchanged by AIDA or MPEP while rhythmic patterns or motoneuron spike wind-up persisted. Disinhibited bursting induced by strychnine + bicuculline was accelerated by DHPG, slowed down by AIDA (which prevented the action of DHPG), unaffected by MPEP and counteracted by the selective group II agonist (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine. The DHPG transformed regular bursting into arrhythmic bursting, a phenomenon also produced by the group II mGluR antagonist (2*S*)-α-ethylglutamic acid. These results indicate that, during fictive locomotion or disinhibited bursting, endogenous glutamate could activate discrete clusters of subtype 1 mGluRs to facilitate discharges. Diffuse activation by the exogenous agonist DHPG of group I mGluRs throughout spinal networks had an excitatory effect overshadowed by its much stronger depressant action due to concomitant facilitation of glycinergic transmission. Irregular disinhibited bursting caused by activation of subtype 1 receptors or block of group II receptors suggests that mGluRs could control not only the frequency but also the periodicity of bursting patterns, outlining novel mechanisms contributing to burst duration.

Introduction

The mammalian spinal cord is an excellent model to study cellular and network properties of rhythmic patterns. One type of rhythmic activity is fictive locomotion (induced by bath application of excitatory agents) which consists of alternating electrical discharges between flexor and extensor motor pools (Kiehn & Kjaerulff, 1998; Butt *et al.*, 2002). This network programme relies on glutamatergic excitatory transmission (Cazalets *et al.*, 1992; Beato *et al.*, 1997) plus glycine- and GABA-mediated inhibition for pattern alternation (Cowley & Schmidt, 1995; Kremer & Lev-Tov, 1997). Repeated stimuli to dorsal root (DR) afferents induce a slow cumulative depolarization of interneurons and motoneurons, with accompanying electrical oscillations (Baranauskas & Nistri, 1995) expressed as alternating motor discharges (Whelan *et al.*, 2000; Marchetti *et al.*, 2001).

A different type of rhythmicity (disinhibited bursting; Bracci *et al.*, 1996a,b) emerges after blocking GABAergic and glycinergic inhibition. This pattern is apparently generated by networks analogous to those responsible for fictive locomotion (Beato & Nistri, 1999) and is due to periodic release of glutamate by intrinsically active interneurons which recruit an extensive circuit through recurrent excitatory collaterals (Tscherter *et al.*, 2001; Darbon *et al.*, 2002).

Notwithstanding the ubiquitous role of glutamate as excitatory transmitter via ionotropic receptors in motor networks (Rekling *et al.*, 2000), this amino acid also activates metabotropic glutamate receptors (mGluRs) comprising three main groups (I, II and III; Pin & Duvoisin, 1995; Schoepp *et al.*, 1999). The mammalian spinal cord mainly expresses group I mGluRs (Valerio *et al.*, 1997; Berthele *et al.*, 1999; Alvarez *et al.*, 2000) which, via intracellular Ca^{2+} release and inositol-triphosphate formation, modulate Ca^{2+} and K^{+} conductances (Pin & Duvoisin, 1995; Anwyl, 1999; Schoepp *et al.*, 1999; Fagni *et al.*, 2000). In the rat spinal cord group I mGluRs increase motoneuron excitability (Dong & Feldman, 1999; Ugolini *et al.*, 1999) together with facilitation of inhibitory transmission (Dang *et al.*, 2002). Therefore, it seems important to understand how such effects may be translated into rhythmic patterns. In the lamprey spinal cord mGluRs generate a slow rhythm with either phase alternation or phase synchrony, perhaps dependent on the extent of modulation of inhibitory

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transmission (Aoki *et al.*, 2001). Such a facilitation is mediated by group I mGluRs because the selective class I agonist (*RS*)-3,5-dihydroxyphenylglycine (DHPG) enhances the postsynaptic membrane action and fictive swimming evoked by *N*-methyl-D-aspartate (NMDA) (Krieger *et al.*, 2000) probably by facilitating Ca^{2+} influx and excitatory synaptic potentials (Takahashi & Alford, 2002), although the extent of presynaptic facilitation remains controversial (El Manira *et al.*, 2002; Kettunen *et al.*, 2002). However, little is known about the role of group I mGluRs in the rhythmogenesis of the rat spinal cord (El Manira *et al.*, 2002), although the subtypes 1 and 5 of group I mGluRs are potential effectors for rhythm regulation in view of their powerful network activity (Marchetti *et al.*, 2003).

Materials and methods

Procedures involving animals were conducted in accordance with NIH guidelines and Italian national law DL 27/1/92 no. 116 following the European Community directive nos 86/609 and 93/88 (Italian Ministry of Health authorization for the local animal care facility in Trieste, D.M. 69/98-B). Full details of the experimental methods can be found in Bracci *et al.* (1996a,b) and Marchetti *et al.* (2001, 2003). In brief, rats were anaesthetized with urethane (10%, i.p.), and the neonatal spinal cord was superfused with Krebs solution of the following composition (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% O_2–5% CO_2; pH 7.4 at room temperature. Most experiments were conducted with DC-coupled ventral root (VR) recordings because of the need to record fictive locomotion and disinhibited rhythms for a long time, as well as to monitor discharge phase coupling amongst various VRs. Experiments with the cumulative depolarization protocol (Barbieri & Nistri, 2001) relied on simultaneous VR and intracellular recordings (from L3–L5 motoneurons) using sharp electrodes filled with either 3 M KCl (30–60 MΩ resistance) or 2 M $KMeSO_4$ (60–120 MΩ resistance). In some experiments QX-314 (20–30 mM; Alomone Laboratories, Jerusalem, Israel) was added to the intracellular solution to block action potential generation. On average, the resting membrane potential and input resistance of motoneurons were -75 ± 6 mV and 47 ± 23 MΩ ($n = 10$ cells).

Dorsal root electrical stimuli, delivered via miniature bipolar suction electrodes, were employed to elicit VR responses recorded from the ipsilateral VR of the same segment. Stimulus intensity (1–10 V range; 0.1 ms duration) was calculated in terms of threshold, defined as the minimum intensity to elicit a detectable response in the homolateral VR (see Marchetti *et al.*, 2001). Cumulative depolarization area and wind-up (expressed as number of spikes in the train) were measured as previously reported (Barbieri & Nistri, 2001).

In general, rhythmic discharges were quantified from extracellular records in terms of their period, defined as the time between the onset of two cycles of oscillatory activity for locomotor-like rhythm or of two bursts for disinhibited rhythm. When period values were averaged for a pool of preparations, data from each spinal cord were calculated as the mean of at least 20 cycles or bursts (unless otherwise indicated). Burst clusters were defined as episodes in which a burst started before the previous burst had returned to baseline. Pause between disinhibited bursts was the interval between the end of a burst and the start of the next burst. Data were quantified as means ±SD with *n* equivalent to the number of preparations or intracellularly recorded cells. Statistical significance was assessed with the Student's *t*-test applied to raw data which were normally distributed or with ANOVA plus Tukey test for nonparametric data. To analyse whether locomotor-like oscillations were alternating amongst pairs of VRs, in each pair their phase between two VRs was defined as the latency for the onset of a cycle in one root during the cycle of the other root, divided by the period and expressed in angular degrees whereby 180° represent complete phase alternation and 0 or 360° full phase coincidence (Kjaerulff & Kiehn, 1996). The Rayleigh test, based on circular statistics, was then used for statistical significance of phase coupling (Drew & Doucet, 1991) where R is the concentration of phase values around the mean phase (Φ; see Marchetti *et al.*, 2001). The accepted level of significance was $P = 0.05$ in all cases.

The following mGluR agonists and antagonists were purchased from Tocris Cookson (Bristol, UK) [full data on their pharmacological selectivity can be found in Schoepp *et al.* (1999)]: (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA; selective antagonist for mGluR1), 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt; selective antagonist for mGluR1), DHPG (selective agonist for group I receptors and equipotent on mGluR1 and mGluR5 subtypes), 2-methyl-6-(phenylethynyl)pyridine hydrochloride (selective antagonist for mGluR5 receptors), (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (selective group II agonist) and (2*S*)-α-ethylglutamic acid (EGLU; selective group II antagonist). *N*-methyl-D-aspartate and D-amino-phosphonovalerate were also purchased from Tocris Cookson while 5-hydroxytryptamine (5-HT), strychnine, bicuculline and nifedipine were from Sigma (Milan, Italy).

Results

Group I metabotropic glutamate receptor activity and chemically induced fictive locomotion

To induce stable fictive locomotor patterns we used the combined application of NMDA and 5-HT, which is a standard regimen to observe this phenomenon in a reliable manner for a persistent time (Cazalets *et al.*, 1992; Kjaerulff & Kiehn, 1996; Kremer & Lev-Tov, 1997; Bracci *et al.*, 1998; Kiehn & Kjaerulff, 1998; Beato & Nistri, 1999). Figure 1A shows an example of a preparation exhibiting a stable, fictive locomotor pattern (note alternating cycles between L2 and L5 VRs on the same side and left and right VRs of the same segment) induced by 5 μM NMDA plus 10 μM 5-HT. Bath application of 5 μM DHPG for 5 min disrupted this rhythm (Fig. 1B), although the few remaining cycles (Fig. 1B) preserved phase alternation between homolateral (L2/L5, $R = 0.86$, $\Phi = 182°$ for DHPG vs. $R = 0.77$, $\Phi = 180°$ in control) and homosegmental (left/right, $R = 0.83$, $\Phi = 185°$ for DHPG vs. $R = 0.97$, $\Phi = 182°$ in control) VRs. Pooled data from four preparations demonstrated the persistence of phase coupling for fictive locomotor patterns during DHPG application (homosegmental left/right, $R = 0.86 \pm 0.04$, $\Phi = 180 \pm 5°$ in DHPG vs. $R = 0.81 \pm 0.16$, $\Phi = 182 \pm 3°$ in control; homolateral L2/L5, $R = 0.84 \pm 0.01$, $\Phi = 181 \pm 1°$ in DHPG vs. $R = 0.78 \pm 0.18$, $\Phi = 178 \pm 13°$ in control). Conversely, fast and slow (usually synchronous) oscillations typical of DHPG application (Marchetti *et al.*, 2003) were not detected during fictive locomotion, although VR depolarization was routinely observed (0.2 ± 0.1 mV; $n = 16$).

The effect of DHPG on fictive locomotor discharges consistently led to pattern suppression ($n = 15/16$ as in one case we observed a 16% rhythm acceleration). Pattern suppression was observed after applying 5–50 μM DHPG and was unrelated to the agonist concentration, indicating that the lowest dose was fully effective in blocking it. In seven of 15 preparations pattern suppression was complete within 4 ± 1 min (although reversible after 10–15 min washout) while, in eight of 15 spinal cords, the pattern suppression was associated with transient locomotor-like cycles (usually episodes of 10 cycles) with period values ($101 \pm 13\%$) similar to control. Lower concentrations of DHPG (300 nM–2 μM) led to slowing down of fictive locomotor patterns without changing their alternation. In particular, rhythm

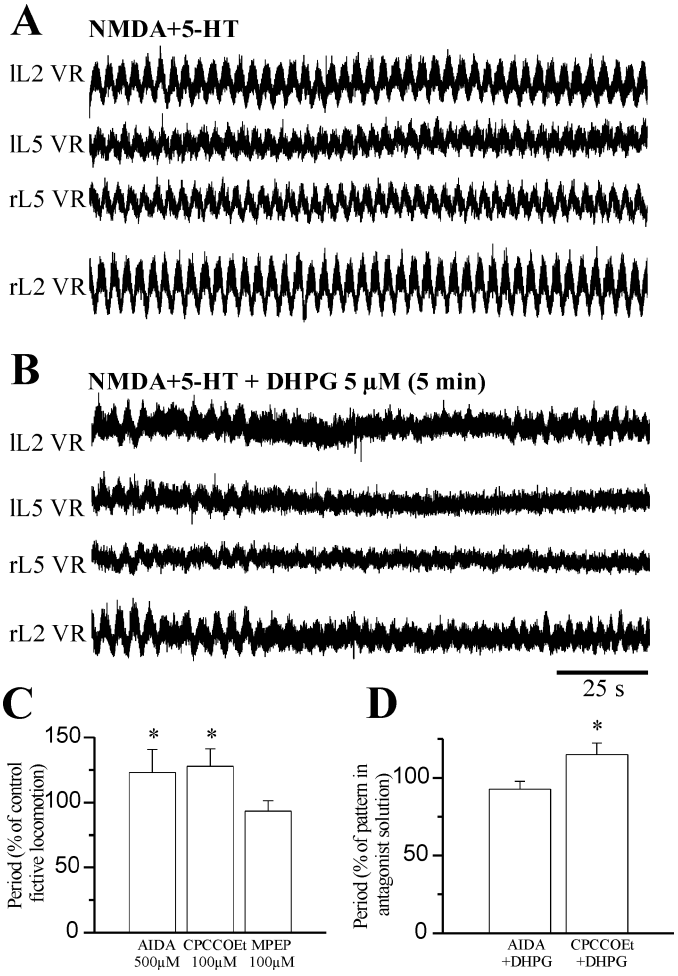

FIG. 1. Role of group I metabotropic glutamate receptors (mGluRs) in fictive locomotion. (A) Example of *N*-methyl-D-aspartate (NMDA) (5 μM) + 5-hydroxytryptamine (5-HT) (10 μM)-induced fictive locomotor rhythm recorded from four ventral roots (VRs) as indicated alongside the trace. (B) Application of (*RS*)-3,5-dihydroxyphenylglycine (DHPG) (5 μM, 5 min) disrupts rhythm and leaves occasional cycles with preserved phase alternation. (C) Mean period of oscillations during fictive locomotor rhythm, evoked in the presence of the mGluR1 antagonists (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA) ($n = 13$) or 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt) ($n = 4$) or the mGluR5 antagonist 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) ($n = 8$). All data are expressed as a percentage with respect to control fictive locomotion; $^*P < 0.01$. (D) In the presence of AIDA DHPG does not significantly alter the mean period of fictive locomotor oscillations while it slightly retards them in the presence of CPCCOEt. In all cases $n = 4$; $P < 0.05$. Data are expressed as percentage period change with respect to the value observed in the presence of antagonist alone. lL, left lumbar (segment); rL, right lumbar (segment). Segment numbers are given as arabic numerals.

retardation was apparent with 300 nM DHPG ($121 \pm 18\%$, $n = 4$, $P < 0.05$) but, although remaining significant vs. control values ($P < 0.05$), it did not intensify with the application of larger doses ($121 \pm 15\%$ with 1 μM, $n = 8$; $124 \pm 23\%$ with 2 μM, $n = 4$). Furthermore, sustained (≥ 20 min) application of 1–2 μM DHPG led to transient pattern suppression as routinely observed with 5–50 μM DHPG. These data indicate that it was difficult to grade either the rhythm deceleration or its suppression even when agonist concentrations were changed by more than 10-fold.

We next investigated whether group I mGluRs might be endogenously activated during fictive locomotion. The subtype 1 antagonists AIDA (500 μM) or CPCCOEt (100 μM) significantly delayed the rhythm period (Fig. 1C) although the fictive locomotor pattern remained stable with unchanged cycle amplitude. Conversely, the subtype 5 antagonist 2-methyl-6-(phenylethynyl)pyridine hydrochloride (10–100 μM) did not alter fictive locomotor patterns (Fig. 1C). When DHPG (20 μM) was applied in the presence of AIDA or CPCCOEt, we never observed pattern suppression. The fictive locomotor rhythm during application of DHPG + AIDA (or +CPCCOEt) had the same period as found in the presence of AIDA alone (Fig. 1D) or was slightly (15%) delayed with respect to that observed with CPCCOEt alone (Fig. 1D). The similar effects produced by AIDA and CPCCOEt suggested that they were mediated by group I receptors (Schoepp *et al.*, 1999). The competitive nature of AIDA antagonism (Moroni *et al.*, 1997) made it more suitable whenever experimental protocols required recovery after antagonist washout. For this reason our subsequent experiments were based on the use of AIDA. In the presence of 2-methyl-6-(phenylethynyl)pyridine hydrochloride, DHPG fully retained its rhythm inhibitory action ($n = 6$; not shown).

We next explored if the disruption of fictive locomotor patterns by DHPG could be reversed by changing the concentration of NMDA or 5-HT. Figure 2 shows an example of disruption of stable locomotor-like rhythm by DHPG (Fig. 2A and B) and subsequent restoration of the pattern when the NMDA concentration was raised from 5 to 9 μM (Fig. 2C). A higher dose (13 μM) of NMDA (Fig. 2D) then abolished the rhythm. This observation was repeated on three preparations. Likewise, decreasing the 5-HT concentration from 10 to 5 μM blocked the rhythm which was restored by adding DHPG ($n = 4$; see example in Fig. 3).

Oscillatory activity evoked by dorsal root stimulation in relation to group I metabotropic glutamate receptor activity

In control solution repeated DR stimulation leads to cumulative depolarization and network oscillations (Baranauskas & Nistri, 1995) with alternating properties typical of fictive locomotion (Whelan *et al.*, 2000; Marchetti *et al.*, 2001). Conversely, DHPG induces synchronous oscillations (Marchetti *et al.*, 2003). We wondered if these two patterns could coexist. In 10 preparations, 5 μM DHPG did not alter the number of locomotor-like oscillations ($97 \pm 26\%$, $n = 10$; $P > 0.05$) due to DR stimuli. Furthermore, the phase alternation of oscillations induced by repetitive DR stimulation was also unchanged in the presence of DHPG as demonstrated by the simultaneous recording from three VRs and one motoneuron (Fig. 4). In this example, 5 μM DHPG-induced oscillations appeared, before the DR stimulus train, on all four records and were synchronous (see trace expansion on a faster time scale in Fig. 4B). In these conditions, repetitive stimulation applied to a single DR induced alternating, locomotor-like discharges (see expansion on a faster time scale in Fig. 4D) while the fast (and slow) synchronous oscillations evoked by DHPG were lost ($n = 10$). The Rayleigh test performed on DR-induced oscillations in the presence of DHPG demonstrated phase alternation, with $R = 0.82$ and $\Phi = 178^\circ$ (see Fig. 4C, bottom). Applying the Rayleigh test to four preparations for analysing phase values of DR-induced oscillations in control conditions or in the presence of DHPG yielded similar results with average R-values of 0.76 ± 0.17 and $\Phi = 142 \pm 27^\circ$ in control vs. $R = 0.77 \pm 10\%$ and $\Phi = 147 \pm 36^\circ$ in DHPG solution ($P > 0.05$). These results clearly demonstrate, during repeated DR stimuli, the persistence of alternating patterns in the presence of DHPG.

(*RS*)-3,5-dihydroxyphenylglycine (5 μM) also affected the area of cumulative depolarization measured from VRs ($80 \pm 30\%$ of control area, $P < 0.005$, $n = 29$) or from single motoneurons ($68 \pm 23\%$, $n = 10$, $P < 0.05$) without significant change in wind-up (number of action potentials evoked during a train and measured from intracellular records; $108 \pm 12\%$, $n = 4$).

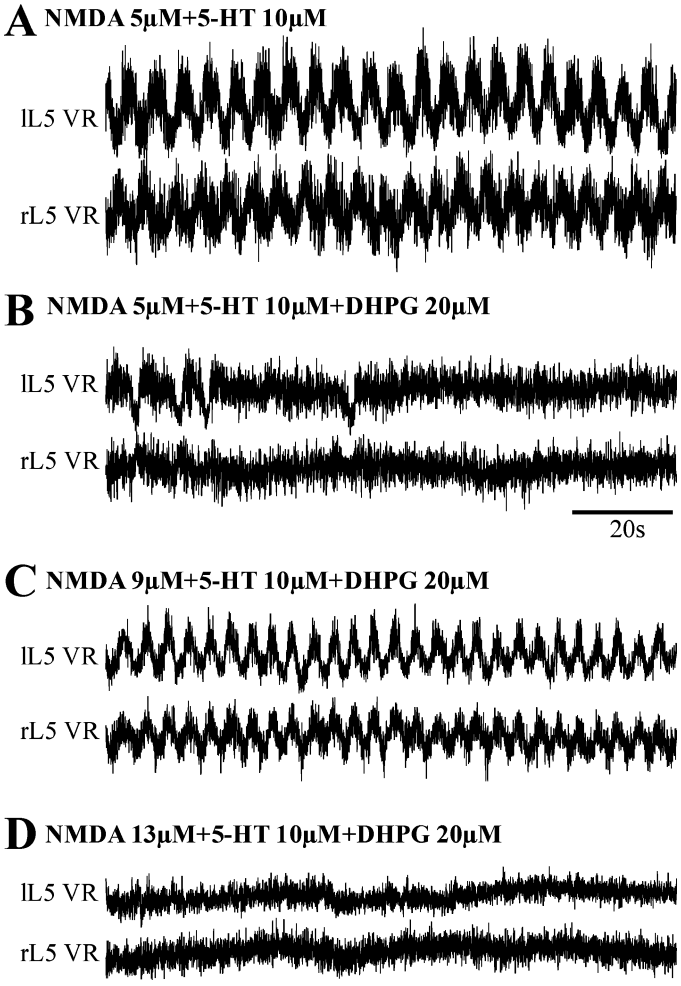

FIG. 2. Reversal of (*RS*)-3,5-dihydroxyphenylglycine (DHPG) inhibition of locomotor rhythm by raising *N*-methyl-D-aspartate (NMDA) concentration. (A) Fictive locomotor rhythm evoked by 5 μM NMDA plus 10 μM 5-hydroxytryptamine (5-HT). Records are from left (l) and right (r) L5 ventral roots (VRs) showing pattern alternation at homosegmental level. (B) Application of 20 μM DHPG (4.5 min) disrupts rhythmicity. (C) Increasing the NMDA concentration to 9 μM restores the locomotor rhythm despite the continuous presence of DHPG. (D) Further increase in NMDA concentration to 13 μM blocks rhythmicity presumably due to excessive network depolarization. All data are from the same preparation. Calibration bar refers to all traces.

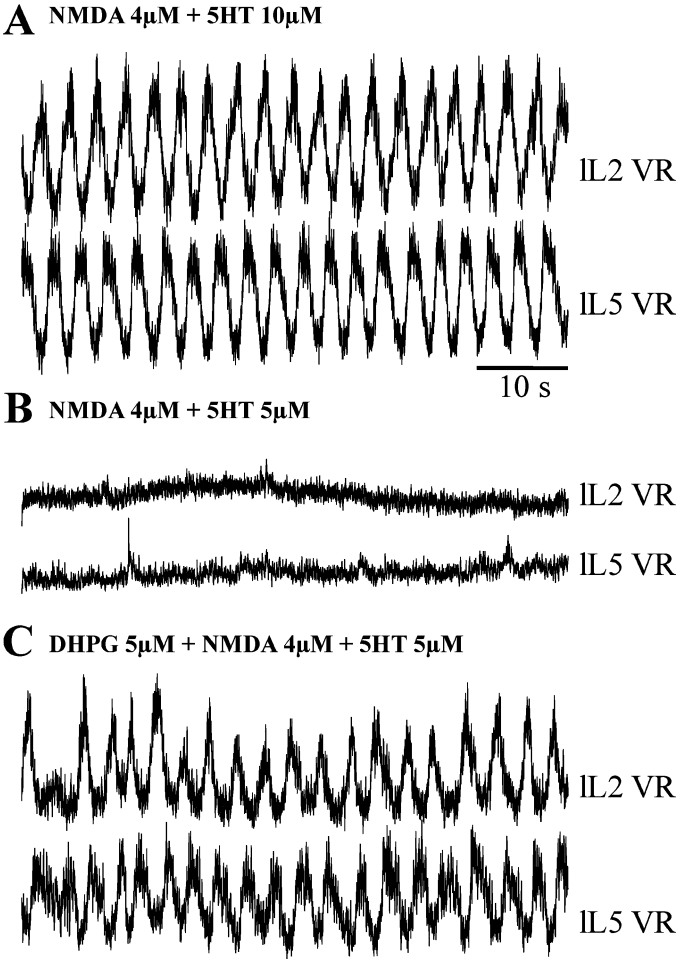

FIG. 3. (*RS*)-3,5-dihydroxyphenylglycine (DHPG) restarts fictive locomotion suppressed by lowering 5-hydroxytryptamine (5-HT) concentration. (A) Fictive locomotor rhythm evoked by 4 μM *N*-methyl-D-aspartate (NMDA) plus 10 μM 5-HT. Records are from left (l) L2 and L5 ventral roots (VRs) showing pattern alternation at intersegmental level on the same side. (B) Decreasing 5-HT concentration to 5 μM blocks rhythmicity. (C) Application of 5 μM DHPG restores the locomotor rhythm despite the low dose of 5-HT. All data are from the same preparation. Calibration bar refers to all traces.

As DHPG facilitates glycinergic transmission (Marchetti *et al.*, 2003), we tested if the glycine receptor antagonist strychnine (1 μM) blocked the depression of cumulative depolarization area induced by DHPG (5 μM). While strychinine *per se* did not significantly increase cumulative depolarization (124 ± 29%; $n = 4$), it fully prevented the action by DHPG (109 ± 43%; $n = 4$). Neither AIDA (500 μM) nor 2-methyl-6-(phenylethynyl)pyridine hydrochloride (100 μM) reduced the cumulative depolarization area measured either extracellularly (93 ± 8%, $n = 6$ and 93 ± 15%, $n = 4$, respectively) or intracellularly (94 ± 3%, $n = 4$ and 99 ± 3%, $n = 4$, respectively).

Metabotropic glutamate receptor I activity accelerated disinhibited rhythms and made them irregular

Figure 5A exemplifies the effect of DHPG (5 μM) which reduced bursting period by 40% without eliciting oscillatory activity during the interburst interval (high gain records analysed in 17 preparations). Pooled data (Fig. 5B and C) indicate that DHPG (5 μM) significantly decreased the burst period with a commensurate shortening of burst duration. Such period reductions were similar (56 ± 11%, $n = 11$) for higher concentrations of DHPG (10–50 μM) and absent (100 ± 16%, $n = 4$) at lower concentrations (0.5–1 μM). These effects of DHPG were blocked by AIDA (500 μM; Fig. 5A) which *per se* significantly lengthened burst period (Fig. 5B) without altering burst duration (Fig. 5C). 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (100 μM) by itself did not change burst period or duration (97 ± 10% and 93 ± 7%, $n = 4$) and left the accelerating activity of DHPG unaffected (35 ± 9% period and 51 ± 9% duration, $n = 4$).

The pharmacological block of Cl^--mediated transmission during disinhibited bursting should have removed the component caused by DHPG-evoked facilitation of glycinergic synaptic activity (Marchetti *et al.*, 2003). Hence, under such conditions it should be possible to examine whether rhythm period acceleration by DHPG was related to any associated network depolarization. Bath application of DHPG during disinhibited rhythm induced VR depolarization (0.20 ± 0.09 mV, $n = 13$) which always accompanied burst acceleration. Periodicity in 5 μM DHPG (plus strychnine and bicuculline) solution was irregular (0.34 ± 0.06 CV, coefficient of variation; $n = 4$) and significantly different ($P < 0.02$) from the highly regular period in strychnine

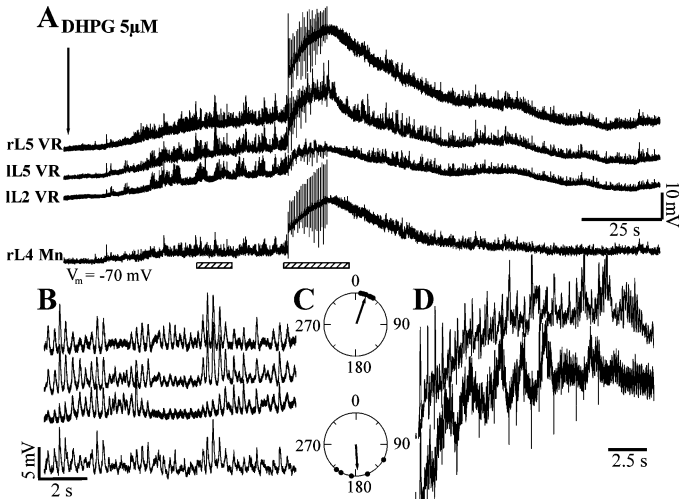

FIG. 4. Rhythmic activity in the presence of (*RS*)-3,5-dihydroxyphenylglycine (DHPG) (5 μM). (A) Sample records of ventral root (VR) responses [from right (r) L5, left (l) L5 and left L2 VRs as indicated alongside their corresponding trace] and right L4 single motoneuron (recorded intracellularly at resting potential $V_m = -70$ mV with an electrode containing QX-314 to block action potential generation). DHPG is applied as indicated by the arrow and induces depolarization associated with appearance of synchronous oscillatory activity. One train of dorsal root (DR) stimuli (2 Hz) generates cumulative depolarization accompanied by appearance of alternating rhythmic discharges which suppress synchronous pattern evoked by DHPG. After the end of the train synchronous oscillations return. (B) Faster time base and higher gain record of trace segments corresponding to the left (short) hatched bar in A. Note synchronous oscillations. (C) Polar plots (for left L2 and left L5 VRs) to compare phase of oscillatory activity induced by DHPG (top plot; note datapoints concentrated near zero to indicate synchronicity) or stimulus train (bottom plot; clustering of datapoints around 180° indicates alternation). (D) Faster time base and higher gain record of two traces taken at the time indicated by the right (long) hatched bar in A. Records demonstrate alternating patterns on left L2 and left L5 VRs during DR stimulus train. Vertical calibrations refer to intracellular records only. Mn, motoneuron.

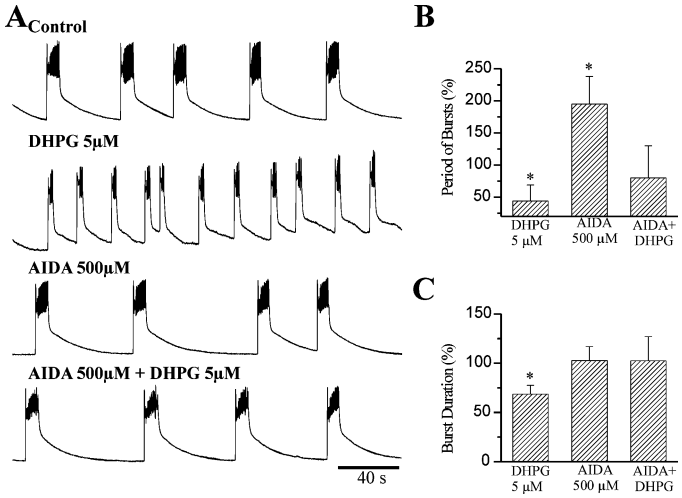

FIG. 5. Action of (*RS*)-3,5-dihydroxyphenylglycine (DHPG) on disinhibited bursting. (A) Disinhibited bursting recorded in control conditions from single lumbar ventral root is accelerated, with loss of regularity, by DHPG (5 μM) and retarded by (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA) (500 μM) which prevents the DHPG-induced acceleration. (B and C) Average data for period of bursting and single burst duration in the presence of DHPG ($n = 11$), AIDA ($n = 11$) or the combination of these ($n = 5$); $^*P < 0.05$.

and bicuculline solution (0.22 ± 0.09 CV). This characteristic action of DHPG was often manifested by the emergence of complex bursts (see example in Figs 5A and 6B) often coalescing into a depolarization cluster. On average, the incidence of clusters per 30 consecutive burst episodes was increased by $17 \pm 12\%$ ($P < 0.005$; nine preparations) in the presence of DHPG. Disinhibited bursts evoked by DR stimulation can be frequency entrained (Bracci *et al.*, 1997). However, in sharp contrast to its modulation of spontaneously occurring bursts, DHPG (5 μM) did not modify bursts evoked by DR electrical stimulation. On average, in the presence of DHPG, burst area was $104 \pm 21\%$ and peak amplitude was $100 \pm 2\%$ ($n = 6$) of control evoked bursts. In these conditions burst entrainment by repeated (0.05 Hz) stimuli could be maintained.

During a relatively long burst episode the oscillatory and depolarizing activity of group I mGluRs (Marchetti *et al.*, 2003) might be expected to be counteracted by coactivation (by endogenous glutamate) of the functionally opposite, depressant group II mGluRs that operate by reducing excitatory transmitter release in the rat spinal cord (Ishida *et al.*, 1993). To test this issue we performed experiments in which we first applied DHPG (5 μM) to induce burst acceleration and then applied DHPG plus the selective group II antagonist EGLU (200 μM). A representative example shows that disinhibited bursting accelerated by DHPG (Fig. 6A and B; 15% burst clustering) was further accelerated by application of EGLU with emergence of stronger burst clustering (Fig. 6C; 34%). Figure 6D and E shows histograms for burst period and duration in those experimental conditions indicating that, in the presence of EGLU, DHPG retained its ability to decrease bursting period and duration. EGLU (200 μM) *per se* reduced bursting period to $54 \pm 11\%$, which became irregular as indicated by the significantly larger CV value (0.34 ± 0.14; $P < 0.05$), and decreased burst duration to $78 \pm 17\%$ ($n = 6$). Coapplication of DHPG (50 μM) and the group II agonist (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (1 μM) did not significantly change bursting period ($121 \pm 19\%$ with an average CV value of 0.18 ± 0.03, $n = 6$), even if (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine *per se* slowed down bursting ($199 \pm 18\%$, $n = 14$).

Discussion

The present report demonstrates that group I mGluRs differentially modulated patterned activity generated by rat spinal networks. Novel findings were that group I receptors activated by endogenous glutamate played a role in fictive locomotion and disinhibited bursting, while exogenous activation of such receptors depressed cumulative depolarization with preserved spike wind-up and associated alternating rhythms and facilitated disinhibited bursting which lost its regular rhythmicity.

Group I metabotropic glutamate receptors and fictive locomotion

Subtype 1 antagonists lengthened the fictive locomotion period, indicating that endogenous glutamate could activate subtype 1 mGluRs with a facilitatory role on this type of spinal rhythmicity. These data fully accord with the observations that, in the lamprey spinal cord, subtype 1 mGluRs support alternating motor patterns via presynaptic (Takahashi & Alford, 2002) and postsynaptic (Krieger *et al.*, 2000; Kettunen *et al.*, 2002) sites of action. It was, therefore, surprising that, in the rat spinal cord, fictive locomotion induced by NMDA plus 5-HT was actually disrupted by DHPG. Analogous data have been obtained with (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (Taccola *et al.*, 2003), although the broad spectrum activation of mGluRs by this drug does not allow identification of the receptor type(s) involved.

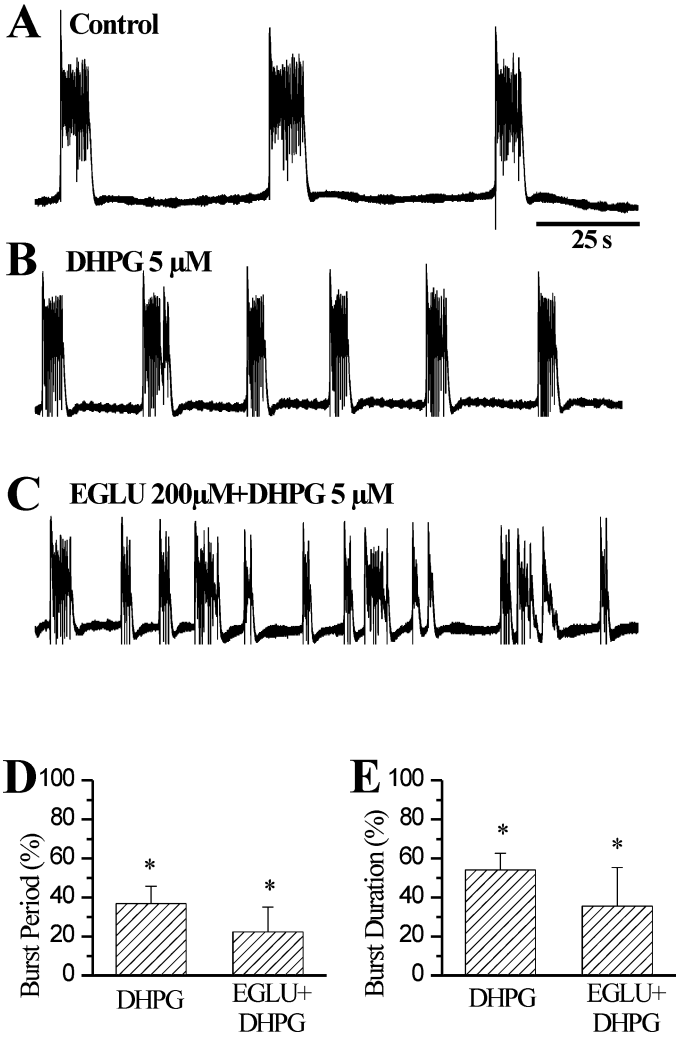

FIG. 6. Disinhibited bursting acceleration induced by (*RS*)-3,5-dihydroxyphenylglycine (DHPG) and (2*S*)-α-ethylglutamic acid (EGLU). (A) Sample record of ventral root responses showing control disinhibited bursting in the presence of strychnine and bicuculline. (B) Application of DHPG (5 μM; 5 min) speeds up bursting which becomes irregular. (C) Coapplication of DHPG and EGLU (200 μM) produces further burst acceleration with arrhythmic bursting. All responses are from the same preparation. (D and E) Histograms of burst period and duration in the presence of DHPG or DHPG + EGLU. In both cases reduction in period and duration is significant ($^{*}P < 0.001$; $n = 4$).

It is noteworthy that fictive locomotion was retarded by applying low concentrations of DHPG, thus producing an effect analogous to that of AIDA. These results suggest that, while the mGluR population activated by endogenous glutamate during fictive locomotion was probably functionally associated with a facilitatory effect (as shown by its block by AIDA), exogenous application of the agonist DHPG acted on a much larger mGluR population with mixed facilitatory and inhibitory actions on fictive locomotion. To support this interpretation it was necessary to clarify the nature of the block of fictive locomotion by DHPG (excessive excitation or enhanced synaptic inhibition).

Increasing the concentration of NMDA was a simple method of raising network excitability which then counteracted the DHPG inhibition, unless the NMDA concentration was so high as to produce excessive depolarization. Had the disruptive action by DHPG been due to excessive excitation, it would then be difficult to understand why producing even more network excitation by rising the NMDA dose should reverse the depression by DHPG. Note that the contrasting action by NMDA and DHPG indicate that potentiation of NMDA receptors by DHPG as described in lamprey spinal cord neurons (El Manira *et al.*, 2002) was unlikely to occur in the neonatal rat spinal cord. If we assume that DHPG could stop the rhythm via enhanced inhibition because NMDA could reverse it, one should conclude that such an inhibitory effect also required concomitant activation of 5-HT receptors because, when the rhythm was arrested by reducing the bath concentration of 5-HT, DHPG could restart it. Application of 5-HT alone has complex effects on the locomotor central pattern generator with concurrent facilitation (mediated by 5-HT_2 receptors; Bracci *et al.*, 1998) and inhibition (mediated by 5-HT_1 receptors; Beato & Nistri, 1998) resulting in stable, regular rhythmicity (Kiehn & Kjaerulff, 1996). These data, therefore, raise the possibility that the outcome of group I mGluR activity on fictive locomotion depended on the functional state of multiple classes of 5-HT receptors.

As a recent report has indicated that depression of synaptic transmission by DHPG requires glycine receptors (Marchetti *et al.*, 2003), glycinergic interneurons are also potentially involved in the effects of DHPG on fictive locomotion. However, this possibility could not be tested directly with a glycine antagonist because blocking glycine receptors fully abolishes fictive locomotion (Droge & Tao, 1993; Cazalets *et al.*, 1994; Cowley & Schmidt, 1995).

In summary, it seems likely that application of DHPG activated a population of group I mGluRs broader than that normally available to endogenous glutamate and exerting a facilitatory as well as an inhibitory function on locomotor networks. The latter role would be predominant when 5-HT receptors are fully activated and perhaps glycinergic transmission is intact.

Coexistence of oscillations due to metabotropic glutamate receptor activity with fictive locomotor patterns?

The synchronous, network-dependent oscillations normally evoked by DHPG (Marchetti *et al.*, 2003) were actually replaced by alternating patterns, which might suggest that the same spinal network was expressing synchronous or alternating patterns depending on the strength of its excitation (see analogous data with stepwise increases in extracellular K^+ Bracci *et al.* 1998). However, DHPG-evoked oscillations are mediated by mGluR5 receptors (Marchetti *et al.*, 2003) predominantly found in the dorsal horn (Berthele *et al.*, 1999), an area not involved in fictive locomotion. Thus, DHPG activated an apparently distinct network, perhaps more limited than the fictive locomotor network, which, once operative, silenced the DHPG-dependent network as in the case of other network activities (Whelan *et al.*, 2000).

It is somewhat unclear why, in the presence of DHPG, alternating patterns usually persisted when elicited by DR stimuli. With repeated DR stimuli, alternating cycles appeared near the top of cumulative depolarization, namely when network excitation was strongly building up to overcome background pattern suppression. Perhaps the facilitation of glycinergic transmission by DHPG (Marchetti *et al.*, 2003) was not sufficiently strong during development of cumulative depolarization to switch off the locomotor central pattern generator which was active due to very powerful cross-excitation (Cowley & Schmidt, 1997; Kremer & Lev-Tov, 1997). Although, in the present experiments, DHPG reduced cumulative depolarization, the motoneuron output expressed as wind-up was unchanged indicating that nonlinear summation of synaptic inputs persisted with actually facilitated stimulus–excitation coupling as, for an overall smaller cumulative depolarization, wind-up and rhythmic patterns were like those in control conditions. As, in the present study, cumulative depolarization was unaffected by subtype 1 or 5 antagonists, it seems that neither receptor subtype was usually activated by endogenously released

glutamate during this phenomenon. This result is consistent with a previous report showing that the broad spectrum mGluR antagonist MCPG (1 mM) induced only a small reduction in cumulative depolarization (Boxall *et al.*, 1996), confirming the limited role of mGluRs in such a phenomenon.

Group I metabotropic glutamate receptor role in disinhibited bursting

This type of rhythmic activity, although not physiological, provides a useful model to investigate the operational properties of spinal networks made up of virtually only excitatory connections (Bracci *et al.*, 1996a,b). As synchronous discharges are typical of the spinal cord at an early stage of development (Demir *et al.*, 2002; Nakayama *et al.*, 2002), disinhibited bursting can additionally help in understanding the mechanisms of immature network operation. Disinhibited bursting was a convenient model to explore the excitatory action of DHPG without the complication of any concomitant facilitation of glycinergic transmission. Thus, in the presence of strychnine and bicuculline, DHPG did not depress electrically induced bursts which could still be fully entrained by DR stimuli, validating the notion that depressant effects by DHPG on synaptic transmission in the rat spinal cord require functionally intact inhibitory networks.

DHPG accelerated spontaneous disinhibited bursting (with shorter burst duration) via subtype 1 receptors mediating network depolarization (Marchetti *et al.*, 2003). Furthermore, it was not unexpected that bursting was insensitive to mGluR5 antagonism because the vast majority of mGluR5 receptors necessary for manifesting DHPG oscillations (Marchetti *et al.*, 2003) are in the superficial layers of the dorsal horn (Berthele *et al.*, 1999; Alvarez *et al.*, 2000), an area playing no major role in disinhibited bursting (Bracci *et al.*, 1996a).

Irregular bursting patterns

One feature of disinhibited bursting in the presence of DHPG was loss of cycle regularity demonstrated not only by the larger period CV but also by frequent burst clusters. It is possible that loss of regularity might have been due to certain changes in intracellular second messengers (inositol-triphosphate and Ca^{2+} Pin & Duvoisin 1995; Schoepp *et al.*, 1999; Fagni *et al.*, 2000) brought about by DHPG. However, very similar effects were also observed with the group II antagonist (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine were coapplied, there was no significant change in burst period or regularity, suggesting that activation of group II mGluRs stabilized the rhythm by blocking the burst facilitation due to group I mGluR activation.

Diffuse depolarization of the spinal network by high extracellular K^+ or NMDA is usually associated with faster, yet regular bursting (Bracci *et al.*, 1996a, 1998). To explain irregular bursting induced by DHPG or EGLU, it is possible to assume that network excitation (by either mGluR1-mediated depolarization or antagonism of inhibitory mGluR II receptors) affected only a limited population of cells endowed with such receptors. A focus of persistently raised excitability might have facilitated rhythmic discharges (burst period decrease) as well as have been responsible for irregular patterns (arrhythmic bursting) because it might have interfered with burst spreading through the network, despite intact mechanisms for burst termination (Ballerini *et al.*, 1997; Darbon *et al.*, 2002). Hence, under these circumstances, disinhibited bursting could shift from a deterministic to a probabilistic process, a property to be considered in building future theoretical models of network operation.

At a very early stage of spinal cord development spontaneous bursting is irregular and only later does it evolve into the synchronous patterns typically found just before birth (O'Donovan, 1999). After birth and spontaneous bursting cessation, disinhibited bursting displays regular discharges owing to the cyclic operation of the Na^+/K^+ pump (Ballerini *et al.*, 1997; Rozzo *et al.*, 2002; Darbon *et al.*, 2003). The transformation of regular into arrhythmic bursting by EGLU suggests that group II mGluRs were complementary to the Na^+/K^+

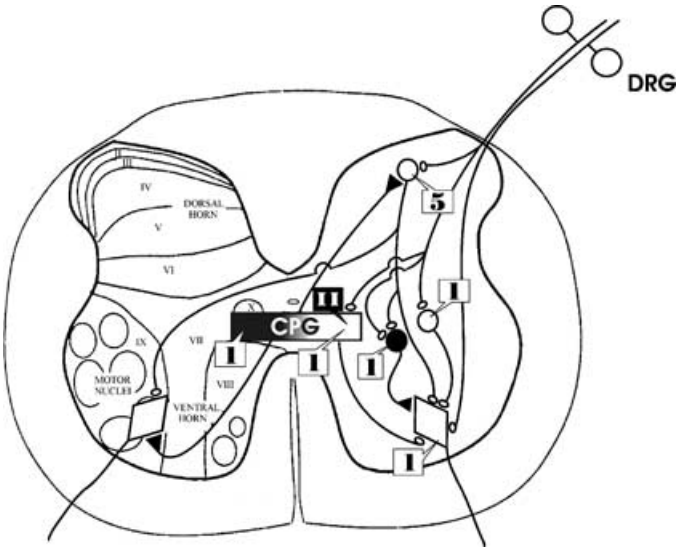

FIG. 7. Idealized wiring diagram of minimal spinal pathways involved in the action of metabotropic glutamate receptors (mGluRs). Cross-section of lumbar spinal cord with outline of main spinal laminae (left). Note that the location of cellular elements (right) is not scaled to the actual size and topography of spinal laminae. The central pattern generator (CPG) is depicted as a box containing interneurons located ventrally to the central canal (Butt *et al.*, 2002) and comprising excitatory (white) as well as inhibitory (black) elements. Motoneurons are identified as rhomboid-shaped structures while interneurons and premotoneurons are hollow circular structures (their inhibitory or excitatory function is identified by black or white colour, respectively). Excitatory synapses have an egg-like open shape while inhibitory synapses are filled triangles. Group I mGluRs are identified on the basis of their subtypes 1 or 5 and are usually located away from the synaptic contacts because they are not normally involved in standard synaptic transmission processes (Marchetti *et al.*, 2003). Group II mGluRs are placed within the CPG although their cellular location remains uncertain. Motoneurons are endowed with subtype 1 mGluRs responsible for the direct depolarization of such cells by (*RS*)-3,5-dihydroxyphenylglycine (DHPG) (Marchetti *et al.*, 2003). As part of the motoneuronal excitation by DHPG is indirectly mediated by glutamate release from interneurons (Marchetti *et al.*, 2003), excitatory premotoneurons are also supposed to bear subtype 1 mGluRs. Some afferent fibres [whose cell bodies, dorsal root ganglia (DRGs), are outside the spinal cord] can make monosynaptic connections with motoneurons. This pathway is inhibited by tonic activation of subtype 1 mGluRs on inhibitory premotoneurons. Other afferent fibres are responsible for polysynaptic motoneuron EPSPs whose amplitude is depressed by activation of group 1 and 5 mGluRs, an effect partly compensated by the facilitation of transmitter release by subtype 1 mGluRs on excitatory premotoneurons. As former work (Marchetti *et al.*, 2003) as well as the present study could not find a role for GABA receptors in mediating the action of mGluRs, the present diagram implies that the inhibitory premotoneurons responsible for mGluR effects are glycinergic. Activation by DHPG of more dorsally located interneurons generates synchronous (see bilateral projection to motoneurons) oscillations mediated by subtype 5 mGluRs (Marchetti *et al.*, 2003 and Fig. 4 of the present study). It might be feasible that the subtype 5-sensitive interneurons correspond to plateau potential-generating cells in the dorsal horn (Russo *et al.*, 1997) and responsible for motoneuronal oscillations. As activation of the locomotor CPG suppresses synchronous oscillations expressed by dorsal interneurons, it is implied that there is a powerful inhibitory input from the CPG to such cells. During fictive locomotion the rhythmic alternating output from the CPG is indicated by inhibitory (left) and excitatory (right) synapses to motoneurons. Within the CPG the functional balance between subtype 1 mGluRs and group II mGluRs ensures correct expression of regular motor patterns and disinhibited bursting.

pump to control rhythm regularity. However, prolonged Na^+/K^+ pump inhibition makes the rhythm erratic and slower (Rozzo *et al.*, 2002), while group II mGluR antagonism made the rhythm erratic, yet faster.

Currently there is much interest in understanding the cellular properties which enable a variety of neuronal networks to express very regular bursting patterns (Agmon & Well, 2003; Israel *et al.*, 2003; Thomas & Bornstein, 2003). The present data provide evidence for a novel role of group II mGluRs activated by endogenously released glutamate in this process and should aid identification of network mechanisms for burst control useful for constructing realistic network models.

Proposed scheme for network action of metabotropic glutamate receptors

Figure 7 shows an idealized wiring diagram of the main network connections mediating the action of group I mGluRs in synaptic transmission, fictive locomotion and disinhibited bursting. While this diagram is, of course, an oversimplification it may help to pinpoint the proposed sites of action for subtypes 1 and 5 of mGluRs and group II mGluRs. In broad terms subtype 1 receptors are supposed to control network activity at various levels in accordance with their more widely distributed expression in the spinal cord (Valerio *et al.*, 1997; Berthele *et al.*, 1999; Alvarez *et al.*, 2000). In this scheme all group I mGluRs are considered to mediate neuronal excitation which can be expressed as either facilitation or block of rhythmic patterns. Their role is, therefore, as gateways to boost inhibition when there is their broad activation by DHPG plus activation of 5-HT receptors and/or intact glycinergic transmission, as well as to facilitate the central pattern generator operation via endogenous glutamate release. Within the central pattern generator, group I mGluR activity is supposed to be functionally balanced by inhibitory group II receptors. Subtype 5 mGluRs are expressed in more restricted spinal areas and are responsible for oscillatory patterns and, partly, for facilitating inhibition (Marchetti *et al.*, 2003). The legend to Fig. 7 provides more details concerning these issues.

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Abbreviations

AIDA, (*RS*)-1-Aminoindan-1,5-dicarboxylic acid; CPCCOEt, 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester; CV, coefficient of variation; DHPG, (*RS*)-3,5-dihydroxyphenylglycine; DR, dorsal root; EGLU, (2*S*)-α-ethylglutamic acid; *Φ*, mean phase; 5-HT, 5-hydroxytryptamine; mGluR, metabotropic glutamate receptor; Mn, motoneuron; NMDA, *N*-methyl-D-aspartate; *R*, concentration of phase values around the mean phase; VR, ventral root.

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Distinct subtypes of group I metabotropic glutamate receptors on rat spinal neurons mediate complex facilitatory and inhibitory effects

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Keywords: dorsal root, GABA, glycine, motoneuron

Abstract

While group I glutamate metabotropic (mGlu) receptors show discrete neuronal distribution in the neonatal rat spinal cord, the functional role of their distinct receptor subtypes remains uncertain. Intracellular recording from lumbar motoneurons together with extracellular recording of ventral root (VR) responses was used to investigate the differential contribution by mGlu receptor subtypes to cell excitability and network activity. The group I agonist DHPG evoked motoneuron depolarization (via the AIDA or CPCCOEt-sensitive mGlu receptor subtype 1) mainly at network level and generated sustained, network-dependent oscillations (via the MPEP-sensitive mGlu receptor subtype 5). DHPG also decreased the peak amplitude of synaptic responses induced by dorsal root stimuli, an effect unrelated to depolarization and dependent on glycinergic transmission. Synaptic responses were insensitive to AIDA or MPEP. The present results can be explained by assuming excitation of discrete classes of interneurons by group I mGlu receptor activity. Thus, the cellular distribution of those mGlu receptors at strategic circuit connections may determine the functional outcome of the network in terms of excitation or inhibition. Even if there was insufficient activation by endogenous glutamate of mGlu receptors during synaptic activity evoked by DR stimuli, it is apparent that such receptors are important pharmacological targets for powerful and rapid up- or down-regulation of spinal signal processing at network level, providing a rationale for the proposed use of mGlu receptor agonists in a variety of spinal pathological conditions.

Introduction

The spinal cord enables detailed studies of neuronal excitability in relation to network function with distinct input and output elements. While, in this area, glutamate has a major role as the principal fast excitatory neurotransmitter (Rekling *et al*., 2000), it is conceivable that it might also modulate neuronal excitability by activating G-protein-coupled metabotropic (mGlu) receptors which comprise three groups, termed I, II and III (Pin & Duvoisin, 1995; Schoepp *et al*., 1999), and whose function remains poorly understood. Group I mGlu receptors are the most abundant receptors in the spinal cord (Valerio *et al*., 1997a) and include the mGlu1 receptor subtype which shows the strongest mRNA signal amongst all mGlu receptors and is widely distributed through spinal laminae (Berthele *et al*., 1999). The mGlu5 receptor subtype transcripts are, however, predominantly located in the superficial dorsal horn (Valerio *et al*., 1997b). Immunohistochemical data indicate that mGlu1 receptors are found in the ventral horn and deep dorsal horn, while mGlu5 receptors are chiefly present in laminae I and II, and absent from motoneurons (Alvarez *et al*., 2000). A very similar distribution is also found in the human spinal cord in which mGlu1 receptors have the highest distribution in lamina VIII and IX, while mGlu5 receptors are mainly in laminae I and II of the dorsal horn (Valerio *et al*., 1997b; Aronica *et al*., 2001). It is interesting that early functional studies of mGlu receptor compounds were conducted on the rat isolated spinal cord (Ishida *et al*., 1993; Jane *et al*., 1994). More recent reports indicate that group I mGlu receptors increase motoneuron excitability at the postsynaptic level (Dong & Feldman, 1999). The effects of group I receptors on rat spinal neurons remain, however, unclear. For example, while DHPG is reported to increase NMDA and AMPA receptor-mediated responses (Dong & Feldman, 1999; Ugolini *et al*., 1999), this result is thought to be due to a generalized enhancement of cell excitability (Jones & Headley, 1995). Although mGlu5 receptor activity is reported to facilitate GABAergic transmission as well as NMDA receptor-mediated responses, it is suggested that the overall action is an inhibitory one (Dang *et al*., 2002).

The present study was initiated to clarify how activation or block of group I mGlu receptors could change motoneuron excitability and network-mediated discharges in the rat spinal cord. Neuronal population responses were recorded from ventral roots (VRs) while changes in membrane potential and input resistance were recorded from single motoneurons which are the readily identifiable output elements of this area. The specific issues addressed here were: (i) can distinct changes in membrane potential or network activity be the hallmark of selective activation of certain group I mGlu receptors?; (ii) could such mGlu receptors be normally activated by endogenous glutamate during synaptic activity or are they pharmacological targets for up- or down-regulation of spinal network activity?

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Materials and methods

Experiments were performed on lumbar spinal cord preparations isolated from neonatal Wistar rats (0–5 days old) under urethane anaesthesia (0.2 mL i.p. of a 10% w/v solution) as previously described (Bracci *et al.*, 1998). These procedures involving animals have been conducted in accordance with NIH guidelines, and Italian national laws DL 27/1/92 n. 116 following the European Community directives n. 86/609 and 93/88 (Italian Ministry of Health authorization for the local animal care facility in Trieste: D.M. 69/98-B).

The spinal cord was superfused (7.5 mL/min) with Krebs solution of the following composition (in mM): NaCl, 113; KCl, 4.5; $MgCl_27H_2O$, 1; $CaCl_2$, 2; NaH_2PO_4, 1; $NaHCO_3$, 25; glucose, 11; gassed with 95% O_2–5% CO_2; pH 7.4 at room temperature. All agents were bath-applied via the superfusing solution at the concentrations mentioned in the text.

DC-coupled VR recordings were obtained with glass suction microelectrodes (containing an Ag–AgCl pellet) filled with Krebs solution. While recording the activity of one or more VRs, in some experiments simultaneous intracellular recordings from functionally identified L3–L5 motoneurons were obtained under current-clamp conditions using sharp electrodes filled with either 3 M KCl (30–60 MΩ resistance), 2 M $KMeSO_4$ (60–120 MΩ resistance) or K-acetate (2 M; pH adjusted to 7.2 with glacial acetic acid; 60–80 MΩ resistance). All signals were amplified, displayed on-line on a chart recorder, and digitally stored on DAT tape (acquisition rate 11 kHz). The input resistance of motoneurons was measured by delivering hyperpolarizing current steps (0.1–0.9 nA, 30–50 ms) through the intracellular electrode; input resistance was 27 ± 17 MΩ ($n = 15$) when recorded with KCl electrodes and 60 ± 20 MΩ ($n = 6$) when recorded with $KMeSO_4$ (or K-acetate) electrodes.

Dorsal root (DR) electrical stimuli, delivered via miniature bipolar suction electrodes, were employed to elicit VR reflexes (recorded from the ipsilateral VR of the same segment). Stimulus intensity (1–10-V range, 0.1 ms duration) was calculated in terms of threshold (Th), defined as the minimum intensity to elicit a detectable response in the ipsilateral VR (on average $Th = 1.8 \pm 0.8$ V, $n = 26$). Low voltage stimuli were classified as those $\leq 2 \times Th$ as they produce activation of low threshold afferent DR fibres (Marchetti *et al.*, 2001). Antidromic VR stimulation (1–5 V, 0.1 ms) was used to elicit antidromic spikes of motoneurons and thus to identify such cells.

Oscillations were measured as deflections at least five times larger than the SD of the baseline noise obtained under resting conditions or in tetrodotoxin (TTX) solution. From each preparation the amplitude of at least 10 oscillatory cycles was measured and averaged. Period (T) was calculated from the beginning of a cycle to the start of the following one or, whenever oscillations had a sinusoidal pattern, between two consecutive troughs. From each preparation period data were calculated as the mean of at least 10 cycles. DR-evoked responses were averages of at least three. For DR-induced responses we measured the initial peak which corresponds to mono- or oligosynaptic components (depending on stimulus strength) and the overall area of the response to quantify the slow polysynaptic component. Data were quantified as means ± SD where *n* is the number of cells or spinal cord preparations. Statistical significance was assessed with Student's *t*-test or ANOVA plus Tukey's test. The accepted level of significance was $P = 0.05$.

Whenever the effects of one mGlu receptor antagonist was examined, the blocker was preapplied for at least 10 min prior to the agonist application and maintained throughout the agonist superfusion period. The following mGlu receptor agonists and antagonists were purchased from Tocris while data concerning their pharmacological selectivity can be found in Schoepp *et al.* (1999): (*RS*)-1-Aminoindan-1,5-dicarboxylic acid (AIDA; selective antagonist for mGlu1 receptors), 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt; selective antagonist for mGlu1 receptors) (*RS*)-3,5-dihydroxyphenylglycine (DHPG; selective agonist for group I receptors and equipotent on mGlu1 and mGlu5 subtypes), 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP; selective antagonist for mGlu5 receptors). N-methyl-D-aspartate (NMDA), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), D-amino-phosphonovalerate (APV) and TTX were also purchased from Tocris while strychnine, bicuculline and nifedipine were from Sigma. N-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium chloride (QX-314) was purchased from Alomone Laboratories.

Results

The database of the present study comprises 120 isolated spinal cords for VR recording and 57 motoneurons with average resting potential (V_m) of -75 ± 4 mV.

Motoneuron depolarization and oscillations evoked by the group I agonist DHPG

Figure 1A shows an example of the action of the group I agonist DHPG (5 μM) simultaneously recorded from an rL5 motoneuron (top trace; KCl-filled electrode), and the contralateral lL2 (middle) and lL5 VRs (bottom). Within a few seconds from the start of the application, a 10-mV depolarization was recorded intra- and extracellularly (Fig. 1A). Membrane depolarization was (mean ± SD) 9 ± 3 mV (range 2–14 mV; $n = 15$ cells) and became larger (22 ± 10 mV, $n = 6$) with 20–50 μM DHPG. Unless otherwise indicated, DHPG was applied at a concentration of 5 μM to avoid excessive depolarization. Oscillatory activity emerged during the DHPG-evoked depolarization and could be observed intracellularly and extracellularly. The period of oscillations was measured from intracellular traces from which oscillations could be resolved in slow ones (see example in Fig. 1A) and fast ones (see example in Fig. 1C). On a random sample of five cells fast oscillations had an average period (T_{fast}) of 180 ± 90 ms, while slow oscillations had a period (T_{slow}) of 4.4 ± 1.3 s. The amplitude of fast oscillations (A_{fast}) varied depending on whether they were occurring at the top (8 ± 4 mV) or the trough of the slow oscillations (14 ± 2 mV). The average amplitude of slow oscillations (A_{slow}) was 15.6 ± 5.6 mV ($n = 5$). At the top of fast oscillations, one or more spikes occurred (Fig. 1C). Emergence of DHPG-induced oscillations did not require motoneurons to generate spikes. In fact, when the recording electrode was filled with QX-314 to block sodium currents and slow inward rectifiers (Perkins & Wong, 1995), DHPG (5 μM)-induced depolarization was 9 ± 4 mV ($n = 11$), a value not significantly different from control (9 ± 3, $n = 15$; $P > 0.05$). Likewise, oscillations also exhibited period values not significantly different from control, although their amplitude was significantly reduced ($A_{fast} = 1.97 \pm 1.02$ mV; $A_{slow} = 1.43 \pm 1.08$, $P < 0.001$; $n = 4$).

Even though VR discharges were complicated by strong asynchronous activity, it was often possible to distinguish oscillatory waveforms as exemplified, on a faster time base, in Fig. 1B. By aligning the peak of fast and slow oscillations it was clear that these responses were all synchronous in 10 preparations out of 19, both ipsilaterally (lL2 vs. lL5 VRs, Fig. 1B) and intrasegmentally (rL5 motoneuron vs. lL5 VR, Fig. 1A). On six preparations, our recording arrangements allowed evaluation of ipsilateral synchronicity only, while three other preparations showed out-of-phase oscillations (one of which completely alternated). Although not shown in Fig. 1, after ≈ 5 min of continuous application of DHPG, the cell membrane potential began to repolarize

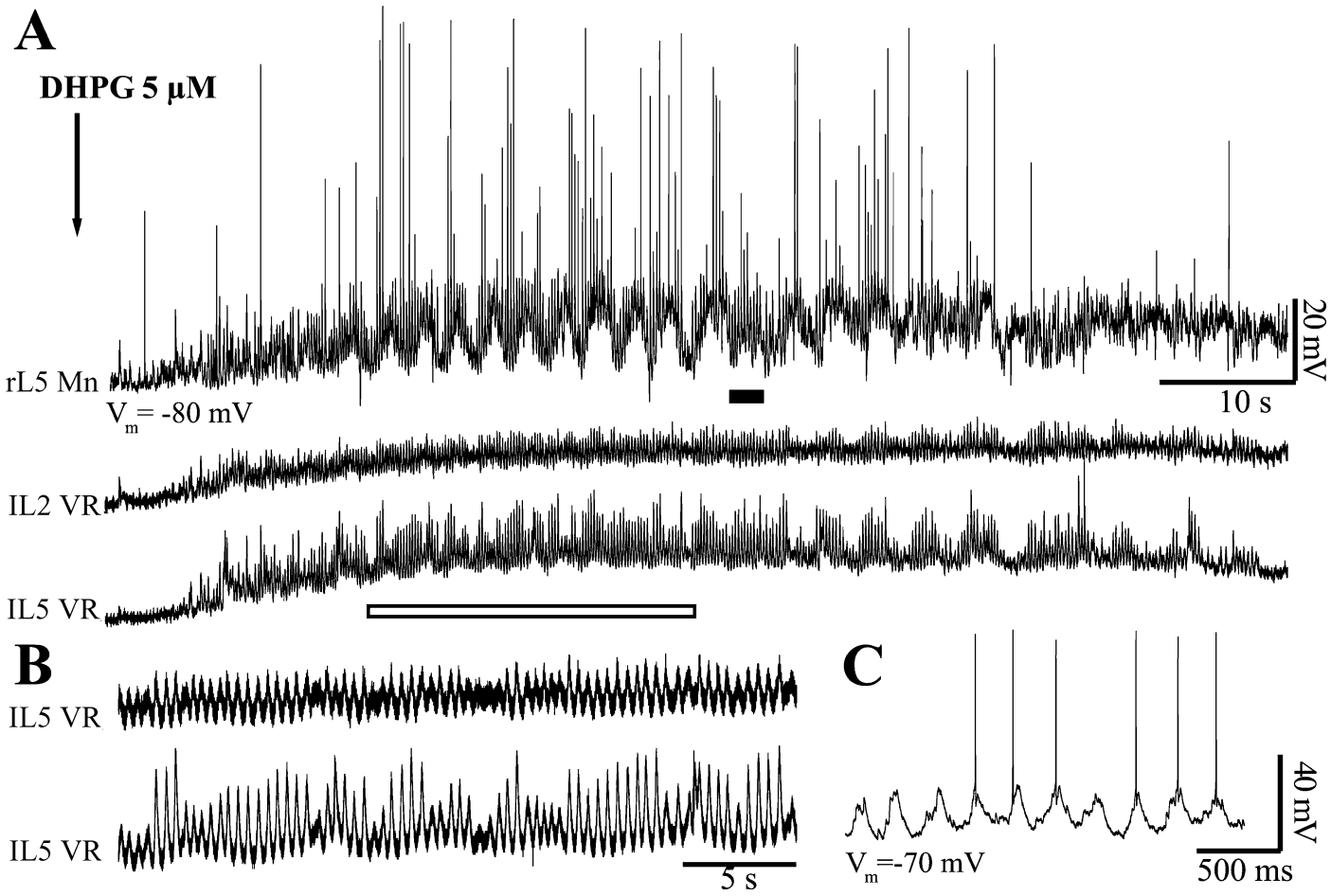

FIG. 1. DHPG induces depolarization and oscillatory activity. (A) Simultaneous recording from an L5 motoneuron (rL5 Mn; top trace), the lL2 VR (middle) and the lL5 VR (bottom). Application of DHPG (from time point indicated by the arrow) induced motoneuron depolarization of 10 mV with superimposed synchronous oscillatory activity (on average, $T_{slow} = 4.4 \pm 1.3$ s) and action potential firing. Depolarization and oscillations are also detected from VRs. In this and the following Figs, V_m is the resting membrane potential. (B) Expansion (gain doubled) on faster time scale of the record (indicated by open bar in panel A) reveals the presence of fast ($T_{fast} = 180 \pm 90$ ms) oscillations recorded extracellularly. (C) Expansion on faster time base of the intracellular record (filled bar in panel A) reveals fast oscillations and spiking on single motoneuron.

spontaneously, while increased firing persisted and oscillations became slower. In all cells tested, spontaneous repolarization took place within 2–5 min from the start of DHPG application, accompanied by oscillations (13/21 preparations) lasting 5–15 min; during such a late phase of DHPG action the oscillation period was 37 ± 5 s (oscillation length, 18 ± 2 s).

In intracellular experiments, the input resistance was not significantly changed in the presence of 5 μM DHPG (27 ± 15 MΩ vs. 27 ± 18 MΩ in control; $n = 7$). Furthermore, current–voltage (I–V) curves obtained from five motoneurons in control conditions and after 5 min application of DHPG (5 μM) were very similar (see example in Fig. 2A). Linear fits showed no significant changes in the slope of I–V curves ($100 \pm 14\%$ for 5 μM DHPG vs. control; $n = 5$). These observations confirm that there was no apparent change in cell input resistance.

The extent of membrane depolarization observed following DHPG (5 μM) application was unrelated to the initial resting potential, as indicated by the plot of Fig. 2B ($n = 25$). In the presence of DHPG (5 μM), at the same membrane potential, neither the amplitude of the antidromic spikes (87 ± 18 mV vs. 84 ± 22 mV in control; $P > 0.05$, $n = 5$) nor their half-width (1.61 ± 0.32 ms vs. 1.55 ± 0.28 ms in control; $P > 0.05$, $n = 5$) was changed.

Is the effect of group I mGlu receptor activation on motoneurons direct or network-mediated?

In control conditions DHPG (50 μM) elicited VR depolarization (560 ± 260 μV, $n = 15$) which, when retested in the presence of 1 μM TTX, was 260 ± 110 μV ($n = 8$, $P < 0.01$; independent t-test), showing that motoneurons as well as the spinal network contributed to VR depolarization. Oscillations were completely abolished by TTX. Direct motoneuron depolarization by DHPG (50 μM), when synaptic and action potential-mediated transmission was blocked (i.e. in the presence of TTX, CNQX, APV, bicuculline and strychnine), was 105 ± 90 μV ($n = 6$, $P < 0.05$ vs. data in TTX only; independent t-test).

Repolarization (by intracellular DC current injection) to the same membrane potential value observed prior to DHPG application did not bring any significant changes in oscillations with respect to control ($n = 4$), confirming that oscillations were not a consequence of motoneuronal membrane depolarization by DHPG.

Subtype receptor selectivity of DHPG-induced response

We tested the sensitivity of DHPG-induced depolarization and oscillations to the mGlu1 antagonist AIDA and the mGlu5 antagonist MPEP.

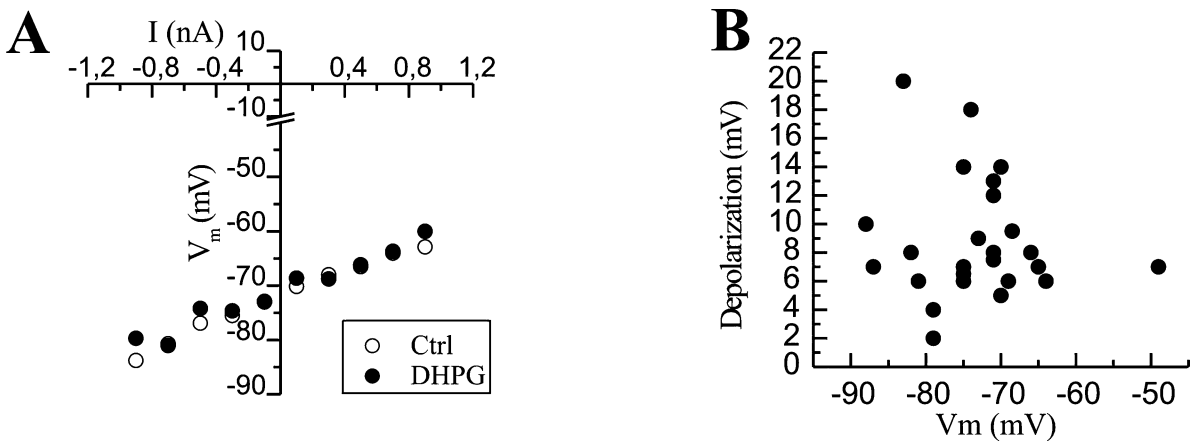

FIG. 2. Dependence of DHPG-induced effects on motoneuronal membrane potential. (A) Example of current–voltage (*I–V*) curve for a single motoneuron, obtained by injecting negative and positive DC current pulses and measuring the electrotonic potentials in control conditions or in the presence of DHPG (5 μM). Linear fits showed no significant changes in slope of *I–V* curves (100 ± 14% for 5 μM DHPG vs. control, $n = 5$), indicating no apparent change in cell input resistance by DHPG. (B) Amplitude of the motoneuron membrane depolarization was independent of resting membrane potential value. Each data point refers to a distinct cell.

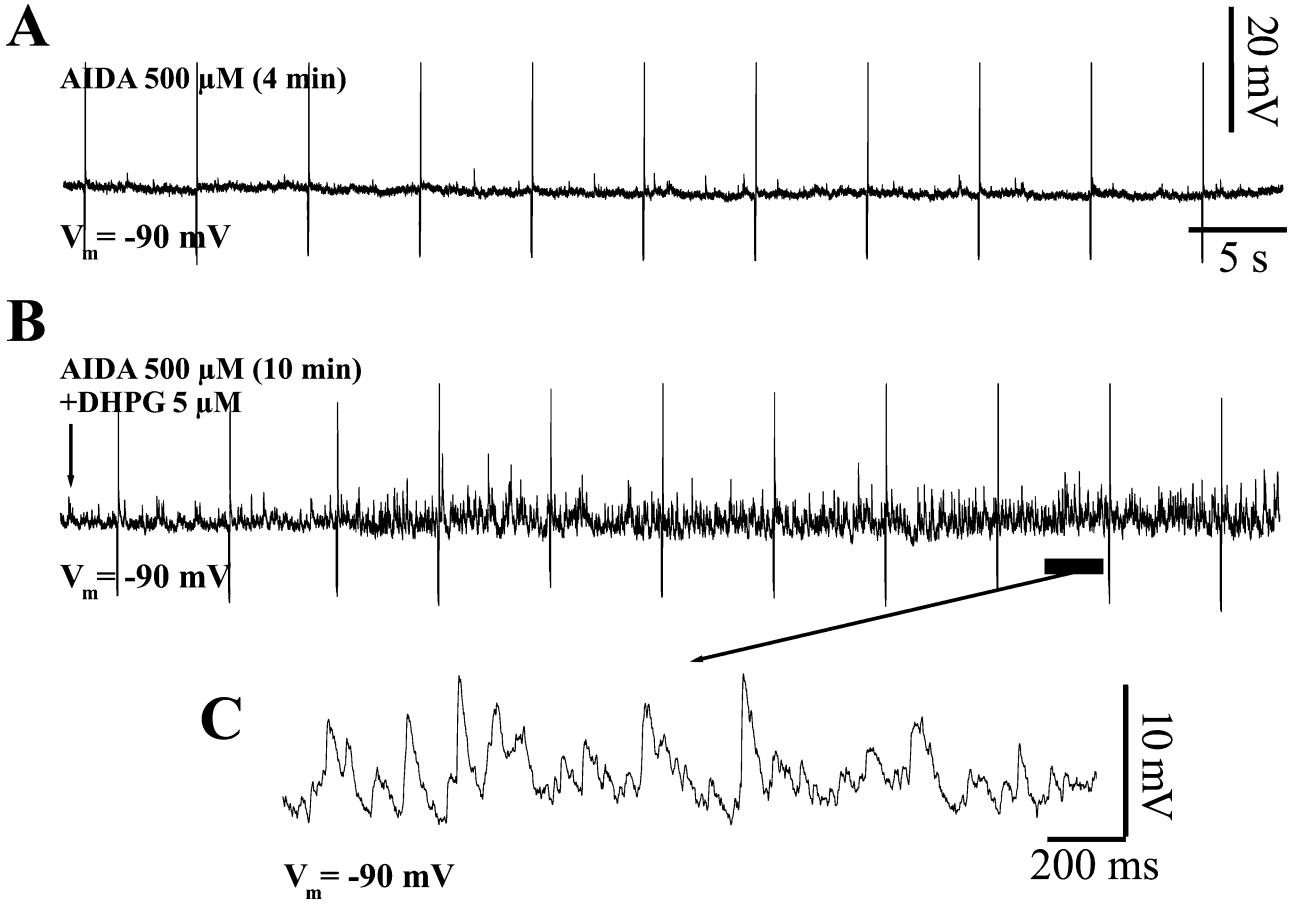

FIG. 3. DHPG-induced depolarization was blocked by the mGlu1 subtype receptor-selective antagonist AIDA. All traces are intracellular records from the same L5 motoneuron. (A) AIDA (500 μM; trace starts after 4 min application) *per se* does not affect baseline membrane potential. Fast deflections are antidromic stimulus artifacts (stimuli were applied to monitor changes in spike amplitude and shape). (B) AIDA prevents 5 μM DHPG-induced depolarization but not oscillatory activity evoked by DHPG. (C) Faster time scale record indicated by the bar in panels B with oscillations persisting in the presence of AIDA.

As exemplified in Fig. 3A, AIDA (500 μM) *per se* did not affect baseline membrane potential (analogous data were obtained from seven motoneurons) but, when coapplied with DHPG, it prevented membrane depolarization by DHPG (Fig. 3B). Lack of DHPG-induced depolarization due to AIDA was observed in 3 out of 4 motoneurons and in all extracellular recordings from four preparations. In these conditions, slow and fast oscillations persisted as shown in the trace in Fig. 3B and its inset. On average fast oscillations exhibited longer

periods ($T_{fast} = 315 \pm 30$ ms, $P < 0.05$; $T_{slow} = 3.5 \pm 1.3$ s, $P > 0.05$; $n = 4$) although A_{fast} and A_{slow} were not different from control.

Previous studies have raised the possibility that AIDA may not be entirely selective for mGlu1 receptors (Krieger *et al*., 1998; Salt *et al*., 1999). To confirm that block of DHPG-induced depolarization with persistence of oscillations was due to mGlu1 antagonism, further experiments were carried out with CPCCOEt (100 μM), another selective antagonist which, unlike AIDA, has a noncompetitive mechanism of block (Schoepp *et al*., 1999). On four spinal cord preparations recorded extracellularly, CPCCOEt blocked the DHPG-evoked VR depolarization by $74 \pm 24\%$ ($P < 0.02$) and left oscillatory activity with T_{fast} period ($148 \pm 55\%$; $P > 0.05$) and T_{slow} period ($121 \pm 58\%$; $P > 0.05$). These observations validate the data obtained with AIDA and confirm the use of AIDA as a suitably selective antagonist which in view of its competitive nature could be washed out more readily than CPCCOEt.

However, in the presence of MPEP (10–100 μM, which *per se* did not change membrane potential; $n = 8$), DHPG (5 μM) still elicited membrane depolarization comparable to control (8 ± 4 mV with MPEP vs. 9 ± 7 mV without MPEP, $n = 5$; paired *t*-test, $P > 0.05$) with very reduced oscillatory activity (compare A with B in Fig. 4). When AIDA and MPEP were coapplied, both membrane depolarization and oscillations induced by DHPG were prevented ($n = 4$; not shown).

In view of the possibility that MPEP might also inhibit NMDA receptors (O'Leary *et al*., 2000) which can elicit oscillatory activity (Hochman *et al*., 1994; MacLean *et al*., 1997; Schmidt *et al*., 1998), we tested motoneuron depolarization evoked by 5 μM NMDA before and after applying MPEP (100 μM); no significant change (16 ± 5 mV with MPEP vs. 17 ± 6 mV without MPEP; $n = 3$) was observed.

Is the effect of DHPG mediated by intrinsic neuronal conductances?

Because activation of mGlu receptors on interneurons or motoneurons can induce bistable properties (Kiehn *et al*., 1996) responsible for repetitive firing via facilitation of L-type Ca^{2+} channels (Morisset & Nagy, 1999), the DHPG-evoked oscillatory behaviour was investigated in the presence of the Ca^{2+} blocker nifedipine (20 μM). As indicated in the example of Fig. 5A and B, nifedipine *per se* did not affect baseline membrane potential ($n = 4$) or the motoneuron depolarization evoked by 5 μM DHPG (8 ± 1 mV vs. 9 ± 1 mV; $n = 3$). Likewise, oscillations persisted with characteristics similar to control (see Fig. 5B).

Because DHPG can facilitate NMDA receptor activity (Holohean *et al*., 1999; Ugolini *et al*., 1999; Dang *et al*., 2002), we tested whether DHPG-induced oscillations were dependent on NMDA receptor activation (Fig. 5C). When DHPG (5 μM) was applied in the presence of APV (50 μM, which *per se* did not alter baseline potential; $n = 3$), motoneuron depolarization was 3.3 ± 1.5 mV ($n = 3$), a value significantly smaller than control ($P < 0.05$). Furthermore, slow oscillations were no longer visible while fast oscillations, though retaining periodicity similar to control ones ($T_{fast} = 160 \pm 40$ ms), had significantly smaller amplitude ($A_{fast} = 2.5 \pm 1.3$, $n = 3$, $P < 0.01$). When DHPG

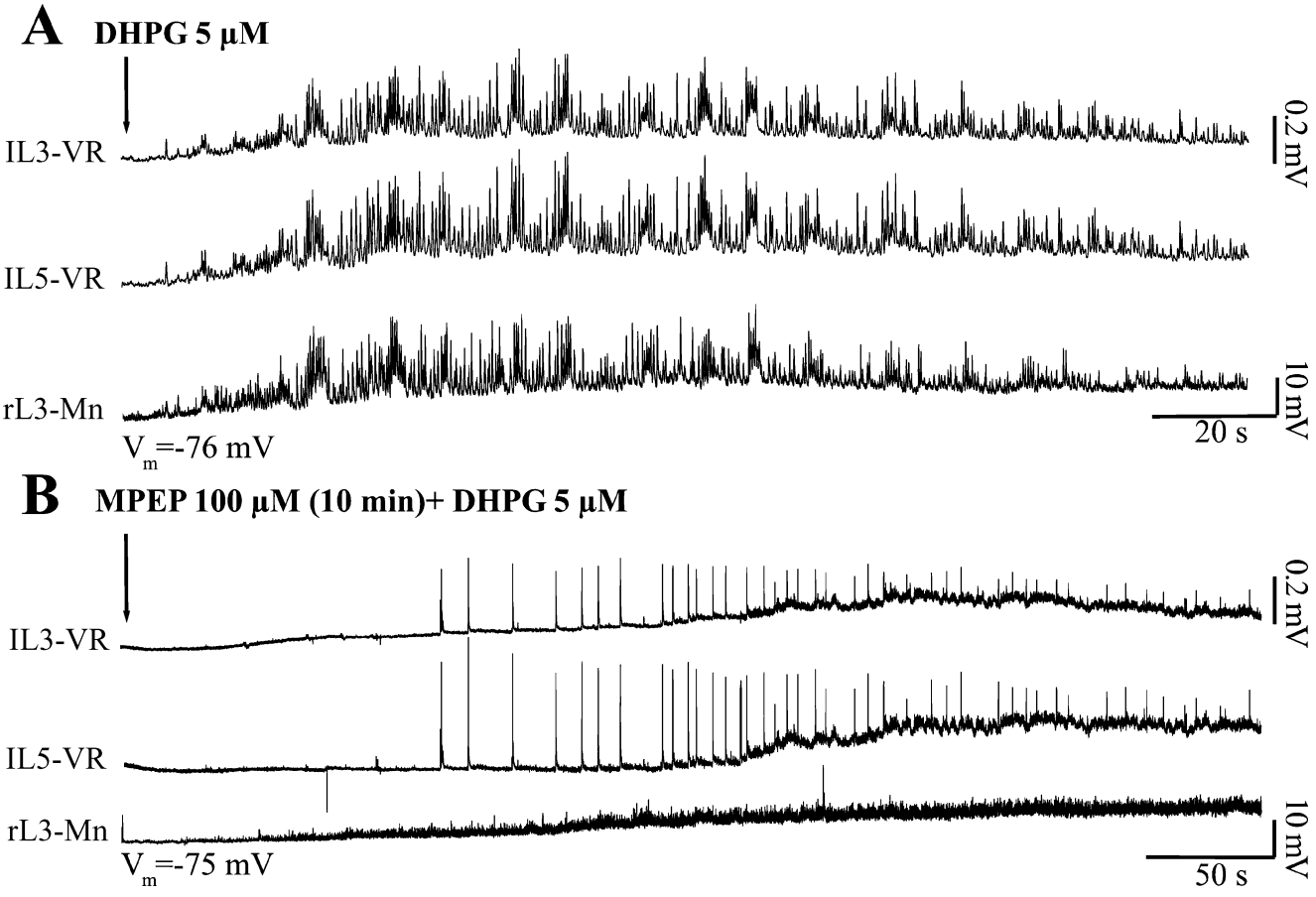

FIG. 4. DHPG-induced oscillations are blocked by mGlu5 subtype receptor-selective antagonist MPEP. In each panel the top two records are VR traces while bottom one is an intracellular trace from a motoneuron. (A) DHPG (applied at arrow) induced depolarization and oscillatory activity. (B) In the presence of MPEP (which *per se* does not change membrane potential), DHPG-induced oscillatory activity was strongly reduced, while depolarization remained similar to control.

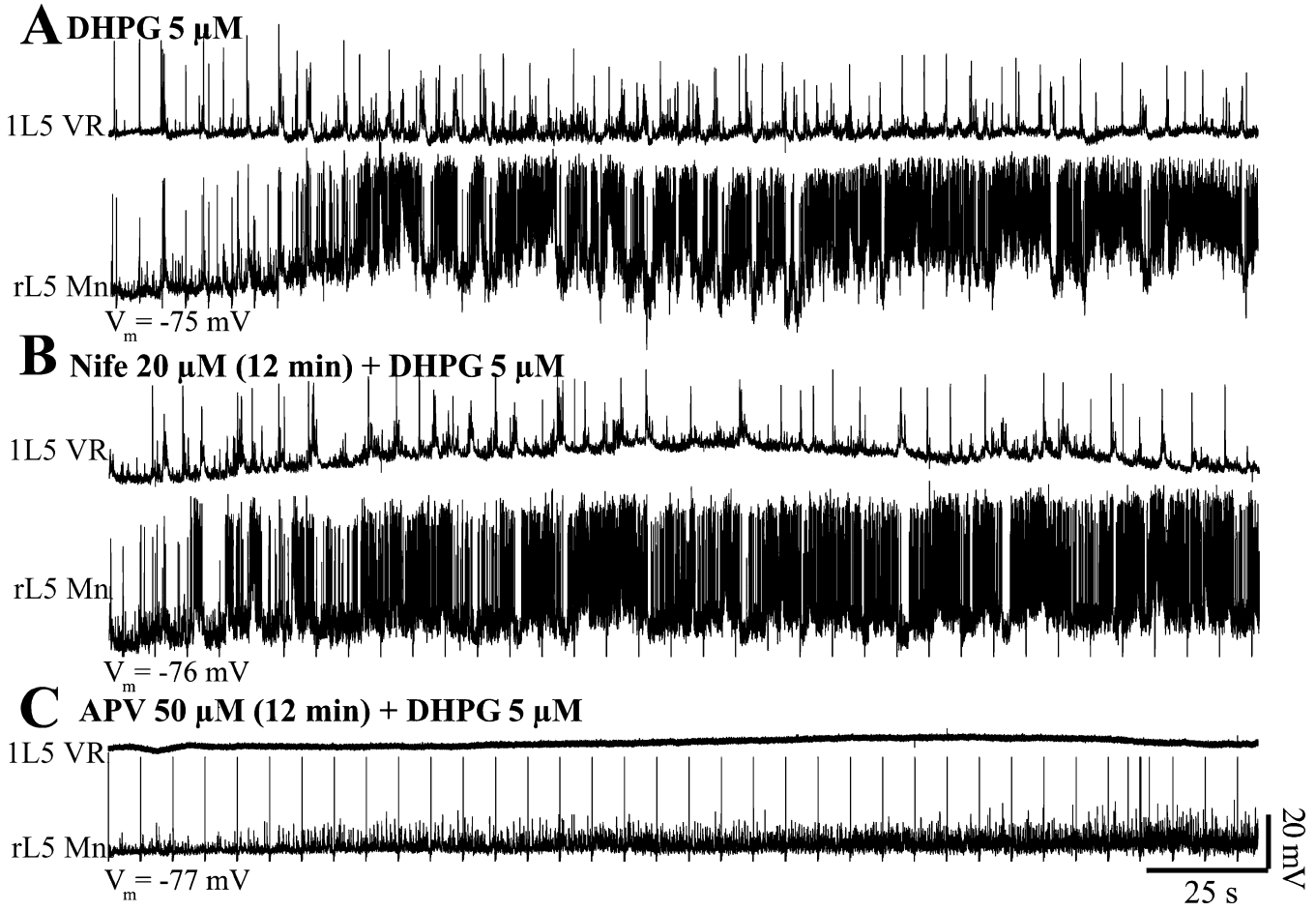

FIG. 5. Role of L-type Ca^{2+} conductances and NMDA receptors in mediating DHPG-induced effects. Paired records (top, extracellular; bottom, intracellular) from the same preparation. (A) DHPG (applied at the start of the trace) induced depolarization and oscillatory activity (simultaneous recording from an rL5 motoneuron and the contralateral lL5 VR). (B) When DHPG was applied in the presence of nifedipine (which *per se* has no effect on baseline membrane potential), it induced depolarization and oscillations comparable to those obtained in control conditions. (C) DHPG application in the presence of the NMDA receptor antagonist APV induced smaller depolarization and reduced oscillatory activity.

was applied in the presence of CNQX (10 μM), slow oscillatory activity was suppressed ($n = 4$) and motoneuron depolarization was decreased (becoming $30 \pm 20\%$ of control; $n = 3$) or, in one case, even absent, while fast oscillations persisted ($T_{fast} = 75 \pm 34$ ms; not significantly different from control in DHPG solution). Combined application of CNQX and APV led to suppression of oscillatory activity.

Modulation of DR-evoked responses by group I mGlu receptors

In addition to its excitatory action consisting of oscillations and neuronal depolarization, DHPG also brought about changes in DR-evoked potentials, recorded either intracellularly or extracellularly. Figure 6A shows examples of average VR responses to single stimuli of intensity $1.5 \times$ Th and $5 \times$ Th (Fig. 6, Aa and Ab, respectively), in control conditions (grey trace) and in DHPG solution (black trace). Synaptic responses were differentially affected by DHPG depending on the stimulus intensity used to generate them. In fact, as shown in Fig. 6, Aa, the peak amplitude and the response area to the weak stimulus became smaller while the response area following the stronger stimulus was unchanged (Fig. 6, Ab) despite a reduced early peak (Fig. 6, Ac). Thus, the decrease in peak amplitude was compensated for by an increase in late synaptic components (with associated oscillatory activity; see Fig. 6, Ab). These phenomena are quantified in the histograms of Fig. 6B for 28 preparations. Each symbol (○) represents the area of DR-evoked responses (averaged and then normalized vs. control) for a single preparation, while the histogram represents the mean group data. In the case of low voltage stimuli (Fig. 6B, first two columns, for intracellular and extracellular recordings, respectively), responses were always significantly depressed ($P < 0.001$). In the case of high voltage stimuli, mean values for synaptic response areas were not significantly different from control, although (as indicated by the datapoint spread) there was considerable variability. Conversely, peak amplitude values were always significantly reduced ($74 \pm 25\%$, $n = 19$, $P < 0.05$) regardless of stimulus strength.

We explored whether the effect of DHPG on DR-evoked responses was related to the VR polarization level which, in the presence of DHPG, first depolarized and then spontaneously repolarized. For this purpose, we monitored the time course of DR-evoked responses and VR depolarization in five preparations, in which we stimulated one DR every 50 s with low voltage stimuli. The average response area (○) and peak (△; both normalized to control values) and average VR depolarization (■; normalized to the maximum value in each preparation) are shown in Fig. 6C. This plot indicates a loose association between VR depolarization and depression of the response area. In fact, when VRs started depolarizing the response area transiently increased, though further depolarization (induced by DHPG) depressed the response. Indeed, when recording intracellularly in the presence of

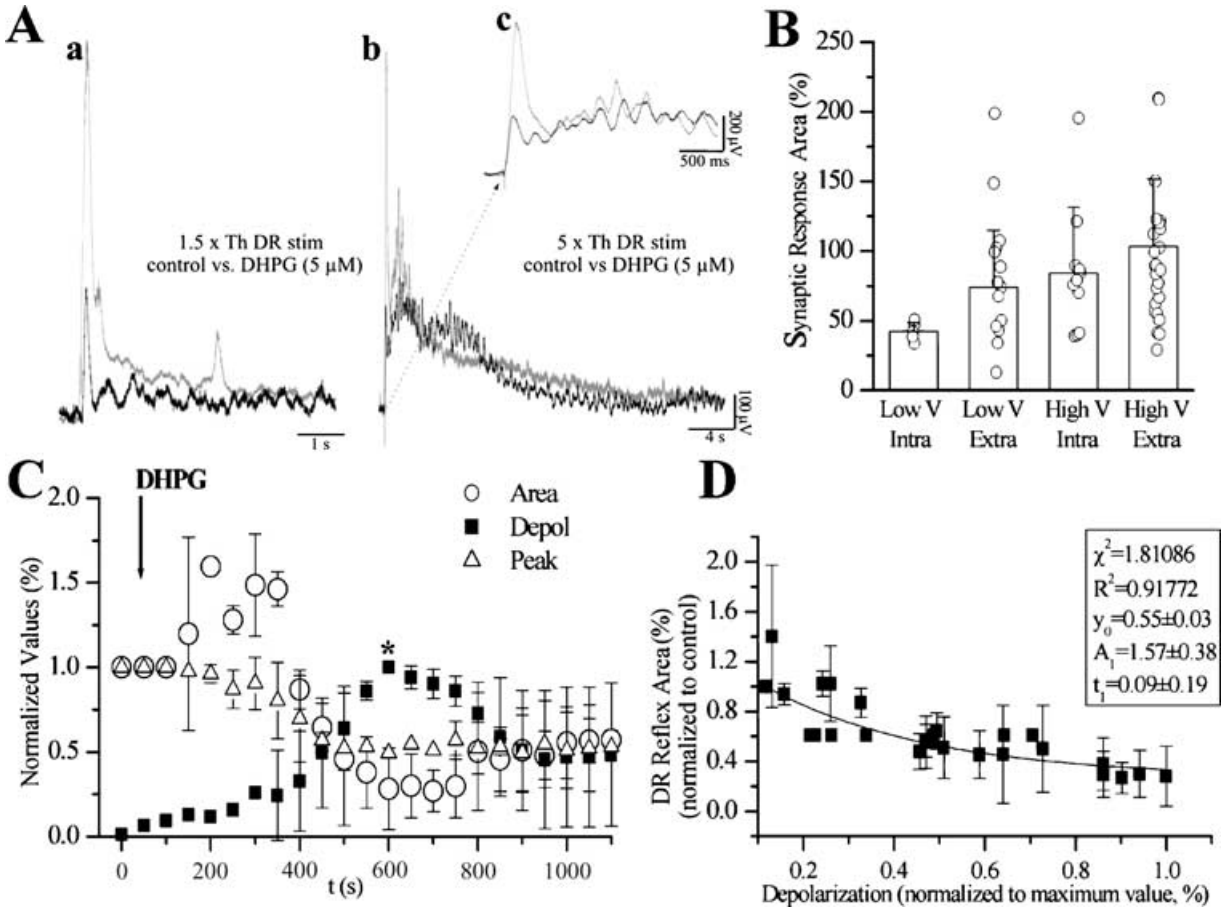

FIG. 6. Action of DHPG on DR-evoked responses. (A) DHPG (5 μM) depresses DR-evoked response (Aa), recorded from an rL5 motoneuron and elicited with 1.5 × Th pulse intensity applied to rL5 DR. On the same cell, DHPG does not depress the synaptic response area evoked by a stronger stimulus (5 × Th) because late components are augmented (Ab) to compensate for the depression of the peak amplitude (Ac). (B) Histograms depicting changes in synaptic response area (DHPG vs. control) measured for 28 preparations for low voltage (Low V) or high voltage (High V) DR stimulus intensity and recorded either intracellularly (Intra) or extracellularly (Extra). For low voltage intracellular data, $n = 5$, $P < 0.001$; for low voltage extracellular data, $n = 8$, $P < 0.001$. Each symbol represents one preparation, while bars represent the population mean. (C) Time course of normalized DR response area (○), peak amplitude (△) and VR depolarization (■) vs. time. Asterisk indicates the maximum depolarization amplitude. Data are the average from five preparations. (D) Plot of DR-evoked response area (normalized values of DHPG vs. control) vs. VR depolarization. Data points can be fitted with an exponential function (parameters shown in box), $n = 5$.

DHPG from motoneurons manually repolarized to resting level prior to delivering a strong DR stimulus, we found that synaptic area depression was still present, although decreased by $15 \pm 6\%$ ($n = 3$). Unlike the biphasic profile of changes in synaptic area, the response peak amplitude was reduced throughout the DHPG application (Fig. 6C).

Plotting the response area vs. VR depolarization (Fig. 6D) for experiments illustrated in Fig. 6C showed that such data could not be fitted linearly. On the other hand, these results could be fitted with an exponential curve ($y = y_0 + A_1^{-x/t_1}$, whose values are shown in Fig. 6D), indicating the complexity of the processes governing depolarization and reflex reduction.

Subtype receptor selectivity for the DHPG effect on DR-induced responses

In intracellular records like the example shown in Fig. 7, Aa and Ab, DR stimuli (2 × Th) evoked a polysynaptic response whose overall area (at the same level of membrane potential) dropped to 40% in the presence of DHPG. The mGlu1 antagonist AIDA (500 μM) had minimal effect on the DR-evoked response (Fig. 7, Ac), but it prevented further depression by DHPG (Fig. 7, Ad).

Figure 7, Ca and Cb shows that, in an intracellularly recorded motoneuron (KCl plus QX-314 electrode; membrane potential level kept at control level throughout), the depression of the synaptic response induced by DHPG was also lost after application of MPEP (100 μM). Figure 7B and D quantifies such observations, showing that either AIDA or MPEP prevented the depressant action of DHPG. These histograms also indicate the neither antagonist *per se* had any significant effect on synaptic responses evoked by DR stimulation, regardless of whether the intensity of stimulation was high (4–6 × Th, $n = 8$ for AIDA and $n = 7$ for MPEP) or low (2 × Th, $n = 5$ for AIDA).

Mechanism of DHPG inhibitory action on DR reflexes

We next investigated whether facilitation of glycinergic or GABAergic transmission could play a role in the inhibitory action of DHPG. The extracellular recording of Fig. 8, Aa and Ab, shows that the DR-evoked reflex (2 × Th stimulus intensity) was depressed (60%) in the presence of DHPG (baseline VR depolarization was 240 μV). In the presence of strychnine (1 μM), DHPG depolarized the VR by 180 μV and induced a distinctive change in the VR reflex shape (Fig. 8, Ac and Ad); in fact, although the response area in this case was 102% of the control in strychnine solution, it became longer and displayed intense oscillatory activity. The reflex peak amplitude was no longer significantly depressed by DHPG (on average $87 \pm 26\%$ vs. control in strychnine, $n = 6$, $P > 0.05$). These results were confirmed with intracellular

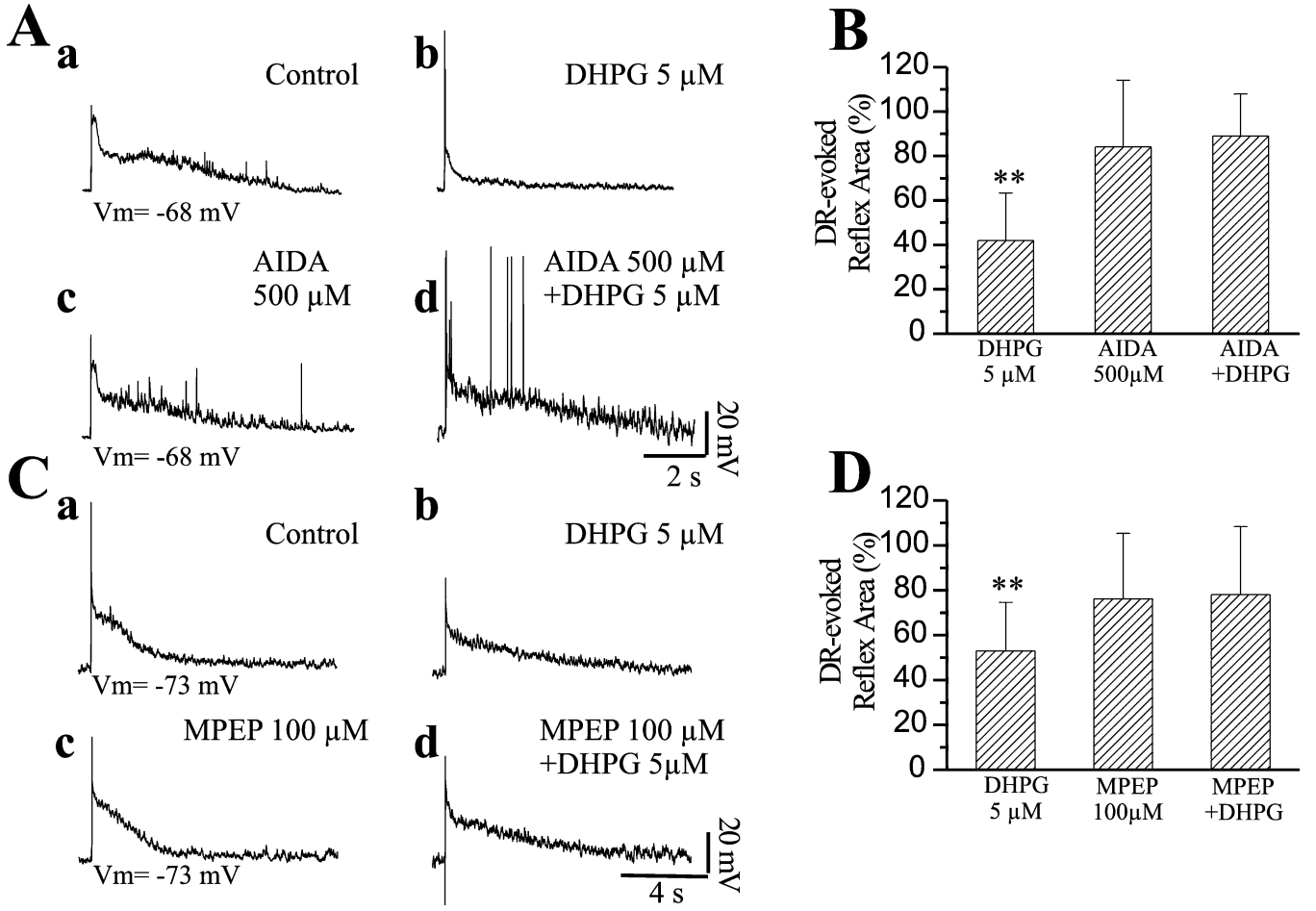

FIG. 7. Subtype receptor selectivity of DHPG effect on DR-evoked responses. All traces are intracellular records from single motoneurons. (A) DR-evoked responses to stimuli of $2 \times$ Th intensity, recorded (with a KCl-filled electrode) at the same membrane potential value: (a) control; (b) in the presence of DHPG (5 μM) the response was depressed by 60%; (c) application of AIDA (500 μM) had minimal (10%) effect on the DR-evoked response; (d) DHPG-induced depression was prevented by AIDA (87% of control). (B) Average values of DR-evoked response areas (normalized to control values) in the presence of DHPG with ($n = 5$) or without ($n = 5$) AIDA application or AIDA alone ($n = 13$). $^{**}P < 0.005$. (C) DR-evoked responses to stimuli of intensity $2 \times$ Th, recorded with a KCl + QX-314-filled electrode at the same membrane potential value: (a) control; (b) in the presence of DHPG, the response was depressed (57%); (c) MPEP (100 μM) did not depress the DR-evoked response (97%); (d) coapplication of DHPG and MPEP did not reduce the DR reflex (99%). (D) Average values of DR-evoked response areas (normalized to control values) for experimental protocols as shown in C. $^{**}P < 0.005$, $n = 6$.

recordings, as shown in Fig. 8B; in this case, DHPG (in the presence of strychnine) first depolarized the motoneuron by 10 mV (a value comparable to the DHPG-induced depolarization in control conditions; not shown). When the cell spontaneously repolarized after 3 min in the continuous presence of DHPG (plus strychnine), the DR-evoked response area and associated firing were increased by 50%. On all six preparations tested (stimulus intensity $2 \times$ Th), in which DHPG had reduced the DR-evoked VR reflex area to $60 \pm 25\%$ of control, such an effect was absent in the presence of strychnine ($140 \pm 80\%$, Fig. 8D). On average, DHPG-induced depolarization in the presence of strychnine was 210 ± 170 μV ($n = 8$).

On the other hand, bicuculline did not counteract the depressant action of DHPG on VR reflex (area in Fig. 8, Cd, was 37% of the one in Fig. 8, Cc, stimulus intensity $3 \times$ Th) while the DHPG-induced depolarization was ≈ 200 μV. Pooled data (stimulus intensity $2 \times$ Th; Fig. 8D) indicate that DHPG retained its depressant action on the reflex area in 20 μM bicuculline solution ($36 \pm 28\%$, $n = 4$, $P < 0.02$).

Indirect involvement of L-type Ca^{2+} channels or NMDA receptors in inhibiting DR-evoked responses seemed unlikely because nifedipine (20 μM), APV (50 μM) or CNQX (10 μM) did not block the depressant effect of DHPG (Fig. 8E). Even if APV or CNQX largely decreased the reflex area (14 ± 8 and $26 \pm 7\%$, respectively), neither of them prevented further depression ($6 \pm 1\%$ and $15 \pm 10\%$, respectively) by DHPG.

Discussion

The main result of the present study was to identify the contribution by certain subtypes of mGlu receptor to neuronal excitability and synaptic transmission in the neonatal rat spinal cord. In particular, activation of group I mGlu receptors induced depolarization of motoneurons (mediated by mGlu1 receptors) with superimposed oscillatory activity (mediated by mGlu5 receptors). Facilitation of glycinergic transmission via mGlu1 and 5 receptors was responsible for depression of the peak amplitude of DR-evoked responses. With the caveat that the current data were obtained from neonatal tissue and that mGlu receptors are subjected to developmental maturation (Valerio *et al.*, 1997b; Berthele *et al.*, 1999), these findings provide a detailed picture of the functional role of group I mGlu receptors in the rat spinal cord.

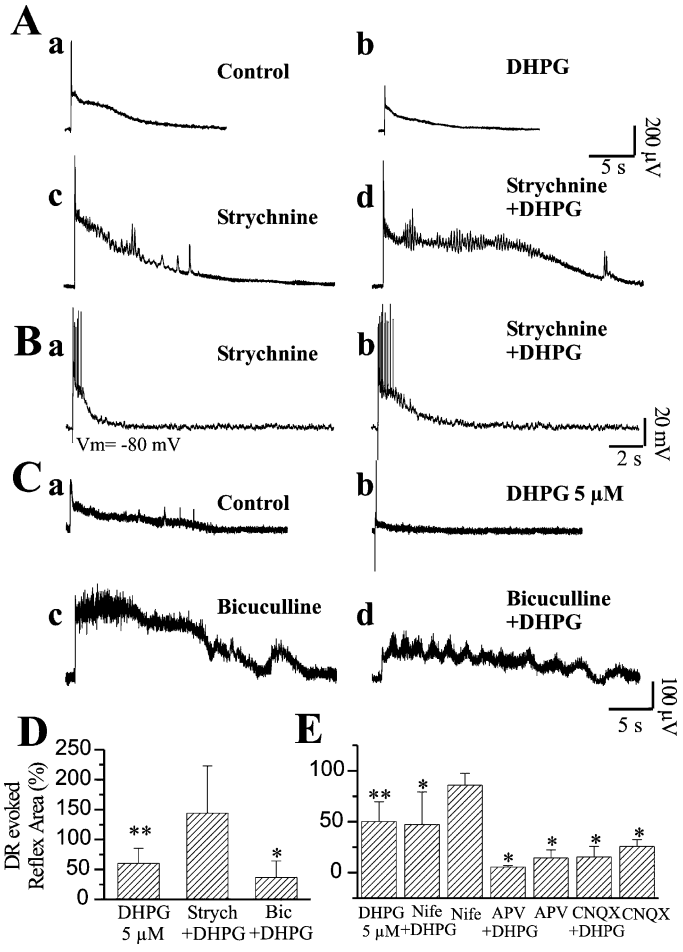

FIG. 8. Mechanisms underlying the DHPG effect on DR-evoked responses. (A) DR-evoked VR responses to stimuli of $2 \times$ Th intensity: (a) control; (b) in the presence of DHPG (5 μM), the response was depressed; (c) application of strychnine (1 μM) enhanced the DR-evoked response; (d) DHPG-induced depression was prevented by strychnine and reflex duration was prolonged. (B) With intracellular recordings the reflex response in the presence of strychnine and DHPG (panel Bb) was not reduced with respect to strychnine alone. (C) DR-evoked VR responses to stimuli of $2 \times$ Th intensity (different preparation from A): (a) control; (b) in the presence of DHPG (5 μM), the response was depressed; (c) application of bicuculline (20 μM) enhanced the DR-evoked response; (d) DHPG-induced depression was not prevented by the presence of bicuculline. (D) Summary of pooled data from six preparations: for each experimental protocol the DHPG data are expressed as a percentage of the response area measured immediately before DHPG. $^{**}P < 0.02$, $^{*}P = < 0.05$. In the presence of strychnine or bicuculline the VR depolarization evoked by DHPG was $88 \pm 23\%$ or $195 \pm 160\%$ with respect to the one in control solution ($n = 6$; data not shown). (E) Histograms show pooled data for percentage changes in VR reflex area in the presence of DHPG, 20 μM nifedipine + DHPG, nifedipine, 50 μM APV + DHPG, APV, 10 μM CNQX + DHPG, or CNQX ($n = 3$).

Excitation and oscillations due to group I mGlu receptor activation

DHPG consistently induced motoneuron and VR depolarization which became substantially smaller in the presence of TTX and blockers of ionotropic receptors. Previous studies have shown that rat motoneurons are depolarized by broad spectrum mGlu receptor agonists (Birse *et al*., 1993; Jane *et al*., 1994) although the extent of the direct depolarizing action remains controversial (King & Liu, 1996). Holohean *et al*. (1999) have shown, on frog motoneurons, such a depolarization to be equally sensitive (≈30% block) to TTX and ionotropic glutamate receptor antagonism. More recent work with DHPG has indicated that this agonist also has direct depolarizing effects on rat (More *et al*., 2002) and lamprey (Kettunen *et al*., 2003) neurons. While our present data broadly confirm such observations, they additionally suggest that a very substantial part of the action by DHPG included network firing and release of depolarizing transmitters comprising glutamate, GABA and glycine liberated on motoneurons. Thus, the simplest interpretation was that premotoneurons and interneurons made the major contribution to the response recorded from motoneurons which, however, possessed a limited number of native group I mGlu receptors. Thus, the lack of change in motoneuron input resistance is likely to reflect the remote origin of the recorded depolarization (or coactivation of opposing conductances). Because the mGlu5 receptor agonist CHPG is completely ineffective on rat spinal motoneurons (Dang *et al*., 2002), it seems likely that the depolarizing action by DHPG was mediated by mGlu1 receptors.

During sustained agonist application, the DHPG-evoked depolarization faded away. While this phenomenon may simply reflect receptor desensitization, biochemical and cytotoxicity data indicate a more intriguing possibility, namely that, during prolonged agonist binding, group I mGlu receptors become phosphorylated and couple to a different G-protein which determines a receptor-functional switch from an excitatory to an inhibitory one (Rodriguez-Moreno *et al*., 1998; Bruno *et al*., 2001). In the present study, depolarization fading was, however, accompanied by oscillatory activity to show that, even if there was a receptor switch, network excitation still prevailed.

DHPG-induced oscillations had network origin and did not arise as a mere consequence of motoneuronal depolarization because they disappeared in TTX solution and persisted when the motoneuron membrane potential was repolarized. Furthermore, oscillations were present (albeit with smaller amplitude) even when motoneuron spiking was suppressed by QX-314. This result confirmed that oscillations indeed originated from network activity and were amplified by intrinsic conductances of the motoneuron membrane. Note, however, that the conductances activated by a single antidromic spike were apparently unaffected by DHPG, which left action potentials unchanged.

Studies with mGlu receptor antagonists helped to dissect out the group I mGlu receptor subtypes mediating the DHPG-induced depolarization and oscillations. The latter were suppressed by the mGlu5 antagonist MPEP while the former was sensitive to the mGlu1 antagonist AIDA. Previous reports have indicated that AIDA may not be fully selective against mGlu1 receptors as antagonism of ionotropic glutamate receptors has been observed when AIDA is used in high concentrations (1 mM, Krieger *et al*. 1998; iontophoretically applied from 50-mM AIDA-containing pipettes, Salt *et al*., 1999). In the present investigation the AIDA concentration did not exceed 500 μM and did not block the control DR-evoked motoneuron responses, making unlikely any significant antagonism of ionotropic glutamate receptors. Nevertheless, it was important to confirm that the same pattern of antagonism by AIDA was produced by CPCCOEt, a noncompetitive mGluR1 antagonist (Schoepp *et al*., 1999), validating the suggestion that mGlu1 receptors were indeed responsible for the DHPG-induced depolarization. The competitive nature of AIDA antagonism (Moroni *et al*., 1997) made it more suitable whenever experimental protocols required recovery after antagonist washout. For this reason our subsequent experiments were based on the use of AIDA.

In view of the discrete laminar distribution of the mGlu1 and 5 subtypes (Valerio *et al*., 1997a,b; Berthele *et al*., 1999; Alvarez *et al*., 2000), our pharmacological data suggest distinct contributions by certain spinal cord regions to one or the other component of the DHPG action.

On rat dorsal horn neurons, group I mGlu receptor agonists produce direct membrane depolarization with intense neuronal discharge (Zhong *et al*., 2000). We reckon that the DHPG-evoked, MPEP-sensitive oscillatory activity detected in the present investigation might have arisen from the dorsal horn area, in view of the spinal distribution of mGlu5 receptors (Berthele *et al*., 1999). This activity was perhaps responsible for generating network-coherent oscillations presumably due to synchronization of discrete clusters of neurons (Streit *et al*., 2001). Because a comparable reduction in the amplitude of motoneuronal depolarization and of oscillatory activity in response to DHPG was observed in either APV or CNQX solution, it seems likely that network-based NMDA and non-NMDA receptors were both necessary to express the DHPG-induced excitation without selective up-regulation of a specific class of ionotropic glutamate receptors. Because DHPG-evoked oscillations were usually synchronous at ipsi- or intersegmental level, it seems likely that group I receptors could not directly activate the network responsible for fictive locomotion.

Synaptic depression due to group I receptor activation

While the peak of the DR-induced synaptic response was always inhibited by DHPG regardless of the stimulus intensity, in the case of high voltage stimulation, prolongation of the reflex could occur so that the overall response area was unchanged. These effects by DHPG were mediated by mGlu1 and 5 receptors. However, as neither AIDA nor MPEP was effective on synaptic transmission, it seems likely that group I mGlu receptors were not normally activated under these circumstances. On spinal slice dorsal interneurons monosynaptic EPSPs are reported to be depressed by group I receptor activation while polysynaptic EPSPs are potentiated (Zhong *et al*., 2000). Previous intracellular recordings from rat ventral horn neurons have indicated that the broad spectrum mGlu receptor agonist ACPD depressed EPSPs induced by weak or strong DR stimuli (King & Liu, 1996), although the mechanism of action remained uncertain. In the present experiments clear potentiation by DHPG was, however, an occasional phenomenon because of overlapping synaptic depression which was the main effect of this substance.

Several hypotheses seem unlikely to explain this depressant action of DHPG. For example, excessive depolarization to inactivate spikes and reduce the EPSP driving force is improbable because there was dissociation between inhibition of synaptic responses and depolarization, and because responses to strong stimuli (eliciting more firing) were not reduced. Persistence of action potential activity plus lack of change in input resistance also make improbable a large membrane shunt at motoneuronal level. Because, in the presence of nifedipine or APV, DHPG could still depress synaptic activity, it seems unlikely that there was any generalized down-regulation of Ca^{2+} or NMDA conductances. Finally, a functional switch of group I mGlu receptor function (Rodriguez-Moreno *et al*., 1998; Bruno *et al*., 2001) seems improbable when accounting for synaptic depression because the depression was related to stimulus strength and occurred before the peak DHPG depolarization. One probable explanation was that group I mGlu receptor activation reduced DR-evoked responses by enhancing the activity of glycinergic inhibitory neurons because strychnine (but not bicuculline) prevented the effect of DHPG. Glycinergic interneurons are widely distributed in the dorsal and ventral horn (Shupliakov *et al*., 1993; Todd, 1996), making them potential targets for the group I mGlu receptor activity. Depression by DHPG of the peak amplitude of fast synaptic responses perhaps implies tonic facilitation of glycine release at or near motoneurons in view of the presence of mGlu1 receptors in this area (Berthele *et al*., 1999). Decreased amplitude of polysynaptic responses might have been due to the activation of a wider glycinergic cell population comprising the one in the dorsal horn as well, in which, in addition to mGlu1 receptors, mGlu5 receptors are amply expressed (Alvarez *et al*., 2000). In the presence of strychnine, reflexes were prolonged perhaps because the DHPG excitatory actions were unopposed by its concurrent inhibitory effects. As Jia *et al*. (1999) have reported rather limited colocalization of mGlu5 receptors with GABA in superficial dorsal horn interneurons, this observation is consistent with the present data showing the DHPG depressant action to be insensitive to bicuculline.

Previous results on the inhibition induced by DHPG are, however, controversial. For example, Zhong *et al*. (2000) found that both strychnine and bicuculline were necessary to remove the depressant action by DHPG on EPSPs of lamina II interneurons. On the other hand, on dorsal horn neurons, group I mGlu receptor activation could induce sustained depression at sensory synapses even in the presence of strychnine and bicuculline, therefore independent of glycinergic and GABAergic transmission (Chen *et al*., 2000b). In the present study synaptic response depression could be simply explained by the activity of glycinergic interneurons depolarized by group I mGlu receptor activation (Dang *et al*., 2002). Excitatory transmission induced by strong DR stimuli was apparently sufficient to overcome enhanced glycinergic transmission.

Role of mGlu receptors in spinal network activity

Because none of the tested mGlu receptor antagonists affected synaptic transmission in the spinal cord, it seems likely that, in this region, group I mGlu receptors do not take active part in standard synaptic processes. This realization does not, however, exclude the possibility that, because of the time lag in the second-messenger-mediated responses triggered by mGlu receptors (Pin & Duvoisin, 1995; Schoepp *et al*., 1999), such receptors play an important role in delayed synaptic plasticity (such as long-term depression; Chen *et al*., 2000b), an issue which deserves further investigation. In addition, mGlu receptors appear to be important pharmacological targets for novel treatments aimed at controlling neuropathic pain with mGlu5 antagonists (Mills *et al*., 2002) or ameliorate the effects of spinal lesions with mGlu1 antagonists (Agrawal *et al*., 1998; Chen *et al*., 2000a; Mills *et al*., 2002). Understanding the cellular basis of the action of these substances should aid the design of more effective strategies to treat such conditions.

Acknowledgements

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Abbreviations

AIDA, (RS)-1-Aminoindan-1,5-dicarboxylic acid; APV, D-amino-phosphonovalerate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CPCCOEt, 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester; DHPG, (RS)-3,5-dihydroxyphenylglycine; DR, dorsal root; mGlu, metabotropic glutamate; MPEP, 2-methyl-6-(phenylethynyl)pyridine hydrochloride; NMDA, *N*-methyl-D-aspartate; QX-314, N-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium chloride; *T*, period; Th, threshold; TTX, tetrodotoxin; VR, ventral root.

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RESEARCH NOTE

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Effect of metabotropic glutamate receptor activity on rhythmic discharges of the neonatal rat spinal cord in vitro

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Abstract To extend our understanding of the network-based properties which enable a neuronal circuit to produce sustained electrical oscillations, we explored the potential contribution of metabotropic glutamate receptors (mGluRs) to generation of rhythmic discharges. The in vitro spinal cord of the neonatal rat was used as a model to find out if electrical patterns characterized by either alternating or synchronous motor pool discharges (recorded from lumbar ventral roots) required mGluR activation or were modulated by it. Alternating patterns of fictive locomotion (induced by NMDA and 5HT) were slowed down and blocked by the broad spectrum mGluR agonist (±)-1-aminocyclopentane-trans-1, 3-dicarboxylic acid (t-ACPD; 5–50 μM) and unaffected by the broad spectrum mGluR antagonist (RS)-α-methyl-4-carboxyphenylglycine (MCPG; 1 mM). The regular, synchronous bursting emerging in the presence of strychnine and bicuculline was accelerated by t-ACPD with a commensurate decrease in single burst length, an effect antagonized by MCPG which per se did not affect bursting. The action of t-ACPD was selectively inhibited by the L-type Ca^{2+} blocker nifedipine which, however, did not change rhythm acceleration evoked by NMDA. These data suggest that neither alternating nor synchronous oscillatory discharges were apparently dependent on mGluR activation via endogenously released glutamate. However, mGluR activation by the agonist t-ACPD modulated rhythmic patterns, indicating that such receptors are a potential target for pharmacological up- or downregulation of spinal rhythmicity.

Keywords Locomotor network · Fictive locomotion · Disinhibited bursting · Spinal network · Glutamate receptors · Oscillations

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Introduction

To study the basic properties of network operation within the central nervous system, it is useful to investigate the functional activity of the mammalian spinal cord because it contains a rhythmic motor programme generated by a cluster of neurons termed central pattern generator (CPG), without the need of external inputs to trigger it. The spinal CPG can produce a range of motor pool discharges like the locomotor pattern which consists of rhythmic alternation of motor signals to flexor and extensor muscles on either side (reviewed by Grillner and Wallen 1999). A very similar pattern can be produced also by the mammalian spinal cord in vitro in the presence of excitatory substances like NMDA and serotonin (5HT; reviewed by Kiehn and Kjaerulff 1998). Because of its accessibility and long-term stability, such a preparation represents a very advantageous model with which to clarify the mechanisms responsible for network operation. When synaptic inhibition is blocked pharmacologically, the spinal CPG generates a synchronous, slow discharge termed disinhibited bursting (Bracci et al. 1996). Even though its lack of alternation is not compatible with locomotor activity, the disinhibited bursting is an important model to help understand how the spinal networks can initiate a discharge, control its length and terminate it. Indeed, it has recently been possible to model such an activity to

dissect out some elementary mechanisms (Rozzo et al. 2002).

Rhythmic discharges produced by the spinal CPG crucially depend on excitatory connections where glutamate acting on ionotropic receptors is the major excitatory transmitter (Rekling et al. 2000). However, the spinal cord also contains a wide population of metabotropic glutamate receptors (mGluRs; Alvarez et al. 2000) which are G-protein coupled and operate by changing intracellular second messenger such as Ca^{2+} and IP_3 (reviewed by Schoepp et al. 1999). Very little is known about mGluR contribution to the CPG operation in the mammalian spinal cord (El Manira et al. 2002) because the role of mGluRs in spinal cord physiology remains incompletely understood. In fact, it is difficult to demonstrate that endogenously released glutamate can activate mGluRs during single or repeated synaptic activity as mGluR antagonists have modest effects on excitatory synaptic transmission (Boxall et al. 1996). Nevertheless, activation of mGluRs with a broad spectrum agonist like trans-ACPD readily modulates synaptic transmission at pre- and postsynaptic level (Cerne and Randic 1992; Cao et al. 1995; Dong et al. 1996; King and Liu 1996), indicating that these receptors play a role in plasticity phenomena like long-term depression (Chen et al. 2000). The mechanism responsible for the modulatory action by t-ACPD remains controversial because some studies suggest selective enhancement of ionotropic glutamate receptor activity (Bleakman et al. 1992; Ugolini et al. 1997) or upregulation of voltage-dependent Ca^{2+} channels (Morisset and Nagy 1996; Russo et al. 1997) while others, instead, propose non-selective changes in neuronal excitability (Jones and Headley 1995).

The present study explored how activating or blocking an ample family of mGluRs with a broad spectrum agonist and a broad spectrum antagonist may affect fictive locomotion and disinhibited bursting in order to find out how these receptors might shape spinal network activity. In view of the need to record for a long-lasting period fictive locomotion and disinhibited rhythms, as well as to monitor discharge phase-coupling amongst various ventral roots (VRs), such experiments were conducted with DC-coupled extracellular VR recordings.

Materials and methods

Full details about the experimental preparation can be found in Bracci et al. (1996), Beato and Nistri (1999), and Rozzo et al. (2002). In brief, lumbar spinal cord preparations from neonatal Wistar rats (0–5 days old) were superfused with Krebs solution containing (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% O_2-5% CO_2; pH 7.4 at room temperature. DC-coupled VR recordings were obtained with suction microelectrodes filled with Krebs solution. Fictive locomotion was induced by continuous bath application of NMDA (5 μM) and 5HT (10 μM) and remained stable for hours. Measurements of fictive locomotor responses were started after a 10-min stabilization period following the initial drug application. Likewise, disinhibited bursting was produced by continuously bath-applied strychnine (1 μM) and bicuculline (10 μM) and remained unchanged for many hours. Disinhibited bursting parameters were measured after a 10-min stabilization period following strychnine and bicuculline application. All rhythmic activity was analyzed on the basis of its period (T) defined as the time between onset of two consecutive discharges. To quantify period values for a group of spinal cord preparations, data from each spinal cord were first calculated as the mean of at least 20 consecutive cycles, and then pooled together to obtain means ± SD. The ratio between the standard deviation and the mean of the period provided the period coefficient of variation (CV), which is an index of regularity of discharges (the lower the CV, the more regular rhythmicity is). The duration of single bursts during disinhibited bursting was measured from the abrupt onset of depolarization from baseline to the time when depolarization returned to noise threshold (25 times the SD of baseline noise; usually 100–150 μV). Statistical significance was assessed with the Student's t-test for normally distributed data, or ANOVA plus Tukey's test for parametric data (accepted level of significance was p=0.05 in all cases). The effects of drugs on disinhibited bursting were expressed as % changes in burst period with respect to the value in control conditions (strychnine plus bicuculline) prior to drug application. All mGluR agents were bath applied via the superfusing solution for 15–30 min. On the same preparation drug applications were usually replicated to ensure reproducibility of results. Whenever mGluR antagonists were tested against the action of an agonist, they were applied after having established that the agonist per se evoked a clear response reversible on washout. Antagonist application started at least 10 min prior to agonist application. Washout of drugs required up to 1 h. The broad spectrum mGluR agonist (±)-1-aminocyclopentane-trans-1, 3-dicarboxylic acid (t-ACPD) and the broad spectrum antagonist (RS)-α-methyl-4-carboxyphenylglycine (MCPG), as well as the selective antagonist for mGluR1 receptors (*RS*)-1-aminoindan-1, 5-dicarboxylic acid (AIDA) and the selective antagonist of group II receptor (2*S*)-α-ethylglutamic acid (EGLU) were purchased from Tocris (full details concerning their pharmacological selectivity can be found in Schoepp et al. 1999). Nifedipine, NMDA and serotonin (5HT) were also from Tocris while tetrodotoxin (TTX) was bought from Affinity Research.

Results

t-ACPD (50 μM) induced VR depolarization (419 ±210 μV, n=10) which persisted in TTX (1 μM) solution, albeit reduced in amplitude (210±125 μV, n=8; p<0.01). Despite its depolarizing action, t-ACPD (5–50 μM) did not induce alternating rhythmic discharges in control solution (n=7). Furthermore, as shown in Fig. 1, t-ACPD (25–100 μM) disrupted the alternating pattern typical of fictive locomotion (induced by application of NMDA +5HT) by first making oscillations irregular (Fig. 1B; note, however, that cycle alternation persisted) and then abolishing them (Fig. 1C) within 4 min from the start of application (n=5). Recovery was observed after 9 min washout (Fig. 1D). MCPG (1 mM) had no significant effect on fictive locomotion nor did it prevent the suppressant action by t-ACPD (n=5).

Since disinhibited bursting is characterized by large and persistent glutamate-dependent depolarizations with superimposed oscillations (Bracci et al. 1996), this process might be more suitable than fictive locomotion for disclosing a role of mGluRs in rhythmicity control. Figure 2A, B shows an example of the effect of t-ACPD (10 μM) which accelerated bursting; this effect was antagonized by MCPG (Fig. 2C, D). On average, t-ACPD strongly accelerated bursting (Fig. 2E) with a concomitant

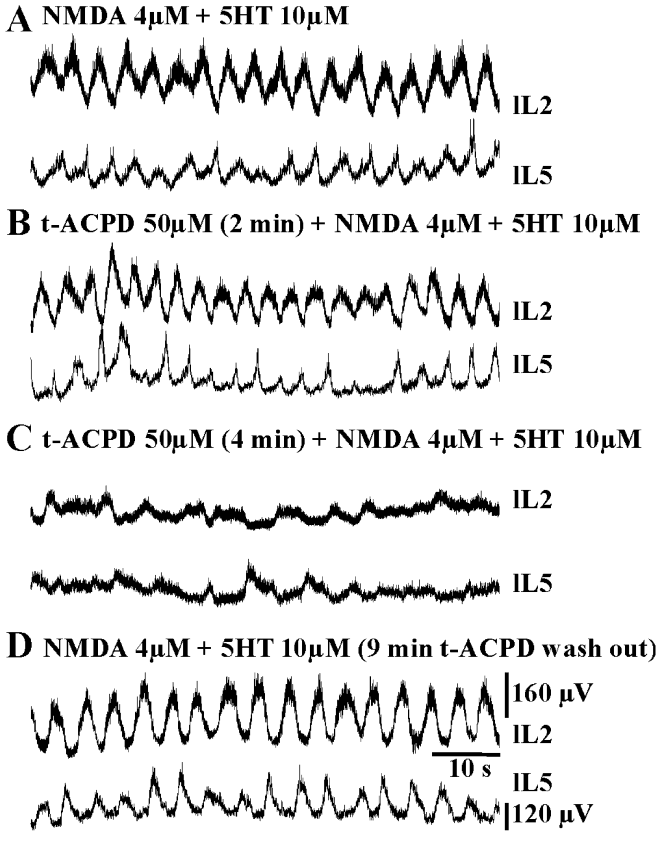

Fig. 1A–D Effect of t-ACPD on fictive locomotor pattern. **A** Alternating pattern is generated by bath-applied NMDA and 5HT. Traces are records from left L2 (*lL2*) and left L5 (*lL5*) VRs which supply flexor and extensor muscles of the homolateral hindlimb. **B** Two minutes application of t-ACPD leads to rhythm irregularity with preserved phase alternation. **C** After 4 min exposure to t-ACPD fictive locomotor rhythm is lost and only occasional alternating cycles are observed. **D** After 9 min washout of t-ACPD fictive locomotor pattern is recovered. Voltage calibrations in **D** refer also to corresponding trace pairs in **A–C**. Time calibration is the same throughout records

increase in the period CV value (0.41±0.02 vs control CV of 0.16±0.07; n=4; p<0.05), indicating that bursting had become more irregular. Lower concentrations of t-ACPD (0.5–5 μM) did not significantly modify such a rhythm (98 ±10%, n=9), while higher ones (25–50 μM) had effects comparable to 10 μM (58±22%, n=5, p<0.001 vs control). Burst duration was also reduced by t-ACPD (Fig. 2E) with a CV of 0.37±0.13 (this was not significantly different from that of the control, 0.17±0.10). Figure 2E, F shows that, on average (n=12), the broad spectrum antagonist MCPG (1 mM) did not significantly alter burst period or duration, but it prevented t-ACPD induced acceleration (n=6; p<0.05 with respect to t-ACPD only). The mGluR1 antagonist AIDA (500 μM; n=11) had no effect on disinhibited bursting and did not block the accelerating action by t-ACPD (70±40% period in AIDA plus t-ACPD vs 55±18% period in t-ACPD solution; n=11). The mGluR-II antagonist EGLU (200 μM; n=9) did not prevent the t-ACPD induced rhythm acceleration (52 ±33% period in EGLU plus t-ACPD vs 43±11% period in t-ACPD solution; n=9). These observations confirm that the action of t-ACPD was mediated by more than just one mGluR class.

Intracellular recording from single dorsal horn interneurons of the turtle (Russo et al. 1997) or rat (Morisset and Nagy 1996) spinal cord has indicated that mGluR activation can facilitate the onset of plateau potentials dependent on nifedipine-sensitive, L-type Ca^{2+} channels and capable of inducing sustained excitation of spinal motoneurons. We tested whether the changes in disinhibited bursting observed after application of t-ACPD might have been due to a similar nifedipine-sensitive mechanism, even though the present recordings restricted to VR responses could not directly demonstrate plateau potentials. The L-type Ca^{2+} channel blocker nifedipine (20 μM) per se did not alter bursting period (Fig. 3G). Nevertheless, nifedipine counteracted the t-ACPD-evoked acceleration of disinhibited rhythm as shown in Fig. 3B, E

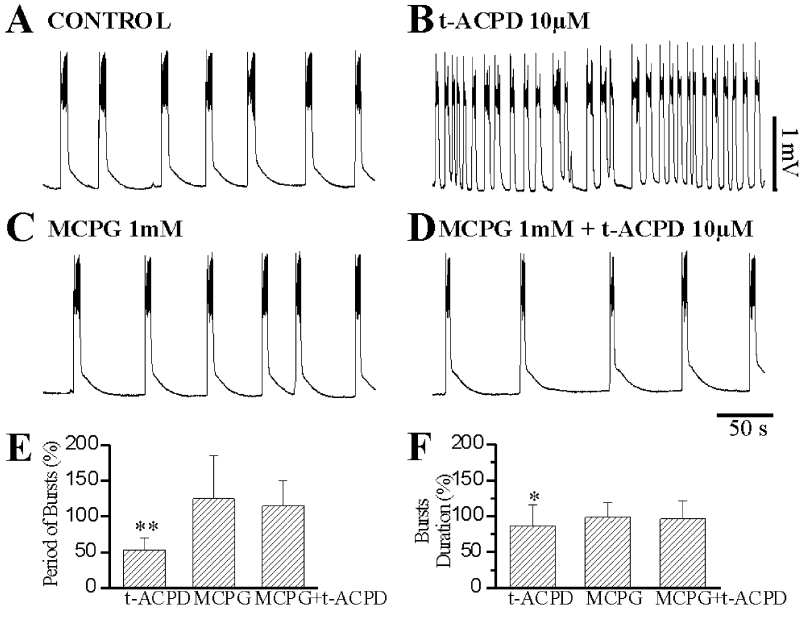

Fig. 2A–F Action of the broad spectrum mGluR agonist t-ACPD on disinhibited rhythm. **A** Example of disinhibited rhythm recorded from single VR in control conditions (strychnine plus bicuculline solution). **B** t-ACPD (10 μM) accelerates bursting. **C** The broad spectrum antagonist MCPG (1 mM) does not significantly alter period or burst duration. **D** MCPG prevents t-ACPD induced acceleration. **A–D** are from the same preparation. **E, F** Average data for period of bursting and single burst duration for t-ACPD (n=23), MCPG (n=12) and MCPG + t-ACPD (n=6); *p<0.05, **p<0.001

(see also Fig. 3G for data summary). This effect was related to mGluR activation since, when the disinhibited rhythm was similarly accelerated by bath-applied NMDA (5 μM), nifedipine could not normalize period acceleration which remained fast (Fig. 3C, F, G).

Discussion

The principal finding of the present report is that activation of mGluRs modulated rhythmic discharges generated by locomotor networks in the rat spinal cord. In particular, the broad spectrum mGluR agonist t-ACPD, acting via nifedipine-sensitive mechanisms, accelerated spontaneous bursting appearing after block of fast synaptic inhibition, and made it more irregular. This observation suggests that the combined presence of t-ACPD and excitatory bursts created a synergistic process probably responsible for facilitation of Ca^{2+} conductances of spinal interneurons (Morisset and Nagy 1999; Russo and Hounsgaard 1999) which modulated synchronous network discharges. Together with rhythm acceleration, the network retained its property to shorten burst duration to avoid burst coalescence into seizure like activity. Hence, the suggested facilitation of high threshold Ca^{2+} conductances (Morisset and Nagy 1999; Russo and Hounsgaard 1999) was apparently compensated (despite block of GABA and glycine mediated inhibition) by other mechanisms which might include ion pumps and synaptic fatigue (Rozzo et al. 2002).

The present investigation employed a simplified experimental model, namely disinhibited bursting, to explore network activity of the spinal cord. Such bursts are due to strong neuronal excitation spread throughout the network via recurrent glutamatergic collaterals (Tscherter et al. 2001; Darbon et al. 2002). This discharge pattern undoubtedly represents an extreme case of network excitability because of concomitant suppression of the main synaptic inhibitory processes (Bracci et al. 1996), but it also provides a suitable tool to explore the most elementary processes which regulate rhythmicity. An additional complication with the present study was caused by bath application of pharmacological agents which may have interacted with a much wider receptor population than normally available to the endogenous transmitter. The discrete localization of spinal mGluRs (Alvarez et al. 2000) should have, however, prevented indiscriminate receptor activation in all spinal laminae. Of course, records of VR responses, which express average neuronal population discharges, are unsuitable to provide detailed information on the cellular mechanisms underlying bursting. However, when multiple VRs are monitored, they do supply useful data on rhythm regularity and synchronicity at various segmental levels so that a multisite view of network operation can be obtained without invasive methods. It is also interesting to note how closely bursts recorded from VRs resemble bursts recorded intracellularly from single motoneurons (Bracci et al. 1996).

Information concerning the role of mGluRs in mammalian fictive locomotor patterns is currently minimal (reviewed by El Manira et al. 2002). The present investigation shows that t-ACPD could not elicit fictive locomotion, and actually blocked it. It is unlikely that excessive network excitation was the cause of this phenomenon, because t-ACPD did not block rhythmicity when the network was in the higher excitability phase of disinhibited bursting (see Rozzo et al. 2002). The concentration of t-ACPD required to block fictive locomotion was higher than the one for modulating disinhibited bursting, suggesting that distinct mechanisms were responsible for such effects. Suppression of fictive locomotion was preceded by a phase of irregular discharges with preserved alternation, indicating the synaptic inhibi-

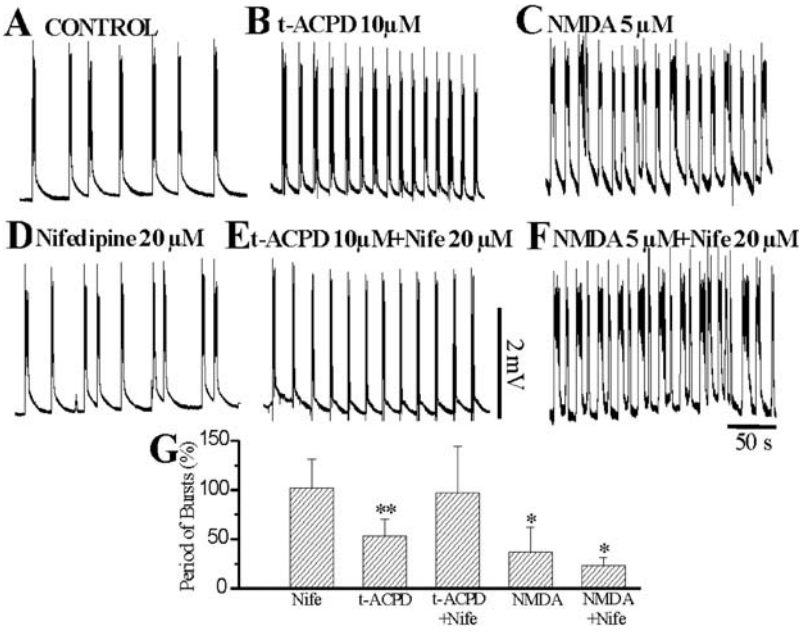

Fig. 3A–G Involvement of L-type Ca^{2+} channels in disinhibited rhythm. **A** Disinhibited rhythm recorded extracellularly from single VR in control conditions. **B, C** Bath application of t-ACPD (10 μM) or NMDA (5 μM) accelerates rhythm. **D–F** Nifedipine (20 μM) per se does not alter disinhibited rhythm, but it slows down to control values the t-ACPD accelerated rhythm, while not affecting NMDA accelerated rhythm. **G** Average data for bursting period in nifedipine (n=7), t-ACPD (n=10) or NMDA (n=4) solution (or combinations thereof); *p<0.05, **p<0.001

tion remained operational. Since mGluRs are expressed by inhibitory interneurons (Jia et al. 2000), it is likely that t-ACPD recruited a larger population of them which eventually blocked generation of fictive locomotion. Relatively rapid recovery after t-ACPD washout is also compatible with a transient functional impairment of pattern expression. Since this mGluR agonist possesses presynaptic inhibitory effects (Cerne and Randic 1992; Cao et al. 1995; Dong et al. 1996; King and Liu 1996), it is possible that presynaptic depression of excitatory transmitter release might also have contributed to arrest of the fictive locomotor network.

In the case of disinhibited rhythmicity the safety factor for pattern operation must have been much higher and only rhythm irregularity hinted at some impairment of excitatory synaptic efficacy which nevertheless generated faster oscillations. The differential effects of t-ACPD on fictive locomotion and disinhibited bursting do not necessarily suggest that such activities are mediated by distinct networks, especially taking into account that previous experiments have indeed shown that both rhythms are apparently generated by common circuits (Beato and Nistri 1999). It is proposed that the present different results are probably attributable to the two distinct experimental conditions, namely intact or blocked synaptic inhibition. This realization emphasizes the usefulness of investigating also the disinhibited bursting model which, in view of its network simplification, helps to dissect out mechanisms hidden within a more complex pattern of circuit action.

The present investigation was a pilot study to find out what would occur by mimicking the action of the natural transmitter glutamate, which, of course, is the natural broad spectrum agonist for all mGluRs in the spinal cord. Further studies are necessary to dissect out the relative contribution by various mGluR subclasses. This issue is demonstrated by the observations with MCPG, which could block the action of t-ACPD on disinhibited rhythms but not on fictive locomotion. t-ACPD is a promiscuous mGluR agonist (Schoepp et al. 1999) activating group I, II and III receptors. Conversely, despite the fact that MCPG is a broad spectrum antagonist, it is ineffective on certain group II and all group III receptors (Schoepp et al. 1999). It is likely that suppression of fictive locomotion by t-ACPD involved receptor classes with a low sensitivity to MCPG. The simplified network responsible for disinhibited bursting was, however, susceptible to modulation by t-ACPD via receptor classes blocked by MCPG.

Onset and maintenance of either fictive locomotion or disinhibited bursting did not appear to require mGluR activation as the broad spectrum antagonist MCPG did not affect either of them. The simplest interpretation is that there was an insufficient amount of endogenous glutamate to activate mGluRs because of either rapid uptake or mGluR remoteness from the sites of glutamate release. Another possibility is that mGluR activation by endogenous glutamate has delayed effects on spinal network activity occurring beyond the observation time of electrophysiological experiments. Since transgenic knockout mice lacking certain mGluR subtypes display locomotor dysfunction (Conquet et al. 1994), one might hypothesize that mGluRs play a role in locomotion. Future experiments based on in vivo recording from normal and transgenic animals should help to understand the involvement of mGluRs in this phenomenon.

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DISCUSSION

In summary, with respect to the aims listed on page 46, the present project was able to clarify the following issues:

1. Activation of group I mGluRs evoked motoneuron depolarization via mGlu receptor subtype 1 and generated sustained, network dependent oscillations via mGlu receptor subtype 5. Neither the group II nor the group III had any effect on lumbar motoneuron membrane potential or input resistance.
- Agonists of group I mGluRs decreased the peak amplitude of synaptic response induced by dorsal root stimuli, an effect unrelated to depolarization and dependent on glycinergic transmission. Agonists of group II and III mGluRs strongly depressed synaptic responses.
- During Fictive locomotion or disinhibited bursting, endogenous glutamate could activate discrete clusters of group I subtype 1 mGluRs to facilitate discharges, while activation of group III mGluRs slowed down fictive locomotor patterns and arrested disinhibited bursting. Finally, group II mGluRs had no significant effect on fictive locomotion, yet blocked disinhibited bursting.

2. 4-AP slightly increased input resistance of lumbar motoneurons (without affecting their action or resting potential) and enhanced synaptic responses without changes in axon conduction.
- Very low doses of 4-AP produced synchronous oscillations on both VRs and DRs.
- DR activity persisted after ablating the ventral region and was preserved in an isolated dorsal quadrant, indicating its dorsal horn origin.
- 4-AP accelerated fictive locomotion and facilitated the onset of fictive locomotion in the presence of subthreshold stimuli. Activation of fictive locomotion reversibly suppressed DR root oscillations induced by 4-AP.

3. At high concentrations TEA evoked large depolarization of motoneurons together with spike broadening.
- Low concentrations of TEA induced an irregular, synchronous rhythm that, at higher concentrations, became alternating.

- Horizontal sectioning of the spinal cord preserved irregular VR root and DR root discharges.
- 4-AP did not accelerate rhythmic alternating patterns elicited by TEA.
- Rhythmic alternating patterns elicited by TEA were relatively stereotypic without interactions with fictive locomotion.

These results will therefore be discussed in a wider context with emphasis on locomotor networks and their operating processes.

1. Excitation and oscillations due to group I mGluR activation

A systematic discussion of all details concerning the data of the present study is available in the `discussion` sections of the enclosed papers. Here, I will summarize the main implications of this research and compare the effects observed with different experimental protocols.

The most important finding of the first part of the present study has been the demonstration of the role played by certain subtypes of mGluRs in neuronal excitability and synaptic transmission in the neonatal rat spinal cord. In particular, once group I mGluRs have been activated, mGluR1 determined depolarization of MNs and mGluR5 elicited superimposed oscillatory activity, that originates from the spinal network. Since MNs possessed a limited number of native group I mGlu receptors, the recorded response was mainly determined by premotoneurons and interneurons.

In accordance with the presence of a discrete laminar distribution of mGlu1 and 5 subtypes (Valerio et al., 1997a,b; Berthele et al., 1999; Alvarez et al., 2000), our pharmacological data suggest that the DHPG action included two components that arose from certain regions of the spinal cord. In fact, in our experiments, the DHPG-evoked, MPEP-sensitive oscillatory activity probably started in the dorsal horn area, in view of the spinal distribution of mGlu5 receptors (Berthele et al., 1999), while mGluR 1 receptors were more abundant in the ventral horn area. Activation of group I mGluRs by endogenous glutamate was minimal during sporadic or low-frequency DR stimulation. The contribution of these receptors may be more significant during high frequency stimulus trains.

2. EPSP and IPSP depression due to group I receptor activation

None of the tested mGluR antagonists affected synaptic transmission in the spinal cord, demonstrating that group I mGluRs were not normally activated under these circumstances. However, the peak of the DR-induced synaptic response recorded from MNs was always inhibited by relatively large contributions of DHPG, regardless of the stimulus intensity.

The synaptic depression induced by DHPG might be explained by group I mGluR-mediated enhancement of glycinergic inhibitory neurons. In fact, strychnine (but not bicuculline) prevented the depressant effect of DHPG.

Recurrent synaptic inhibition of lumbar MNs was inhibited by activation of group I mGluRs, probably because of block of cholinergic transmission, which thereby deprived Renshaw cells of their input drive. In addition, if Renshaw cells (like other ventral horn interneurons) expressed group I mGluRs (Berthele et al., 1999; Alvarez et al., 2000), their direct depolarization by DHPG could evoke inactivation of voltage-dependent Na^+ (and perhaps Ca^{2+}) channels with impairment in their ability to release inhibitory transmitter.

Significant depression of recurrent IPSPs on lumbar MNs may explain the emergence of synchronous oscillations between MN pools triggered by mGluR activation in the rat spinal cord. In fact, it is widely accepted that glycinergic interneurons in the ventral horn support the alternation of electrical discharges between distinct groups of MNs (Butt et al., 2002). Therefore, the impairment of Renshaw cell activity may have contributed to the onset of a synchronous rhythm that did not, however, prevent the appearance of fictive locomotor patterns or their modulation.

3. Role of group I mGluRs in fictive locomotion

The present study shows that group I mGluRs can modulate, in several ways, the patterned activity elicited by rat spinal networks. Our findings demonstrated that group I receptors, activated by endogenous glutamate, could play a role in fictive locomotion and disinhibited bursting. Exogenous activation of such receptors depressed cumulative depolarization, while preserving spike wind-up and associated alternating rhythms, and facilitated disinhibited bursting, which lost its regular rhythmicity.

mGluR 1 antagonists slowed down fictive locomotion, indicating that endogenous glutamate could activate subtype 1 mGluRs with a facilitatory role in this type of

spinal rhythmicity. It was, therefore, surprising that, in the rat spinal cord, fictive locomotion evoked by NMDA plus 5-HT was suppressed by a large dose of DHPG.

A likely explanation is that, while the restricted mGluR population activated by endogenous glutamate during fictive locomotion was probably associated with a facilitatory effect, exogenous application of the agonist DHPG acted on a much larger mGluR population with a mix of facilitatory and inhibitory actions on fictive locomotion. Facilitation would be predominant only when glycinergic transmission was probably unaffected by using low concentration of DHPG.

During locomotor-like activity, the synchronous oscillations evoked by DHPG were replaced by alternating patterns, indicating that these two rhythms were mutually exclusive.

However, DHPG-evoked oscillations were presumably mediated by the mGluR5 receptors that were mostly present in the dorsal horn (Berthele et al., 1999), an area not involved in fictive locomotion. This led us to suppose that DHPG was working via an apparently distinct network, perhaps more limited than the fictive locomotor one. In fact, once this latter one had become operative, it silenced the DHPG dependent one, like in the case of other network activities (Whelan et al., 2000).

4. Role of group I mGluRs in disinhibited rhythm

DHPG sped up the disinhibited bursting induced by application of strychnine plus bicuculline via subtypes 1 receptors, that also mediated network depolarization. Furthermore, it was not surprising that this type of bursting was insensitive to the mGluR5 antagonism, because the great majority of mGluR5 receptors needed for DHPG oscillations are present in the superficial layers of the dorsal horn (Berthele et al., 1999; Alvarez et al., 2000), which is not an important area for the development of disinhibited bursting (Bracci et al., 1996 b).

One characteristic of disinhibited bursting in the presence of DHPG was the loss of cycle regularity, demonstrated not only by the wider period CV, but also by frequent burst clusters. It seems probable that this loss of regularity had been caused by discrete modifications in intracellular second messengers (IP3 and Ca^{2+}; Pin & Duvoisin 1995; Schoepp et al., 1999; Fagni et al., 2000) brought about by DHPG interaction with group I mGluRs. This phenomenon was also observed with the application of a group II antagonist, indicating that multiple classes of mGluRs controlled glutamatergic transmission and network excitability once synaptic

inhibition was blocked. Ca^{2+} and Ca^{2+}-dependent conductances may control the release of glutamate necessary for burst initiation and the intrinsic ionic mechanisms regulating burst duration.

5. Role of group II and III mGluRs in the spinal cord

Under standard conditions, there was an insufficient level of ambient glutamate to tonically activate group II or III receptors on network cells impinging upon MNs.

Nevertheless, agonists of group II and III mGluRs could potently depress synaptic transmission, as shown by the reduction in DR-evoked reflexes, via presynaptic inhibition of transmitter release (Pinco and Lev-Tov 1993; Cao et al. 1995, 1997; Dong and Feldman 1999).

Group II and III antagonists did not affect fictive locomotion, indicating that, despite the need for endogenous glutamate release (Cazalets et al. 1992; Beato et al. 1997), the rhythm was not crucially dependent on activation of these mGluRs.

Conversely, disinhibited bursting was more dependent than fictive locomotion on the recruitment of mGluRs. In fact, the present study observed that activation of group II or III mGluRs consistently inhibited bursting, a phenomenon antagonized by their class-selective blockers. It seems likely that long-lasting bursts typical of this rhythm are associated with a sustained, large release of glutamate adequate to reach and activate group II and III mGluRs.

6. Low micromolar concentrations of 4-AP facilitate fictive locomotion

Another important finding of this work was that low micromolar doses of 4-AP, together with appropriate paradigms of concurrent CPG stimulation, could activate and facilitate the function of the locomotor CPG in the rat spinal cord.

In the absence of even subthreshold stimulation of the CPG, low concentrations of 4-AP (aimed at blocking discrete classes of K^+ channels rather than a broad spectrum of K^+ conductances) *per se* generated a synchronous pattern. Such rhythm was elicited by a wide spinal network that needed intact glutamatergic transmission and was strengthened via electrotonic coupling among spinal neurons, since the gap junction blocker carbenoxolone substantially attenuated discharges. Since 4-AP-evoked discharges were synchronous on all VRs, such electrical signals, even if produced by motor pools, were incompatible with locomotion.

However, in conjunction with appropriate stimuli, 4-AP facilitated locomotor-like oscillations with a clear alternation among appropriate motor pools and simultaneous disappearance of the synchronous patterns typical of 4-AP alone.

7. Characteristics of the electrical oscillations evoked by 4-AP on dorsal DRs

A further notable finding was the demonstration that DR oscillations evoked by low micromolar doses of 4-AP and originating within dorsal horn areas, did not interfere with the fictive locomotor program. It is likely that DR discharges induced by 4-AP required a local dorsal horn network comprising glutamatergic, glycinergic and GABAergic transmission, and that they were not just determined by transient changes in extracellular K^+ (Kremer and Lev-Tov, 1998).

8. Fictive locomotor patterns generated by TEA

A further result of the present project was the demonstration that the broad spectrum potassium channel blocker TEA activated spinal rhythmogenic networks. This drug induced alternating electrical oscillations from homosegmental and homolateral VRs. Hence, TEA induced effects distinct from those of 4-AP.

The effects of TEA were likely to involve multiple classes of potassium channels as even careful titration of this substance cannot ensure selectivity towards a particular potassium conductance (Rudy, 1988). Nevertheless, it is important to point out that there was a TEA dose-dependent change in spinal network excitability with a consequent rise in synchronous VR discharges, finally converted into alternating motor patterns. In fact, millimolar concentrations of TEA surprisingly generated rhythmic, alternating patterns with all features (period, phase, regularity etc.) typical of fictive locomotion. Such an activity was relatively stereotypic in its period, demonstrating that TEA could activate the CPG, but it could not modulate it. This suggestion is in accordance with the model proposed by Lafreniere-Roula and McCrea (2005) that predicts a regional and functional distinction between the networks responsible for generation and those for modulation of locomotor commands.

TEA induced repeated DR activity, although slower, irregular, longer, and of smaller amplitude then the one elicited by 4-AP. Furthermore, once dorsal and ventral areas of the spinal cord had been surgically separated, the ventral horns generated irregular

and asynchronous discharges (unlike the effect of 4-AP), while the dorsal ones produced asynchronous oscillations.

9. Comparisons between effects due to mGluR activity and K^+ conductance block

Table 3 shows a comparison of the action of the substances, acting on spinal circuits, and used during this project. In all cases, effects were primarily the result of activation of the interneurons composing the CPG and premotoneurons. In fact, when inputs coming from the CPG were abolished, either through suppression of ionotropic glutamatergic transmission or application of the Na^+ channels blocker TTX, modifications in membrane properties of single MNs were few, if any.

This fact suggests that any modulatory effect of these drugs on MNs is not essential for determining rhythm development.

Some of the tested compounds could *per se* generate rhythmic oscillations that, except for high doses of TEA, resulted incompatible with locomotion, because of synchronicity across all VRs. The reason why high concentrations of TEA can elicit locomotory oscillations, unlike DHPG and 4-AP, could come from in the fact that large doses of TEA determined a significant increase in MN input resistance. Interestingly, DHPG and 4-AP, that actually have no effect on MN membrane properties, needed gap junctions to develop synchronous rhythms.

This confirms that, when neuronal excitability is elevated, a circuit will produce a rhythmic pattern. In accordance with this view, Yvont and his colleagues (2005) have recorded intrinsic rhythmic activity even from dissociated primary cultures neurons as long as network excitability was raised.

The implication of these findings is that even very basic circuits are intrinsically rhythmic regardless of the way in which neurons are wired together. What matter most in these cases is to have a sufficiently large number of cells to generate a pattern.

The agents that were tested during the present study had different effects on synaptic transmission in the spinal cord. Moreover, while the block of K^+ channels determined a potentiation in neurotransmitter release at central and peripheral synapses, mGluRs agonists reduced it, with the most dramatic effect caused by the III group mGluR agonist.

The only substance to facilitate the alternated rhythm in the presence of subthreshold stimuli was 4-AP.

Nevertheless, all these substances determined an acceleration in disinhibited bursting, showing that the locomotor CPG has sites sensible to all these chemicals, but, at the same time, that the processes to translate CPG activation into a pattern are diverse and have a different sensitivity to these drugs.

	DHPG	DCG-IV	L-AP4	4-AP	TEA	
					low doses	high doses
Physiological target	mGluRs I	mGluRs II	mGluRs III	predominantly I_A current	predominantly I_A current	I_A current + delayed rectifiers
MN depolarization	✓	✗	✗	✗	✗ (●)	✓
Presence of VR oscillations	✓ Synchronous	✗	✗	✓ Synchronous	✓ Synchronous	✓ Alternated
Changes in MN input resistance	✗	✗	✗	✓ Small increase	✓ Small increase (●)	✓ Increase
Rhythm dependency on gap junctions	✓ Suppression (●)			✓ Slow down		✗
Rhythm dependency on ionotropic glutamate receptors	✓ Suppression			✓ Suppression		✓ Conversion into shallow and asynchronous
Synaptic transmission	↓	↓	↓↓	↑	↑ (●)	↓
Effect on stable F.L.	✓ Suppression	✗	✓ Slow down	✓ Acceleration	✓ Acceleration	✓ Suppression (●)
Facilitation of F.L. in presence of subthreshold stimuli	✓			✓	✗	
Acceleration of disinhibited bursting	✓	✗ Slow down	✗ Suppression	✓	✓ (●)	✓ (●)
Presence of DR oscillations	✗ (●)			✓ Synchronous with VRs		✓ Asynchronous with VRs

Table 3. Synopsis of main network characteristics of oscillations induced by mGluRs agonists, 4-AP and TEA; (●, indicates unpublished data by G. Taccola; F.L. = Fictive Locomotion).

As opposed to DHPG, application of 4-AP and TEA was capable of inducing *per se* rhythmical activity from DRs. The resulting oscillations were generated by a similar mechanism, in both of which primary afferent depolarization took a fundamental part.
Data obtained from ablation experiments have shown that, in the case of dorsal discharges caused by 4-AP and TEA, the cellular components responsible for this rhythm were located in the dorsal horns only.
Since dorsal oscillations evoked by 4-AP were inhibited during fictive locomotion, their presence was not necessarily a deleterious phenomenon as they could be expression of augmented presynaptic inhibition.
The differences in the action of the various tested chemicals suggested several pharmacological targets and different cellular mechanisms. Would it be useful to coapply some substances to synergize their individual characteristics. In this regard, we tried to induce an alternating rhythm with the application of TEA alone and then to modulate its frequency by adding 4-AP. From this series of experiments, it was, however, impossible to accelerate, with 4-AP, the locomotor-like rhythm evoked by TEA.
Further studies are warranted to investigate the possibility of drug synergy.
Final proof for a common motor network responsible for standard fictive locomotion and alternating rhythm elicited by high concentrations of TEA is still lacking.
However, shared features like alternating pattern period and regularity, appear to support this hypothesis. On the other hand, the inability by TEA to facilitate fictive locomotion in the presence of subliminal concentrations of NMDA and 5-HT, or subthreshold electrical stimulation, supports the idea that both rhythms take origin from physically separated elements that converge onto identical unit pattern formation.
Moreover, the existence, in the alternating rhythm induced by TEA, of deletions that are not present in the stable fictive locomotion evoked by neurochemicals may be an interesting topic for further studies. Temporary interruptions of alternating oscillations among all VRs bring TEA rhythm closer to the model proposed by Lafreniere-Roula and McCrea (2005) than the locomotor-like pattern induced by NMDA and 5-HT.

CONCLUSIONS

The present results may be relevant to future pharmacological approaches to augment CPG operativity, for the functional recovery of SCI patients.

These data confirm the usefulness of studying spinal circuitry in the isolated neonatal rat spinal cord. Such a preparation can inform possible therapeutic strategies to be eventually transposed to human studies.

Among the used agents, 4-AP was the only one to increase CPG operation in the presence of subthreshold stimuli. This is the situation presented by incomplete spinal cord lesions, where any residual, although insufficient, cortical input travels in the few descending fibers spared by the trauma.

Conversely, potassium conductances blocked by TEA seemed to be responsible for the activation of the locomotor-like pattern, alternated between flexor and extensor muscles in the same limb and between left and right leg. By virtue of these characteristics, the application of substances like TEA could find some applications, even in complete spinal lesion scenarios, to activate the intact areas of CPG caudal to lesion site.

Instead, certain mGluRs agonists could find a possible clinical utilization in order to limit spinal neurons hyperexcitability following SCI. In fact, impairment of spinal inhibition after lesion can cause severe and debilitating collateral effects, such as spasticity and chronic pain. In this regard, group II and III agonists were able to depress excitability, even in the presence of strychnine and bicuculline, that excluded the contribution of glycine and GABA, classic inhibitory neurotransmitters in the spinal cord.

To be able to best exploit the interesting hints emerging from this study for future clinical applications, it will be necessary, on one hand, to use of more selective substances and, on the other, to include pharmacological therapies into a wider therapeutic plan, that provides new neurorehabilitative techniques and recent discoveries in the bioengineering field.

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