An *in vitro* functionally mature mouse spinal cord preparation for the study of spinal motor networks

BY

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Thesis for the degree of Master of Science

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ABSTRACT

An *in vitro* isolated whole spinal cord preparation has been developed in "motor functionally mature" mice: that is, mice of developmental maturity sufficient to weight-bear and walk. Using a live cell-dead cell assay, it is demonstrated that there are many viable cells throughout the lumbar spinal cord. Administration of strychnine elicited synchronous motor activity bilaterally as recorded in lumbar ventral roots. Rhythmic alternating locomotor-like activity could be produced by application of a combination of serotonin, NMDA, and dopamine in functionally mature animals. The viability of descending pathways was demonstrated with stimulation of the mid-thoracic white matter tracts. In addition, polysynaptic segmental reflexes could be elicited. Although usually absent in whole cord preparations, monosynaptic reflexes could invariably be elicited following hemisection, supporting the existence of an active crossed pathway producing presynaptic inhibition of Ia afferent terminals.

Once the preparation was proved successful, its advantage was further proved by using it to study the postnatal changes of the pharmacology of the mouse spinal cord. Intrinsic membrane properties are important in the regulation of motoneuronal output during such behaviours as locomotion. Activation of L-type calcium channels may be an essential component in the transduction of motoneuronal input to output during locomotion (Hounsgaard and Kiehn, 1989). Thus, experiments were conducted to investigate the post-natal development of L-type calcium channels in mouse motoneurones. Rhythmic bursting was produced by bath application of NMDA and strychnine to the isolated spinal

cord at various post-natal developmental stages. The L-type calcium channel blocker nifedipine was then added to the bath. Nifedipine has no effect on ventral root bursting in animals younger than postnatal day 6, but it reversibly and significantly reduces the amplitude of this activity in animals greater than postnatal day 7. The results demonstrate that the L-type calcium channel profile develops during early postnatal development. This is supported by the immunohistochemical evidence obtained in our laboratory demonstrating a change in the cellular profile of the α_{1C} and α_{1D} subunit of the L-type calcium channel during postnatal development. The density of these subunits increases with age and approximates the adult pattern by postnatal day 18. These studies demonstrate the applicability of this new preparation. With the availability of various transgenic mice, this preparation potentially should be very useful to the future elucidation of the mammalian spinal motor system.

ABBREVIATIONS

CNS	Central nervous system
CPG	Central pattern generator
DHP	1,4-Dihydropyridine
DMSO	Dimethyl sulfoxide
EMG	Electromyograph
ENG	Electroneurograph
EPSPs	Excitatory post-synaptic potential
FDA	Fluorescein diacetate
F-I relation	Frequency current relation
5-HT	5-Hydroxytryptamine
5-HTP	5-Hydroxytryptophan
HVA	High voltage activated
LDP	Locomotor drive potential
LVA	Low voltage activated
NMDA	N-methyl-D-aspartate
P	Postnatal
PI	Propidium iodide
TTX	Tetrodotoxin
VLF	Ventrolateral funiculus

INTRODUCTION

General review of spinal motor network

The spinal cord consists of a complex network of neurones which is capable of producing reflex responses and sophisticated motor behaviours such as locomotion. Walking in tetrapods requires the co-ordination of all four legs. In each leg, different muscles must contract and relax at the appropriate time. A great number of muscles are involved with the activity of each muscle carefully timed to the overall pattern of co-ordination (Grillner, 1981). Sherrington (1910) and Brown (1911) demonstrated that the spinal cord can generate some types of alternating motor activity. However, it took half a century to firmly establish that the mammalian spinal cord has the capacity to produce locomotion in the absence of both the brain and sensory inputs (reviewed by Grillner, 1975). It has been shown that a variety of vertebrates with transected spinal cord at cervical level can still produce locomotor movements even after the caudal part of the spinal cord has been isolated from the rest of the central nervous system including afferent feedback. The species studied include cat (Forssberg et al., 1980a; Forssberg et al., 1980b; Forssberg and Grillner, 1973), rat (Stelzner et al., 1975), chicken (Jacobson and Hollyday, 1982), turtle (Lennard and Stein, 1977), mudpuppy (Wheatley and Stein, 1992), dogfish (Grillner, 1994), lamprey (Grillner et al, 1981) and monkey (Fedirchuk, 1998).

The establishment that motor rhythms can be generated in the spinal cord has led to intensive investigation of its mechanisms. The network responsible for producing the

rhythmic alternating activity has been termed the central pattern generator (CPG) and its elucidation has been called "one of the most important problems in this field" (Grillner, 1981).

Our knowledge of the organization and mechanisms of this spinal CPG is still very limited. Even its nature is still under hot debate: some investigators claimed that the CPG of hindlimb is localised in one or two segments of the spinal cord (Cazalets et al., 1995) while others showed evidence that the CPG is distributed throughout the lumbar spinal cord (Cowley and Schmidt, 1997; Kjaerulff and Kiehn, 1996; Kremer and Lev-Tov, 1997). The first hypothesis on the organization of the spinal CPG was the so called "half-centre" theory proposed by Brown (1911). It assumes that two symmetric, mutually inhibitory interneuronal centres drive flexors and extensors respectively. Lundberg and colleagues revised this model and were able to simulate an alternative rhythm (Jankowska et al., 1967). By observing the treadmill walking behaviour of decerebrate cats, Grillner and his colleagues (1981) found that when one hind limb is prevented from moving on the treadmill, the other limb continues to step normally. They thus concluded that more than one CPG exists in the spinal cord. In addition even within one limb, the control of the individual muscle groups may be partly independent. For instance, the burst activity in one group of muscles may stop despite a maintained tonic activity in these muscles and continued bursting in other muscles (Grillner, 1981). Based on these experiments, Grillner suggested that the spinal cord CPG is composed of CPGs of each limb which in turn can be subdivided into several subunits that he called 'unit burst generators' (reviewed by Grillner, 1981). The burst generators were considered

composed of a network of specialised spinal interneurones, which would produce the bursting activity in certain small group of muscles. Although these two models are the primary models for spinal locomotor CPGs, other proposals have been made, including the ring model and the off-switch model and others. (reviewed by Grillner, 1981). Further, although presented as discrete models, they need not be exclusive (for extensive discussion, see Brownstone et al., 1994)

All of these early models assume that the CPG is composed of a group of neurones with fixed properties. As our knowledge of the spinal motor network increases, a hardwired locomotor CPG seems more and more unlikely (Getting, 1989). Instead, it is likely that the mammalian spinal motor network is a dynamic system similar to that of lower vertebrate as proposed by Harris-Warrick (1991). For example, in cat, single spinal interneurone can be involved in many different motor behaviours and their role changes accordingly (Baldissera et al, 1981; McCrea, 1992; Getting, 1989). Moreover, spinal interneurones are not a homogenous group: they have intrinsic properties that differ from each other and are likely to be modulated differently by descending and/or segmental mechanisms (Noga et al., 1995). Every neurone involved in the behaviour would be predicted to shape the final output to a certain extent. Full understanding of the CPG therefore requires a thorough understanding of the cellular properties of its individual components, including how each component is modulated during behaviour. Motoneurones, being the final output and the easiest identifiable component of the spinal motor network, have been investigated intensively, and are discussed further below.

The intrinsic properties of motoneurones are complex and help to shape the output of the spinal motor network

The spinal motoneurone was called the "final common path" by Sherrington (1906), for it is the final and only motor output of the spinal cord. All sensory and descending pathways involved in motor output converge on motoneurones and influence when and how the motoneurone will fire action potentials to produce muscle contraction. In the early 1950s, through a remarkable series of experiments using intracellular microelectrode recording of cat spinal motoneurones, Coombs, Eccles and Fatt (1955a,b,c) outlined the ionic conductances underlying action potentials. They also established the notion that the cell membranes of spinal motoneurones are passive and allow a "linear" summation of all the converging synaptic signals at the burst generating area, the initial segment of the axon. When the net excitatory current reaches a certain threshold, it will trigger an action potential. Stronger synaptic currents will results in a repetitive discharge of action potentials (also see Kolmodin and Skoglund, 1958).

In the 1960s, Granite, Kernell and co-workers conducted experiments investigating the repetitive discharge of motoneurones (Granite et al., 1966a,b; 1963a,b,c; Kernell,1965). They mimicked the synaptic input with intracellular current injection. The plot of firing frequency against the amount of current injected into the cell reflects the response of a motoneurone to a given input or its input-output relation (Kernell, 1965; Brownstone and Hiltborn, 1992). This frequency-current (f-I) relation was shown to be linear or bi-linear. The slope of this curve reflects the gain of the motoneurone. This slope depends on the intrinsic properties of the neurone, such as the input resistance and afterhyperpolariztion

(Granit et al, 1966a,b). For example, the smaller the cell, the higher the input resistance and the steeper the slope (Kernell, 1965). It was thought that the response of an individual cell to a given amount of synaptic input (or synaptic current) would always be the same. These results were assumed to apply to all cells in the central nervous system (CNS). The consequence was that complexity in the mammalian brain was believed to be attained by the connectivity of the nerve cells of similar static properties, rather than by the elaboration and modulation of their individual electrophysiological properties (reviewed by Llinas, 1988).

Today, it is known that many cortical, subcortical and spinal neurones do not behave in this "linear" or "bi-linear" fashion. Furthermore, These "basic electrophysiological properties" can be modulated. Even the spinal motoneurone, in some cases shows a non-linear input-output relation. Three examples of motoneurone non-linearity are the expression of plateau potentials, voltage-dependent excitatory postsynaptic potentials (EPSPs) and NMDA-induced voltage oscillations. These are each discussed in turn below.

The first description of non-linear behaviour in mammalian spinal motoneurones was the identification of "plateau potentials". Sherrington (1906) observed that the reflex response recorded in motor nerves sometimes outlasted the stimulus. This phenomenon was further studied in 1975 when Hultborn et al observed that a short train of brief stretches to the soleus muscle, in the decerebrate cat, triggered a sustained increase in the EMG activity. A reverberating loop located in the spinal cord was hypothesised to

explain this phenomenon. Such a reverberating loop would require a positive feedback reflex pathway. However, further investigation demonstrated that the prolonged excitability increase still appeared after the brief stretch stimulation after eliminating the feedback reflex pathway by sectioning the soleus nerve (Hounsgaard et al., 1986). Intracellular recordings directly from spinal motoneurones were used to clarify the origin of the long lasting excitability increase. As seen in the EMG and ENG, the motoneurone showed a long last firing which outlasted the stretch stimulation. Unexpectedly however, the injection of a short lasting hyperpolarising current into the motoneurone, stopped the firing and revealed that the underlying EPSP ended soon after the stimulus. After blocking the action potentials with depolarising current, it was shown that the brief stretch stimulus triggered a long lasting membrane depolarisation. This depolarisation was shown to be self-sustained, and a brief hyperpolarising current pulse injected into the cell would terminate it at any time. The argument that this sustained depolarisation to a brief synaptic input results from intrinsic membrane properties was further strengthened by single cell current injection. Instead of stimulation by a brief stretch which will activate many cells in the spinal cord, intracellular injection of a brief depolarising current was also shown to trigger a long-lasting depolarisation which could be terminated by a hyperpolarising current pulse (Hounsgaard et al., 1984). Furthermore, if a bias depolarising current was injected to produce low-frequency repetitive firing, a brief stimulation of Ia afferents resulted in sustained firing at a higher frequency. The excitability of motoneurones therefore could be shifted between two stable states by either brief current pulses or afferent stimulation. This membrane behaviour was termed "bistability". The sustained depolarised potentials were called "plateau potentials". The

plateau potentials and bistable firing behaviour could not be evoked after acute spinal transections, unless followed by intravenous injection of the serotonin precursor 5-hydroxytryptophan (5-HTP; Hounsgaard et al., 1988). It was further demonstrated that the generation of the plateau potential involves a region of negative slope conductance in the I-V relationship that is produced by a slowly inactivating inward current. This negative slope conductance was first described in cat motoneurones by Schwindt and Crill (1980) who later suggested that calcium might be the charge carrier underlying this inward conductance (Schwindt and Crill, 1984). Such a negative slope conductance is critical for the production of plateau potential, because it make the two stable zero current points in the I-V curve possible. Subsequent studies on turtle spinal motoneurones showed that serotonin-induced plateau potentials could be blocked by the L-type calcium channel antagonist nifedipine (Hounsgaard and Kiehn, 1989). The functional aspect of bistable properties of motoneurones was strengthened by studies of single motor unit recordings in unrestrained intact rats (Eken and Kiehn, 1989). Two stable firing rates were recorded from the rat soleus muscle when the animal stood quietly. Short stimuli to Ia afferents shifted the firing from low to high frequencies, while inhibitory skin afferents reversed this shift (Eken and Kiehn, 1989).

The second non-linear behaviour in mammalian spinal motoneurones is produced by the so called "voltage-dependent EPSPs"(Brownstone and Hultborn, 1992). Fast EPSPs are produced by the opening of ligand gated channels usually allowing passage of small cations (Na^+ and K^+) (Coombs et al., 1955a). As a rule, the amplitude of these EPSPs usually decreased as the membrane potential depolarises and approaches the equilibrium

potential of the EPSPs. However, in motoneurones in acute spinal cats treated with L-dopa and nialamide (which can produce a "pre-locomotor" or "locomotor" state; Jankowska et al., 1967), the EPSPs underlying the long-latency long-lasting flexor reflexes increase rather than decrease in amplitude as the membrane is depolarised (Brownstone et al., 1994). This phenomenon was also observed during MLR-induced fictive locomotion (Brownstone et al., 1994). During locomotion, the motoneurones receive phasic excitation and inhibition to produce what are called locomotor drive potentials (LDPs; Shefchyk et al., 1985). While the f-I relation is linear during the inhibitory phase of the locomotor cycle (Fedirchuk et al., 1998), the f-I relation is not linear during the excitatory phase (Brownstone et al., 1992). It was shown that during this phase, intracellular injection of depolarising currents did not increase the firing frequency while a very small amount of hyperpolarising current totally abolished the firing (Brownstone et al., 1992). Whole cell patch recording directly from spinal motoneurones of neonatal rat during neurochemical induced locomotion also showed that the amplitude of LDPs and rhythmic excitatory current increase with membrane depolarisation from -80mV to -40 mV (Hochman and Schmidt, 1998). These findings showed convincingly that during functional motor behaviour, the motoneurone input-output relation is not linear. This was studied further following action potential block with intracellular QX314 (a lidocaine derivative which blocks fast sodium channels). It was shown that there is a voltage-dependent enhancement of the excitation of the motoneurones by outputs from spinal locomotor centres (Brownstone et al., 1994). Extracellular iontophoresis of 2-APV (an NMDA receptor antagonist) could reduce this voltage-dependent effect (Brownstone et al, 1993), but the relative contribution of

NMDA receptors and L-type calcium channels to the production of this voltage dependent excitation has not been determined (Brownstone et al., 1994).

A third non-linear behaviour seen in mammalian spinal motoneurones is the NMDA dependent membrane potential oscillation. This was first reported in lamprey, where in the present of NMDA and the fast sodium channel blocker, tetrodotoxin (TTX), the membrane potential of motoneurones exhibited rhythmic oscillations (Wallen and Grillner, 1987). It was shown later that neonatal rat spinal motoneurones also showed membrane potential oscillations in the presence of NMDA and TTX (Hochman et al., 1994). This phenomenon was further investigated and it is shown that while some cells do not exhibit this oscillatory behaviour initially, bath application of 5-hydroxytryptamine (5-HT) could induce this phenomenon and 5-HT receptor antagonist could block the oscillation (MacLean et al., 1998). These oscillations were shown to require the voltage dependent properties of the NMDA receptor (Nowak et al., 1985) because removal of magnesium ions from the bath would abolish the oscillations (MacLean et al., 1998). In addition, it was shown that both the NMDA receptor blocker 2-APV and L-type calcium channel antagonist nifedipine were shown to be able to block the NMDA-induced oscillation in adult turtle spinal motoneurones, suggesting the involvement of NMDA receptors and the L-type calcium current (Guertin and Hounsgaard, 1998), as well as 5-HT receptors.

The above three phenomena observed in mammalian spinal motoneurones may be due to similar underlying mechanisms or there may be different ionic conductance

involved in each one of them. It seems that the non-linear properties of spinal motoneurones play essential roles in shaping the spinal motor output (Brownstone et al., 1991, 1994). More evidence is needed to elucidate the precise ionic conductances underlie these non-linear behaviours.

The neuronal voltage-gated calcium channels

The neuronal voltage-gated calcium channels are one of the most diversified classes of all voltage-activated ion channels and have been studied extensively. As discussed above, it seems that voltage-gated calcium currents may play an important role in regulating motor output.

The existence of calcium permeability in excitable cells was first described by Fatt and Katz in the 1950s. However, for many years, the biophysical and biochemical analysis of Ca channels were seriously hampered by experimental problems, particularly because of their low density (Tsien, 1983). The introduction of the gigaseal patch clamp recording technique in 1981 by Neher, Sakmann and colleagues channels (Hamill et al., 1981) made it possible to record activity from individual calcium (Nowycky et al., 1985). Using this technique, researchers soon found out that voltage gated calcium channels exist in almost all excitable cell membranes (Hille, 1992). It has been demonstrated that these channels play two major roles: an electrogenic role, as in the generation of the plateau phase of the action potential in cardiac cells or the plateau potentials of spinal motoneurones as discussed above; and a metabolic or messenger role by transiently and possibly locally raising the

intracellular calcium concentration. The intracellular calcium ion is one of the major intracellular signalling molecules used by almost all cells.

The diversity of the calcium channels was first suggested by Llinas and colleagues (1981), who showed that at least two types of voltage-gated calcium channels exist in the mammalian CNS: "low threshold" and "high threshold" channels. Carbone and Lux (1984) demonstrated corresponding low voltage-activated (LVA) and high voltage-activated (HVA) calcium currents in chick dorsal root ganglion neurones. 1,4-Dihydropyridine (DHP) was found to either enhance or block the high voltage gated calcium conductance. Tsien's group confirmed these two types of channels and demonstrated a third class of calcium channels which open at high voltage but is insensitive to DHP. They named the LVA channel T-type calcium channel ("T" for transient), the long-lasting HVA channels L-type ("L" for long lasting) calcium channel and the third group N-type ("N" for non-L non-T) calcium channels (Nowycky et al., 1985). The subsequent finding that a toxin from the venom of pacific cone shell *Conus geographus*, ω-conotoxin GVIA blocked the N-type channels, made it possible to isolate an additional type of calcium channel. A third type of HVA calcium channel was first found in cerebellar Purkinje cells, and was thus named the P-type calcium channel. These channels activated at high voltage, and were insensitive to both DHP and ω-conotoxin GVIA (Llinas et al., 1992). A peptide toxin from the venom of the funnel web spider *Agelenopsis aperta*, ω-agatoxin IVA was shown to selectively block this channel.

The molecular dissection of neuronal Ca^{2+} channels was initially made possible by the biochemical and molecular characterisation of the skeletal muscle L-type calcium channel. This channel is a heteroligomeric complex consisting of five subunits (α_1, $\alpha_2\delta$, β, and γ), with the α_2 and δ subunits being derived from the same gene and proteolytically cleaved (Campbell et al., 1988; Dunlap et al., 1995). In a number of testing systems the α_1 subunits of several L-type channels have been shown to function as both the voltage sensor and calcium selective pore (Takahashi and Catterall, 1987). This led Snutch et al (1990) to identify four different α_1 subunits in the mammalian nervous system and named them $\alpha_{1A\text{-}D}$. It was agreed that the α_{1A} gene encodes the above-mentioned P-type calcium channel, the α_{1B} gene encodes the N-type calcium channel and α_{1C} and α_{1D} genes both encode L-type calcium channels. Interestingly, the insertion of α_{1A} subunits into *Xenopus* oocytes yielded yet another type of voltage-gated calcium channel that is different both kinetically and pharmacologically from the P-type calcium channel. This was named Q-type calcium channel. Furthermore, many researchers reported that in various neurones, after blocking all T-, L-, N-, and P/Q- type channels, there remained some calcium current. The calcium currents that can not be blocked by any of these known calcium-channel blockers are named R-type (R for residual) calcium channel (Dunlap et al, 1995).

The in vitro preparations available for the study of spinal motor network

The study of calcium channels is best carried out in *in vitro* preparations. The advantage of such preparations include: (1) the extracellular environment can be easily altered; and (2) neurochemicals can be applied directly to the bath without the concerns of their effects on other systems or of delivery to the CNS across a blood-brain barrier.

As mentioned above, the traditional preparation for the spinal motor system is the in vivo cat. Several preparations have been developed including the in vitro lower vertebrate as well as mammalian spinal cord. The *in vitro* lamprey spinal cord was developed in the end of 1960s (Rovainen, 1967a,b) and was used for the study of spinal motor systems after Grillner et al (1981) successfully induced locomotion by bath application of NMDA. The first in vitro mammalian spinal cord was introduced by Otsuka and Konishi in 1975 using the neonatal rat. It was rarely used (Walton and Fulton, 1986a; Fulton and Walton, 1986b) until 1987 when Kudo, Yamada and Smith, Feldman respectively induced locomotor activity from the isolated neonatal rat spinal cord following bath application of NMDA. This model has since been studied extensively and has provided much valuable information on the mechanisms of the spinal motor networks as well as motoneurone intrinsic properties (Schmidt, 1994; Cowley and Schmidt, 1994b; Cazalets et al., 1995; Bracci et al., 1996; Kjaerulff and Kiehn, 1996; Kjaerulff and Kiehn, 1997; Kremer and Lev-Tov, 1997; MacLean et al., 1997; Bracci et al., 1998; Hochman and Schmidt, 1998). This preparation has been

limited in most cases to the study of brainstem and spinal cord mechanisms in the first week of postnatal life. However, as discussed in detail below, both the rat and the mouse are born in relatively immature states. At birth, they are unable to perform mature behaviours such as over-ground locomotion. The contribution of spinal cord factors which develop in the postnatal period to the production of mature motor outputs has not been adequately addressed.

Post-natal development of spinal motor networks in mammals

The development of spinal motor networks depends on the maturation and functional integration of many subsystems. Profound developmental changes have been found to occur during the early postnatal period in the rat spinal cord. For example, using retrograde and anterograde labelling technique, it has been shown that the corticospinal tract reaches the lumbar spinal cord by the fifth postnatal day and further growth and myelination occurs during the subsequent three weeks (Joosten et al., 1987; Schreyer and Jones, 1988). Other descending pathways thought to be of importance to the functional behaviour of the adult animal such as the serotonergic and adrenergic pathways also develop postnatally. In the case of serotonin, it has been shown that the 5-HT immunoreactivity within the cord increases with time until the adult pattern and density is obtained by 14 days in the cervical cord and 21 days in the thoracic and lumbar cord (Bregman, 1987). The maturation followed rostral to caudal and ventral to dorsal gradients. Studies of specific motor pools such as quadriceps femoris motoneurones (Q-MNs) showed that 5-HT immunoreactive fibres were presented in the ventral horn of the lumbar spinal cord at birth (Tanaka et al., 1992).

The density of these fibres increased with age. At birth only a few 5-HT terminals and varicosities showed close apposition with about half the Q-MNs examined. At 5-days postnatally, such close apposition was found in all Q-MNs. By the end of the first two postnatal weeks, the 5-HT innervation to Q-MNs appeared to have been established (Tanake et al., 1992)

The noradrenergic system has been studied using tyrosine hydroxylase as a marker. At postnatal day 1, tyrosine hydroxylase immunoreactive fibres were observed only in that portion of the ventral horn medial to Q-MN pools. Subsequently, tyrosine hydroxylase immunoreactive fibres increased and were distributed throughout most of the gray matter at postnatal day 14 (Tanaka et al., 1996).

Besides the development of the descending pathways, the connection between motoneurones and muscles also changes in the early postnatal period. Early muscle innervation is polyneuronal. The adult pattern of mononeuronal innervation is established during the second half of the second postnatal week (Jansen and Fladby, 1990).

In considering the above developmental data, it is clear that to a certain extent, the adult spinal motor network must develop postnatally. However, it is also clear that the spinal CPG exists in neonatal rats and are able to generate rhythmic output like swimming (Bekoff et al, 1979) and locomotion (Kudo and Yamada, 1987). Although there are studies on prenatal development of spinal motor system (Iizuka et al., 1998;

Kudo et al., 1998), the significance of postnatal developmental changes on the production of functional motor output has not yet been investigated.

Westerga and Gramsbergen (1990) studied the development of locomotion in rat with video recording and computer analysis. They found that from postnatal day 11 (P11) onwards, a gradual transition occurs from crawling with the ventral body surface in contact with the floor to free walking. The adult pattern of walking appeared at P15 when they found a change in stride frequency and length. While the weak muscle strength may account for some of the early characteristics of immature locomotion, the change of stride frequency and stride length could also be explained by the development of the spinal motor system. As discussed above, voltage-gated calcium channels play an important role on motoneurone output, giving the motoneurone at least some of its non-linear properties. There is evidence which demonstrates that the calcium channel profile develops in the embryonic and early postnatal period. McCobb et al (1989) showed that during embryonic development of chick motoneurones, the HVA calcium current increases while the LVA calcium current decreases. Mynlieff and Beam (1992b) revealed the same phenomenon in cultured postnatal mouse spinal motoneurones, and Umemiya and Berger (1994) reported this in postnatal rat hypoglossal motoneurones. Taken together with the behavioural development during the same period, it is reasonable to consider there might be a relation between them. To date, such a relationship has not been demonstrated.

Rationale

The traditional preparation for the study of spinal motor systems has been the in vivo cat spinal cord. Over the last decade, much has been learned about networks in the neonatal rat spinal cord. Why, then, should a different mammalian preparation be developed when the building blocks are actively being discovered in the neonatal rat spinal cord? In fact, the motor activity studied in neonatal rat spinal cords may not accurately reflect the adult spinal motor processes. It would therefore be useful to have an in vitro preparation in which one could study both the neonatal and mature motor networks and the mechanisms underlying the production of functional motor output. Some progress has been made in utilising older animals including mice (Bagust and Kerkut, 1981; Fulton, 1986; Biscoe and Duchen, 1986; Somjen and Czeh 1989), rats (Long et al., 1989), and hamsters(Bagust et al., 1985). Increasing myelination and spinal cord size in the older animals is thought to impede oxygen diffusion into the centre of the cord; therefore, most of these investigations have used hemisected preparations. The hemisected cord is adequate for the study of intrinsic neuronal properties and some simple reflex circuits, but it can not be employed in the study of spinal cord motor networks, especially those involving left-right co-ordination. To date, success of isolated non-neonatal whole spinal cord preparations has been reported only in the hamster (Bagust and Kerkut, 1987). To date, no locomotor-like activities has been reported using this preparation.

The primary motive, which underlies the development of the in vitro mouse spinal cord preparation described in this thesis, has been to make feasible the study of spinal

motor networks in vitro from animals which have mature motor functions. In addition, the use of mice in the application of transgenic technology has widened interest in this species. However, although smaller than that of rats, it seems to be more difficult to maintain a viable mouse spinal cord *in vitro*, in particular for the study of motor systems. It has been reported that in spinal cords taken from mice weighing more than 15 g, it is "difficult to evoke much activity"(Bagust, 1993). Droge and his colleagues were the first to utilise an in vitro neonatal mouse spinal cord and hindlimb preparation to study spinal motor patterns (Hernandez et al., 1991; Tao and Droge, 1992; Droge and Tao, 1993). They monitored the spinal motor activity by recording EMGs from tibialis anterior and Gastrocnemius muscles. However, the data presented showed no convincing rhythm of any kind and no relations between the activities of the two antagonist muscles. Recently, another group reported spontaneous rhythmic activity in the in vitro neonatal mouse spinal cord (Bonnot et al., 1998; Bonnot and Morin, 1998). However, no pharmacological induction of rhythmic activity was shown in the spinal cord without spontaneous activity. Rhythmic activity similar in quality to that shown in the isolated neonatal rat spinal cords has not been shown in the mouse.

In the first part of this thesis, an *in vitro* whole spinal cord preparation in mice at a stage which is defined here as being "motor functionally mature" was developed. Over ground walking and weight bear are used as criteria for the maturation of the spinal motor system. The mouse spinal cords of functionally mature age (older than postnatal day 9) were successfully isolated. Both descending and segmental reflex

pathways remain functional in this preparation. By using a combination of neurotransmitters that differs from those used to evoke rhythmic alternating activity in the neonatal rat spinal cord, locomotor-like rhythmicity could be elicited in spinal cords from motor functionally mature mice.

As discussed earlier, the intrinsic membrane properties of the motoneurones are critical to the final motor output. With the "functional mature" mouse spinal cord preparation at hand, the effects of nifedipine, a L-type calcium channel blocker, on a spinal motor output in animals of different age groups can be studied. Bath application of strychnine has been shown to produce synchronous motor bursting in many preparations including lamprey (Harris-Warrick, 1984), cat (Noga, 1993), neonatal rat (Cowley and Schmidt,1995; Braci et al., 1997) and neonatal mouse (Droge and Tao, 1993). This strychnine induced synchronous activity could also be observed in the "functionally mature mice"(Jiang et al., 1999). The long lasting activity and the similarity in the quality of the activity between neonatal and older animals made this an ideal rhythm for the developmental aspect of this study. It is demonstrated here that this rhythm is nifedipine sensitive in animals older than postnatal day 7, but nifedipine insensitive in the younger animals. This not only parallels the maturation of the motor pattern seen in the behaving animal, but also adds evidence to the hypothesis that, in the study of at least some aspects of spinal motor networks, animals past the neonatal stage must be used. These have been published in Jiang et al.(1999a) and submitted in Jiang et al.(1999b).

MATERIAL AND METHODS

Eighty-six balb/c mice of either sex were used. The date of birth was designated as postnatal day 1(P1) and animals used were from P1 to P22. Intraperitoneal injection of ketamine (100 mg/kg) and xylazine (30 mg/kg) were used to anaesthetise the animal in part to reduce the excitotoxic effect mediated by NMDA receptors during dissection.

After anaesthetisation, the mouse was submerged in ice water with the head up in the air (Somjen and Czeh, 1989) with oxygen blown into the nose of the mouse through a plastic tube. After approximately 3-4 minutes, the animals were decapitated. The spinal column, pelvic girdle and hind limbs were immediately cut free, immersed in ice cold dissecting solution (see below) and pinned to the bottom of a Sylgard-lined petri dish with the ventral surface up. The dissecting solution was continuously bubbled with 95% O_2/ 5% CO_2. The tissue was initially kept cold by placing the dish on a bed of ice. Under a dissecting microscope, the spinal cord from the mid-cervical to sacral levels was exposed by removing the vertebral bodies. The dura mater was then cut open and the anterior spinal artery was dissected and removed. The remaining spinal canal was stretched open with dissecting pins so that spinal roots could be visualised. The spinal roots were then cut close to both sides of the spinal cord except for the lumbar roots, which were cut just proximal to the dorsal root ganglia. The spinal cord was floated out of the spinal canal by dissecting all further connective tissues. The tissue block was removed from the dish leaving only the

spinal cord. The dorsal dura mater and any remaining adherent connective tissues were carefully removed. The cord was then secured to the bottom of the dish by dissecting pins through the dorsal roots. The whole procedure took less than 30 minutes. After sitting on ice for another 5 minutes, the bath solution was replaced with cold (4°C) recording solution and the dish was removed from the ice bed and allowed to acclimatise to room temperature (20^0C-22^0C) for at least one hour prior to recording.

Dissecting and recording were both performed in one 30ml Sylgard-lined dish. To minimize excitotoxic effect during dissection, the solutions were carefully modified with the replacement of most sodium ions with sucrose and high concentration of magnisum. The compositions of the two solutions were as follows (in mmol/l). *Dissecting solution*: sucrose 188, NaCl 25, KCl 1.9, $MgSO_4$ 10, $NaHCO_3$ 26, NaH_2PO_4 1.2, Glucose 25. *Recording solution*: NaCl 127, KCl 1.9 – 3.9, NaH_2PO_4 1.2, $CaCl_2$ 1.5-2.4, $MgCl_2$ 1.3, $NaHCO_3$ 26, Glucose 10.

In two animals, the viability of the spinal cord was examined using a live cell-dead cell assay (Jones and Senft, 1984). Fluorescein diacetate (FDA, Sigma; 25μg/ml) can be used as a marker for live cells, as it passes through the membrane and is then hydrolysed by intracellular esterases to produce fluorescein (Jones and Senft, 1984). Propidium iodide (PI, Sigma; 3μg/ml) can be used as a marker for dead cells as it passes through damaged membranes and interacts with the DNA and RNA to

fluoresce red (Jones and Senft, 1984). After a typical experiment lasting 5 – 8 hours, the lumbar spinal cord was isolated and embedded in 2.5% agar in regular aCSF. After allowing the agar to gel, a block of tissue in agar was mounted in a Leica VT 1000E vibrating microtome and cut into 200μm slices. The slices were transferred to a 5 ml dish filled with oxygenated regular aCSF. Both FDA and PI were added to the dish and incubated for 10 minutes. The slices were then washed with oxygenated regular aCSF at least 3 times. Photomicrographs using fluorescein (green, live cell) or rhodamine (red, dead cell) epifluorescence were used to examine the viability of the spinal cords.

NMDA, 5-HT and dopamine were prepared as 10 mM stock solutions with distilled water. They were stored in 1 ml vials in the freezer. For each experiment, only fresh vials were used. Strychnine was prepared at 0.125 mM stock solution and stored in a 4^0C refrigerator. Nifedipine was dissolved in ethanol, dimethyl sulfoxide (DMSO) or acetone. The final vehicle concentrations were less than 1:1000 in the bath and controls were performed by adding the same amount of vehicles into the bath before or after the application of nifedipine. .

Drugs were applied directly to the bath through a pipette. Concentrations given are the final concentration in the bath solution. Washout was achieved by the complete exchange of the bath contents with regular recording solution at least three times at 2 to 5 minute intervals to allow diffusion of the drug out of the tissue.

Suction electrodes were used to record activity from the lumbar ventral roots. The diameter of the electrode tip ranged from 80 μm to 160 μm. The signals were high pass filtered between 1 and 30 Hz and low passed filtered at 3 kHz. They were then digitised using a Digidata 1200B and Axoscope software (Axon Instruments) at sampling rates of 2 – 20 kHz and saved in the hard disk of a Pentium computer. Data were then analysed using Axoscope software and Custom software developed in our Department. Statistics was performed using Microsoft Excel as outlined in the text.

In the developmental study, ventral root activity was elicited by bath application of 10 μM strychnine (Sigma) and 10 μM NMDA (Sigma). Control data were recorded 40 minutes after application of these chemicals. The effects of nifedipine were recorded 30 minutes after its application. Washout recordings were made 15 minutes after the solution exchange.

Segmental reflexes were elicited by stimulating the dorsal lumbar roots using constant current stimulation (0.2-1 mA, 200 μsec, < 0.5 Hz; Isolator-11 Stimulus Isolation Unit; Axon Instruments) through bipolar tungsten electrodes (World Precision Instruments). Descending pathways were tested by inserting the bipolar tungsten electrode through the pia of the ventrolateral funiculus (VLF) of the mid-thoracic cord.

Longitudinal midline hemisection of the spinal cord was performed in some experiments. This was achieved by using a dissecting pin to gently and repeatedly scratch the ventral midline of the spinal cord until the two halves of the spinal cord fell apart. After hemisection, one half of the spinal cord was pinned down to the bottom of the dish with the cut surface up. The recording and stimulation proceeded after 30 minutes recovery time.

RESULTS

Behavioural Observations

Balb/c mice (n=30) from P7 to P15 were observed to determine the age at which their motor systems are functionally mature, as defined above. At P8, 5 of 5 mice were unable to weight bear and walk with their abdomens suspended, even in response to stimuli such as tail-pinching. At P9, 4 of 6 animals could walk for a few (3 – 4) steps with their abdomens off the ground in response to tail pinch. At P10, 4 of 4 mice could walk in response to a mild touch to the skin. All 15 mice older than P10 could walk independently.

Live cell dead cell assay

FDA and PI have been used as indicators for live cells and dead cells respectively (Jones and Senft, 1984). FDA-positive live cells fluoresce green under blue light, while in PI-positive dead cells, the nucleus fluoresces red under yellow light. Fig. 1 shows an example of a 200 μm slice from a 20 day old mouse lumbar spinal cord 6 hours after sacrifice. This demonstrates that the majority of cells even following blocking and sectioning the spinal cord remain viable. Note that the distribution of the live cells are quite even throughout all laminae. There is high background labelling with PI (figure 1B), but only the small nuclear labelling represents dead cells. As described in Methods, this labelling was performed following blocking and sectioning of the spinal cord into 200 μm slices. It is likely that the number of dead cells overestimates the number prior to this relatively stressful procedure.

Descending and segmental reflexes

The VLF has been shown to contain an important descending pathway for the initiation of locomotion in decerebrate cat (Steeves et al., 1980) and neonatal rat (Magnuson and Trinder, 1997). For this reason, experiments were conducted to demonstrate that this pathway remains viable in this preparation. Stimulation of the VLF in the mid-thoracic cord produced short- and long-latency responses in the lumbar ventral roots (Fig. 2A). The short latency response appears at about 3 msec after stimulation and was very constant. We consider it as monosynaptic response while the long latency response as polysynaptic response. Both monosynaptic and polysynaptic responses were elicited in the ipsilateral ventral root (Fig. 2A). Following bath application of strychnine, the same stimulation produced an enhanced ipsilateral polysynaptic response as well as a contralateral polysynaptic response (Fig. 2B).

Segmental reflexes were also elicited. Fig. 3 shows an example of segmental reflexes before and after (Fig. 3A) bath application of strychnine. These reflexes typically had latencies of about 20 msec – far too long to be considered monosynaptic. In only 1 of 6 preparations in which segmental reflexes were investigated could a constant short latency response be elicited. In the remaining 5 animals, only polysynaptic reflexes were recorded. There was no difference in the age or preparation of these latter spinal cords compared with the one in which monosynaptic reflexes were recorded. Bagust and Kerkut (1987) reported that in isolated hamster spinal cords, only polysynaptic

reflexes could be elicited in some of the older animals. The fact that monosynaptic reflexes have been demonstrated in reports from hemisected spinal cords led us to hemisect 3 of the 5 whole cords in which no monosynaptic reflex was produced. In all three animals, monosynaptic reflexes were seen following hemisection (Fig. 3B). In none of these animals, however, could the monosynaptic reflex be produced at lower stimulating strengths than the polysynaptic reflexes.

Neurochemical induced activities

Pharmacological activation of spinal cord networks was produced by the application of various neuroactive substances to the bath. Activity could be recorded for at least 12 hours, and would sometimes appear to improve over time. In the *in vitro* neonatal rat preparation, bath application of serotonin and NMDA consistently produces alternating rhythmic (locomotor-like) activity in the ventral roots (see Cowley and Schmidt, 1994; Kudo and Yamada, 1987). In our preparation, bath application of serotonin (5-HT; 10 - 100 μM) induced tonic ventral root activity with the amplitude increasing in a dose-dependent fashion (n=28). N-methyl-D-aspartate (NMDA; 5 - 50 μM) elicited irregular bursting in the ventral roots (n=22). However, no alternating rhythmic activity could be produced by 5-HT alone (up to 100 μM) or by NMDA alone (up to 50 μM). The combination of 5-HT and NMDA (n=27) at concentrations used in the neonatal rat preparations failed to elicit rhythmic activity (n=27). High concentrations of these two neurotransmitters (60 μM 5-HT and 50 μM NMDA) inconsistently produced some rhythmic activity as shown in the example in Fig. 4

(n=2). The M-type cholinergic receptor agonist muscarine (40 μM; n=2) could elicit some burst activity but no regular rhythm.

To elucidate whether the failure to induce locomotor like activity by 5-HT and NMDA in functionally mature mice is due to development or differences in species, neonatal mice were used. In these mice (up to postnatal day 5), the application of various concentrations of these two drugs does not evoke regular rhythmic alternating activity either (Fig 5A left panel). However, the application of dopamine (50 μM), in addition to serotonin (10 μM) and NMDA (5 μM) produced very consistent (about 2 hours), rhythmic alternating locomotor-like activity (Fig. 5, right panel; 2/2 animals). The combination of any two of the three chemicals failed to induce rhythmic activity (Fig. 5, left panel). It appears that, therefore, there are differences in the neurotransmitter profiles in the spinal cord between rat and mouse. The combination of NMDA, 5-HT and dopamine could also induce rhythmic alternating firing at P9 (n=1), and in 4/10 animals older than P10 (functionally mature). In 2/4 of these animals, sustained alternating rhythmic activity could be elicited from bilateral ventral roots (Figure 6). In the remaining 6/10 animals, tonic activity only was produced.

Development of L-type calcium channels

Similar to the neonatal rat preparation (Cowley and Schmidt, 1995), the glycine receptor antagonist strychnine together with NMDA consistently produced low

frequency synchronous bursting bilaterally (n=10; Fig. 7). In most cases, the burst intervals were irregular. Increasing the dose of NMDA usually increases the firing frequency, however, this has not been systematically investigated. The synchronised bursting activity elicited by bath applied NMDA and strychnine was used to study the development of the spinal motor network. Although the repetitive bursting produced was not always as regular as that seen in the neonatal rat (Cowley and Schmidt, 1995), this combination consistently produced qualitatively similar bursting in the ventral roots of mice of different ages (Figure 5). Once the strychnine-induced rhythm stabilised, 15 μM nifedipine was added to the bath, and the influence on the bursts observed.

In mice less than 5 days old, bath application of nifedipine had little or no noticeable effect on the bursting (Fig.8A). In mice older than postnatal day 7, however, the strychnine-induced bursting was dramatically reduced in amplitude (Fig. 8B). Because of the irregularity of the bursts, changes in inter-burst interval were never convincingly demonstrated: the number of bursts over five minute periods was not significantly altered following application of nifedipine. Washout of nifedipine partially reversed the reduction in amplitude (Fig.8B). Application of vehicle alone did not result in any change in amplitude or frequency of the bursts.

To quantify the reduction in amplitude, the data were digitally rectified and low pass filtered (5 Hz), with the area under the curves taken to reflect the total number of

action potentials in each burst. Ten consecutive bursts in each animal were measured and averaged prior to, during, and after washout of nifedipine (Fig. 9). In animals at postnatal day 7 or greater (n=7), nifedipine significantly reduced this area ($p < 0.01$; ANOVA, single factor analysis). This effect was partially reversible. There was no significant effect of nifedipine in younger animals (n=4).

The rectified, low pass filtered data were also used to assess whether this change in burst area was due to a change in amplitude, burst duration, or both. Bursts in 100 second periods were averaged and overlaid (Fig. 10). In 6/7 animals older than 7 days, there was a reversible reduction in mean peak amplitude with nifedipine. This was never observed in animals younger than 6 days. In 6/7 of the older animals, there was also a reduction in burst duration. In 2 of these 6 animals, this reversed completely with washout (Fig. 10), whereas in the other 4 there was incomplete or no reversal following washout. In one animal, there was reduction in peak amplitude but not in burst duration, and in one there was a reduction in burst duration but not in peak amplitude.

To confirm that the actions of nifedipine are through its effect on calcium channels, the extracellular calcium concentration was altered. In normal calcium concentrations (2.5 mM), all animals over day 7 showed statistically significant reduction of the area under the curve. Conversely, with 'low' extracellular calcium (1.5 mM), although bursting activity could be produced, it was not affected by nifedipine (Table 1). The

effects of low calcium on the ventral root motor output however has not been systematically investigated.

TABLES

Table 1: The reduction in burst area by nifedipine depends on the calcium concentration. The calcium concentration is 1.5 mM in the low and 2.5 mM in the normal calcium groups. Nifedipine effect refers to the reduction of the average area under the curve of 5 consecutive bursts before and after the application of nifedipine (NS: not significant, $P>0.5$ in student's t-test).

Age	Low Calcium			Normal Calcium		
	Number	Nifedipine effect		Number	Nifedipine effect	
		NS	p<0.01		NS	p<0.01
P2 - P5	1	1	0	4	4	0
P7 - P14	4	4	0	7	0	7

DISCUSSION

This study describes an isolated *in vitro* mouse spinal cord preparation which can be used to study spinal cord networks in a motor functionally mature mammal and the utilisation of this preparation to study the postnatal development of the spinal motor network.

Motor funtionally mature mice

The term "motor functionally mature" mice is defined as being those mice which can weight bear and walk with the ventral surface of the body suspended above the ground. In balb/c mice, this occurs at postnatal day 10. This is comparable with rats which can weight bear and walk over ground by postnatal day 11 (Westerga and Gramsbergen, 1990). In rat, this time line has been proved by many studies to be important for the development of spinal motor network. For example, the innervation of muscle from lumbar motoneurones changes at the second half of the second post natal week (Jansen and Fladby, 1990); Walton et al (1992) found rats of P8-13 are most sensitive to the manipulation of enviroment and they called this time the "critical period" for the development of motor behaviour. The ability to bear weight itself may be determined by many maturation processes other than that of the spinal motor network such as the maturation of muscle strength, and the change of muscle innervation from polyneuronal to mononeuronal and etc. But studies on rat has shown that the time of weight bearing coincides with many qualitative changes from neonatal to mature. For instance, descending pathways such as the cortical spinal

tract, reaches the rat lumbar spinal cord by postnatal day 5 (Joosten et al., 1987; Schreyer and Jones, 1988). Serotonergic and noradrenergic pathways also mature at the first two postnatal weeks (Tanaka et al., 1992, 1996). Serotonergic receptors undergo significant developmental changes during the same period (Tally et al., 1997). The pattern of locomotion of rat changes from neonatal crawling to adult like walking in the same period of time (Westerga and Gramsbergen, 1990). No systematic analysis of motor behaviour and postnatal development of the spinal motor system is available yet in mice. With the evidence found in rat and the similarities between these two species, it seems likely that the ability to bear weight and perform overground locomotion is a cornerstone of the maturation of spinal motor system.

However, this is not to say that motor maturation is complete at the time the animals can weight bear and walk. For example, in rat, there is a significant change in stride length and frequency between day 14 and day 15 (Westerga and Gramsbergen, 1990). The change is considered to be due to the further maturation of the spinal motor network We have therefore defined the observable point of being able to weight bear and walk overground as being "motor functionally mature".

The necessity of this preparation

Isolated *in vitro* mammalian spinal cords were first described in the neonatal rat (Otsuka and Konishi, 1974). This preparation has since been used extensively to study spinal cord networks (Kudo and Yamada, 1987; Smith et al., 1988; Cowley and Schmidt, 1995; Cazalets et al., 1995; Bracci et al., 1997; Kremer and Lev-Tov, 1997;

Hotchman and Schmidt, 1998; MacLean et al., 1998), but seems to be limited to rats less than about 7 days of age – animals which are not yet capable of weight bearing or walking. The concern about the developmental changes raises some questions regarding the data obtained from these neonatal animals.

For more than a decade, researchers have been trying to develop a mature in vitro mammalian spinal cord preparations. To date, hemisection of the spinal cord seems necessary to keep older in vitro spinal cord alive, and has been performed on various animals including rat (Long et al., 1989), hamster (Bagust, 1993) and mouse (Biscoe and Duchen, 1986; Fulton, 1986a; Bagust, 1993). But the use of hemisected spinal cord preparations precludes the study of evoking bilateral co-ordinated activity such in locomotion.

Bagust and Kerkut (1987) were successful in developing a whole isolated spinal cord preparation from juvenile hamsters weighing up to 40 grams. However, only dorsal root-ventral root and dorsal root-dorsal root reflexes were demonstrated in this preparation. No co-ordinated rhythm was shown. In fact, even isolated whole spinal cords from neonatal mice have rarely been used (Smith et al., 1993; Bonnot et al., 1998a). The preparation we described here thus supplements the existing animal models for the study of mammalian spinal motor systems. The use of mice will also enable the use of transgenic animals in the future (Smith et al., 1993).

Methodology of the preparation

The fact that hemisected but not whole spinal cords of older animals could survive in vitro suggests that the ability of oxygen and nutrients diffusion is vital. The use of mouse in this preparation was because they are the smallest rodents and the spinal cord is the thinnest. During dissection, the major insult to the spinal neurones is hypoxia and excitotoxicity due to excess intracellular calcium. To minimise this problem, several measures were taken: (1) ketamine, a blocker of NMDA receptor, is used as an anaesthetics; (2) hypothermia and oxygenation is then used to decrease the metabolic need and increase oxygen supply; (3) A modified dissecting solution was used that contains low sodium, no calcium and high magnesium ions. The major concern after dissection is the diffusion of both oxygen and nutrients in to the centre of the spinal cord. To improve diffusion, the anterior spinal artery is removed during dissection so that its exposed intra spinal cord branches could possibly increase diffusion.

The live cell /dead cell assay

FDA has been shown to be able to perfuse through cell membranes into the cytoplasm where it is oxidised by the enzymes of live cells. The oxidised FDA fluoresces green. PI can not penetrate the healthy cell membranes but will stain the nucleus of dead cells bright red (Jones and Senft, 1985). They were used to evaluate the viability of the preparation anatomically. The live cell/dead cell assay was performed on two animals. The results were quite similar. The photograph of the assay from the older animal (22days postnatally) is shown (Fig 1). It shows that a

large number of cells stained positive with FDA. It is not surprising that there were many cells stained positive with PI considering the extended experimental procedures of blocking and slicing. Moreover, the FDA stained live cells are distributed evenly throughout all the laminae of the spinal cord suggesting adequate diffusion was achieved.

The spinal reflexes

The segmental spinal reflex pathways are important units of the spinal motor networks (Baldessera et al., 1981; McCrea, 1992; Jankowska, 1992). In this preparation, stimulation of the dorsal root can induce segmental reflexes on the ipsilateral ventral root. Reflexes on contralateral ventral roots could be induced after bath application of strychnine, a glycine receptor antagonist, which also enhances the ipsilateral reflexes. The ventrolateral funiculus (VLF) has been shown to be essential in mediating the MLR-induced locomotion in cat (Steeves et al., 1980), and stimulation of it could induce locomotor-like activity in neonatal rat (Magnuson and Trinder, 1997). In our preparation, stimulation of VLF could induce responses in the bilateral lumbar ventral roots.

In 5 out of 6 preparations, a constant and short latency (presumably monosynaptic) reflex could not be elicited by dorsal root stimulation in the whole cord. This is similar to the data from juvenile hamsters (Bagust and Kerkut, 1987) where monosynaptic reflexes could be elicited only in 3 of 9 animals studied. In studies using hemisected cords, monosynaptic reflexes were seen (Bagust et al., 1985; Fulton,

1986). To explore this phenomenon further, spinal cords were hemisected longitudinally. Following this, monosynaptic reflexes appeared (Fig. 3B). These observations support a crossed tonic inhibitory control of the monosynaptic reflex, however more direct evidence is needed to confirm this possibility.

Locomotor-like activity

Locomotor-like activity can be induced following bath application of different neurotransmitters such as 5-HT, NMDA and Ach in the isolated neonatal rat spinal cord (Cowley and Schmidt, 1994a; Kudo and Yamada, 1987; Smith et al., 1988). Dopamine has been shown to be able to elicit rhythmic activity in neonatal rat spinal cord (Kiehn and Kjaerulff, 1996), but the rhythm is slow and irregular. In this preparation, the locomotor-like activity could not be induced by 5-HT, and/or NMDA, but could with the combination of 5-HT, NMDA and dopamine (Fig. 5, 6). This cocktail does not change between neonatal and old mice. However in older animals, the rhythm is much more difficult to be induced. There are several possible explanations for the lower success rate in functionally mature animals. Firstly, the mature spinal cord is likely more vulnerable to traumatic and hypoxie insult because of the thicker pia and higher degree of myelination. Secondly, recording ventral roots activity may not accurately reflect the actual hind limb flexor-extensor activity. Even in the neonatal rat, where it has been shown that the L2 and L3 ventral roots are mainly extensor and the L5 root is flexor, there is still significant variations in this and the use of ventral roots recordings to monitor locomotor activity is therefore considered to be "not reliable" (Cowley and Schmidt, 1994b, but also see Kiehn and

Kjaerulff, 1996). There is no information available regarding the anatomical distribution of motor axons in the lumbar ventral roots of mice. From our own experience, there are definitely variations among each cord in terms of the appearance of the ventral roots. In many cords, the lumbar ventral roots are not symmetrical, with one side having a typical thick L4 ventral root and a thin L5 ventral root and the other side only have one thick conjoint ventral root. Ventral root recordings also showed that sometimes when one side fired rhythmically, the other side fired tonically. This could be explained by the fact that there were both flexor and extensor axons in the same ventral root.

The postnatal development of L-type calcium channels

The early developmental changes of calcium channels in motoneurones were first reported by McCobb et al (1989), who showed that during embryonic development of chick motoneurones, the HVA calcium current increases while the LVA calcium current decreases. Mynlieff and Beam (1992b) revealed the same phenomenon in cultured postnatal mouse spinal motoneurones, and Umemiya and Berger (1994) reported this in postnatal rat hypoglossal motoneurones. Given that plateau potentials have not been demonstrated in neonatal rat motoneurones, and the above mentioned postnatal development of calcium channel profiles, experiments were undertaken to examine the potential role of L-type calcium channels in the production of rhythmic motor output in the neonatal versus the motor functional mature mice.

Application of strychnine has been shown to induce bilateral synchronous bursting in flexor and extensor nerves in lamprey (Cohen and Harris-Warrick, 1984), fetal rat spinal cord (Kudo, 1991), neonatal rat spinal cord (Cowley and Schmidt, 1995) and the adult cat (Noga et al., 1993). Strychnine induced synchronous bursts could also be elicited in neonatal and functional mature mice (Fig. 7). The nature of this strychnine induced synchronous rhythm is still under investigation. It has been postulated that this rhythm is caused by the removal of reciprocal inhibition of the two half-centre CPG (Cohen and Harris-Warrick, 1984; Cowley and Schmidt, 1995; Kudo, 1991; Noga et al., 1993). This activity persisted for long periods of time, and was qualitatively similar in different age groups. It was therefore used to examine the development of contribution of L-type calcium channels to a rhythmic output. The disadvantage of this rhythm in our preparation is that it was often not very regular, thus making changes in frequency difficult to evaluate.

As mentioned above, L-type calcium channels are important for the spinal motor output in adult turtle for they mediate the so-called plateau potentials. While these plateau potentials also presents in adult cat and rat, the contribution of L-type calcium channels is still not clear due to the limitation of the in vivo preparations. On the other hand, however, the plateau potentials have never been shown in the neonatal rat preparation. With the evidence that the voltage-gated calcium channels profiles changes during early postnatal period, we hypothesised that the L-type calcium channels might not be adequate to contribute significantly to the total motor output, but that they will play more and more important roles as the animals mature.

In mice older than postnatal day 7, nifedipine consistently reduced the area of ventral root bursts (fig. 8). If the role of L-type calcium channels were to produce plateau potentials, it would be expected that the blocking of L-type calcium channels would decrease the duration of the ventral root bursting. This reduction was seen in 6/7 of the animals studied, in the other animal, only the amplitude was decreased. The observed reduction in ventral root burst amplitude in response to nifedipine (6 of 7 animals) could be because L-type calcium channels enhance the recruitment of motoneurones by central networks, or that they increase the frequency at which the motoneurones fire in response to a given input (Brownstone and Hultborn, 1992).

There are some concerns about the specificity of nifedipine at the dose of 15 μM (Hume, 1985; Yatani and Brown, 1985; Jones and Jacobs, 1990). In the whole spinal cord preparation, however, the concentration of nifedipine that actually reached the neurones also depends on diffusion. The actual local concentration is not known but could clearly be lower than the bath concentration. In guinea pig brain stem slice preparation, Hsiao et al (1998) used 10 μM nifedipine while in turtle spinal cord slice preparation, Hounsgaard et al (1988) used up to 20 μM nifedipine to block L-type calcium channels. Further, as myelination increases with age, it may be more difficult for the drug to diffuse through the spinal cord of older animals. That nifedipine exerted its actions preferentially on the older, motor functional mature spinal cord, supports observed differences independent of possible drug diffusion problems. In addition, the effect of nifedipine in functionally mature mice is calcium dependent (Table 1). Thus the observed actions are likely due to its effect on L-type calcium channels. To confirm the time-dependent development of L-type calcium channel, immunohistochemical techniques were performed in our lab with antibodies against

two neuronal L-type calcium channel α_1 subunits, α_{1C} and α_{1D}. The results showed that both these two subunits developed in the early postnatal period and support the electrophysiological data presented here.

These data which showed that L-type calcium channels develop dramatically during the first two weeks of postnatal life in mice and become significant component in determine the final motor output also corresponds with the development of descending serotoninergic pathways and spinal serotonin receptors, as well as the time of overground locomotion. Taking all these together it is likely that, both the spinal network and its modulation is far from mature at these early stages, even though the basic motor networks seem to be present and active in embryonic and neonatal rats and mice (Cowley and Schmidt, 1994a; Kjaerulff and Kiehn, 1997; Bonnot et al., 1998a; Bonnot and Morin, 1998b).

Using the functionally mature mouse spinal cord preparation, as described here, the developmental changes of L-type calcium channels during the early postnatal period can be addressed. Similar studies could also be done to investigate the role on motor output of other voltage-dependent ion channels or different receptors of various neurotransmitters. The availability of many transgenic mice could also be used to study the effects of single gene knockouts to the functional motor behaviours during early postnatal periods. This preparation thus holds great promise for the study of the mammalian spinal motor network.

SUMMARY

The present study describes a new in vitro mammalian whole spinal cord preparation in the "motor functional mature" balb/c mouse. Using a live cell/dead cell assay technique, it is shown that in the spinal cord isolated from animals as old as postnatal day 22, a large number of spinal cells remains alive after 6 hours of experiments and after the extended procedure of slicing and staining. The distribution of live cells is even throughout all laminae of the spinal cord. Descending and segmental reflexes could be elicited from these preparations. Bath application of various neurochemicals could induce tonic firings on the lumbar ventral roots. One important finding of the present study is that the induction of alternating rhythmic activity (the locomotor-like activity) in mouse requires the presence of dopamine in addition to 5-HT and NMDA. The developmental change of spinal L-type calcium channels was studied using this preparation. It was shown that the contribution of L-type calcium channels in spinal motor output increases with age in the early postnatal period. This preparation will be useful to the future study of mammalian spinal motor networks.

Figure **1**. Live cell-dead cell assay in the ventral horn of a 200 μm slice taken from a 22 day old mouse spinal cord after a 6 hour recording session. Fluorescein diacetate labels the cytoplasm of live cells green (**A**), while propidium iodide stains the nucleus of dead cells red (**B**). Scale bar is 20 μm.

A. Fluorescein diacetate

B. Propidium iodide

Figure 1

Figure **2**. Descending reflexes. Each trace shows five superimposed sweeps. stimulation of the right mid-thoracic ventrolateral funiculus (VLF; 1 mA, 0.2 msec) produces mono- and polysynaptic responses in the right L2 ventral root (lower trace) of a 15 day old mouse spinal cord, but no response contralaterally (upper trace; **A**). Following addition of 10μM strychnine (**B**), the ipsilateral reflex is enhanced, and a polysynaptic reflex is seen contralaterally in response to the same stimulation. Scale bar is 10 msec.

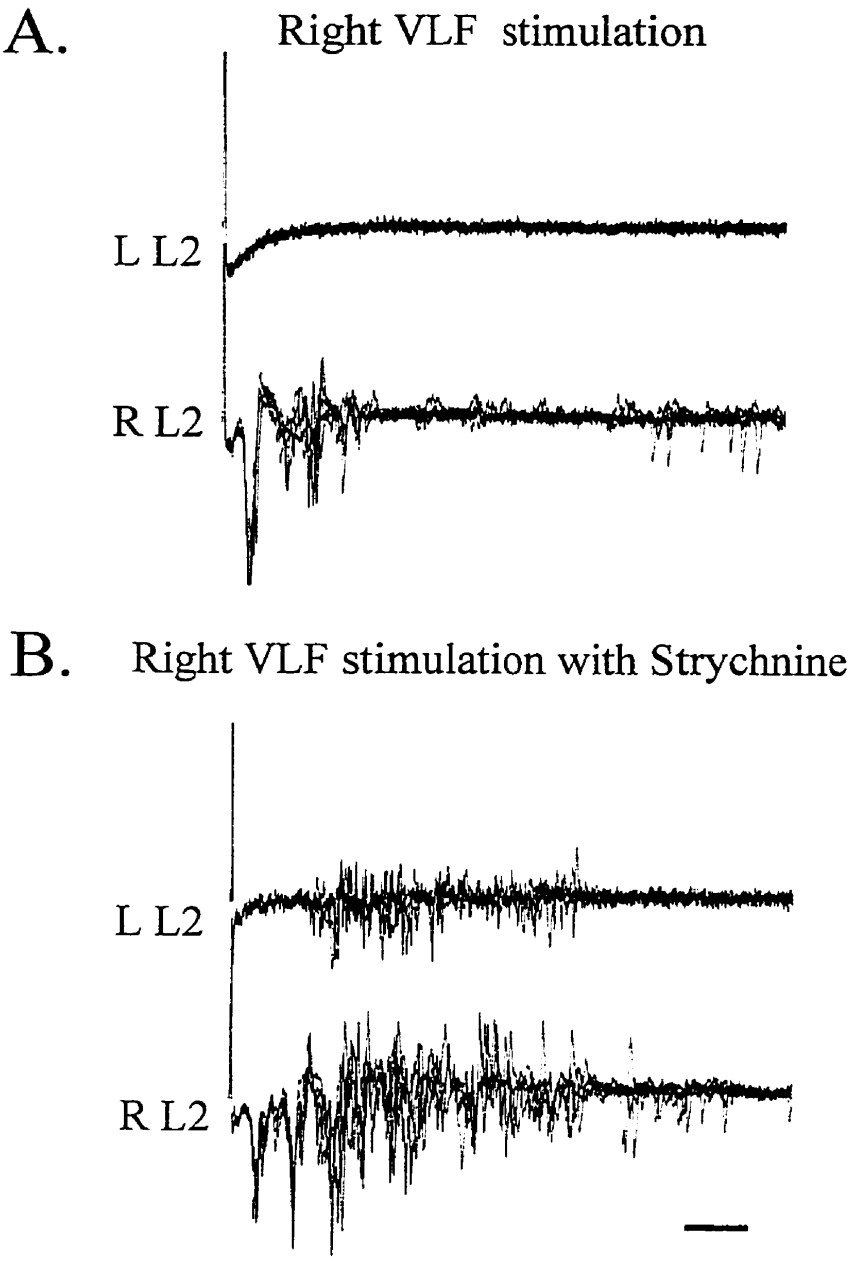
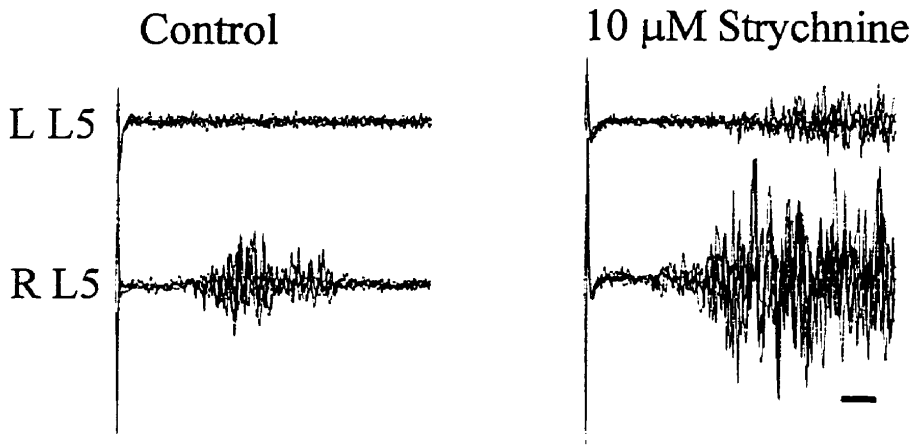

A.
Right VLF stimulation
L L2
R L2
B.
Right VLF stimulation with Strychnine
L L2
R L2

Figure 2

Figure 3. **A**. Fourteen day old mouse. Stimulation of the right L5 dorsal root (0.2 mA, 0.2 msec) produces a polysynaptic response ipsilaterally. Following the addition of 10 μM strychnine, this response is enhanced and a contralateral polysynaptic response is seen. **B**. Fifteen day old mouse. Stimulation of the ipsilateral L5 dorsal root (1 mA, 0.2 msec) in the whole cord produces a polysynaptic response in the right L5 ventral root (upper trace). Following hemisection, a short-latency response (presumable monosynaptic) is seen at the same stimulation strength. Scale bar: 10 msec in **A;** 5 msec in **B**.

A. R L5 dorsal root stimulation



B. R L5 dorsal root stimulation

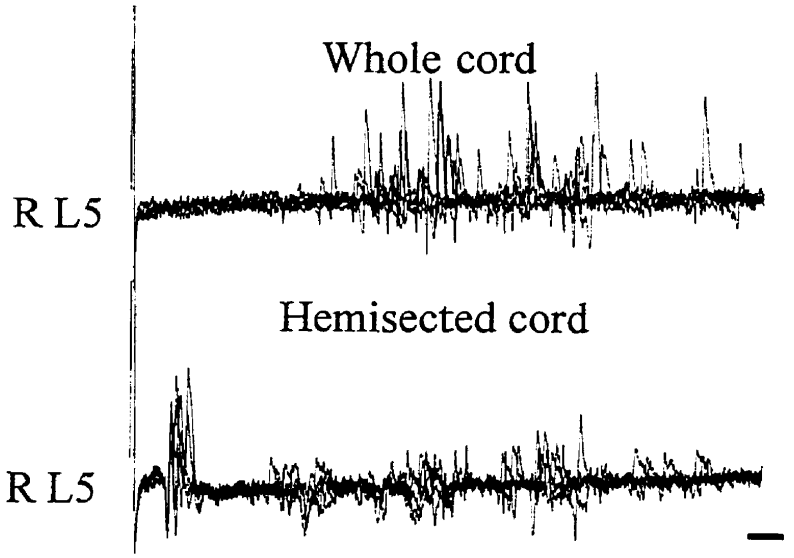


Figure 3

Figure **4**. Drug-induced activity. Sixty micromolar 5-HT and fifty micromolar NMDA elicits tonic activity in left L3 (upper trace) and rhythmic activity in right L3 (lower trace) in a 15 day old mouse. Washout and reapplication of the drugs reverses the activity. Scale bar: 4 seconds.

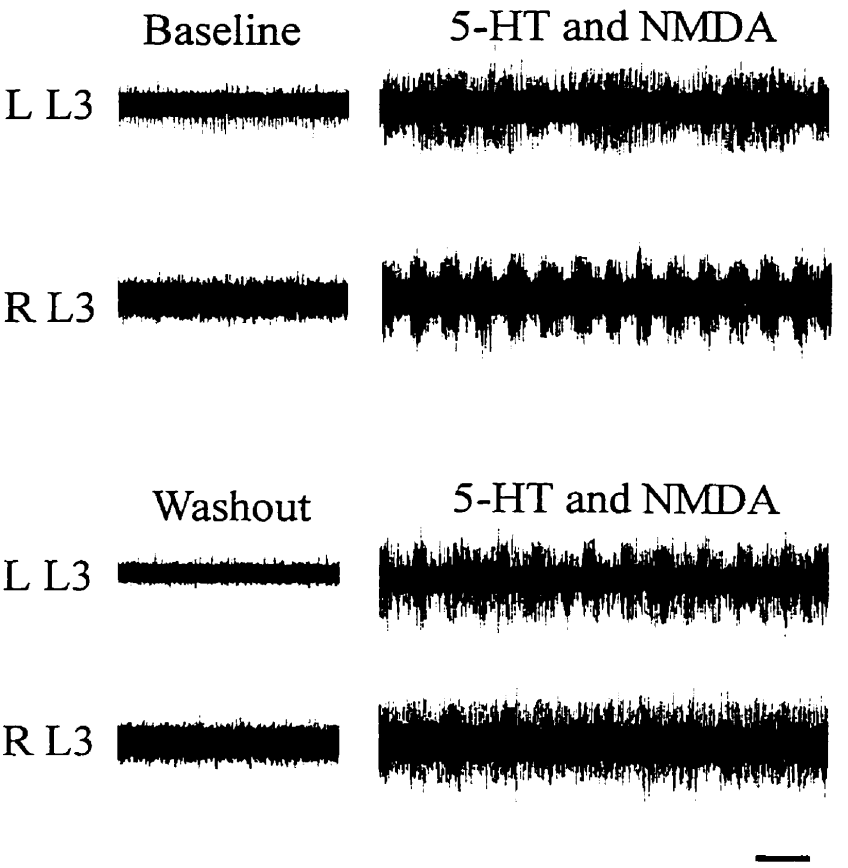


Figure 4

Figure **5**. Locomotor-like activity induced by a combination of dopamine (50 μM), serotonin (10 μM) and NMDA (5 μM) in a 5-day old mouse spinal cord. To induce rhythmic alternating activity, it is necessary to have all three substances present. All solutions were washed out with aCSF between **A**, **B**, and **C**. Scale bar is 30 seconds.

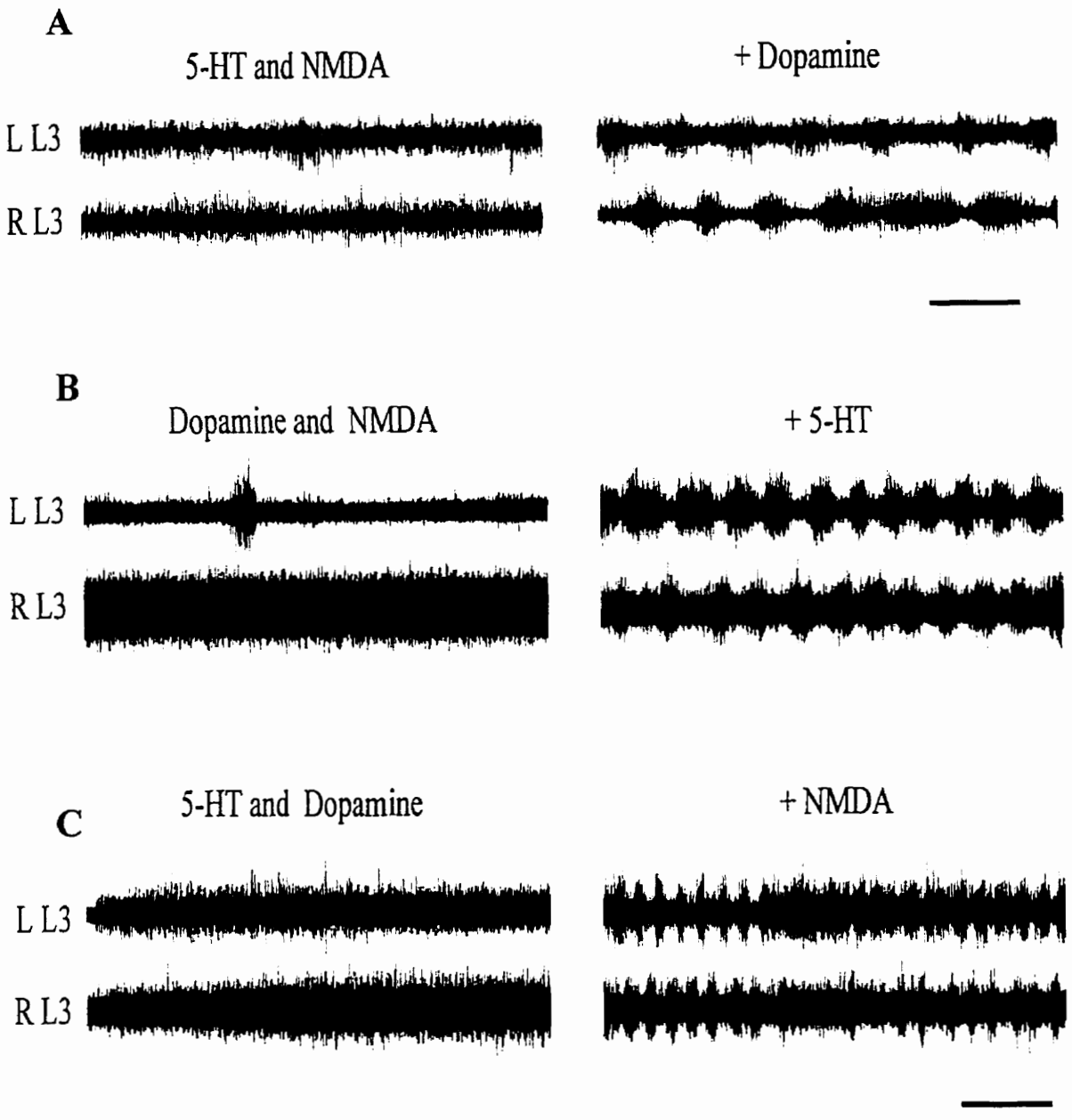

A
5-HT and NMDA
+ Dopamine
L L3
R L3
B
Dopamine and NMDA
+ 5-HT
L L3
R L3
C
5-HT and Dopamine
+ NMDA
L L3
R L3

Figure 5

Figure 6. Locomotor-like activity in a 12-day old mouse spinal cord produced by application of dopamine (50 μM), serotonin (20 μM), and NMDA (5 μM). A. The rhythmic activity is sustained. Traces have been rectified and integrated. B-1. A short portion of the raw trace shown in A. B-2. Same trace as shown in B-1 after rectification and integration. Scale bar is 20 secs in A and 4 seconds in B.

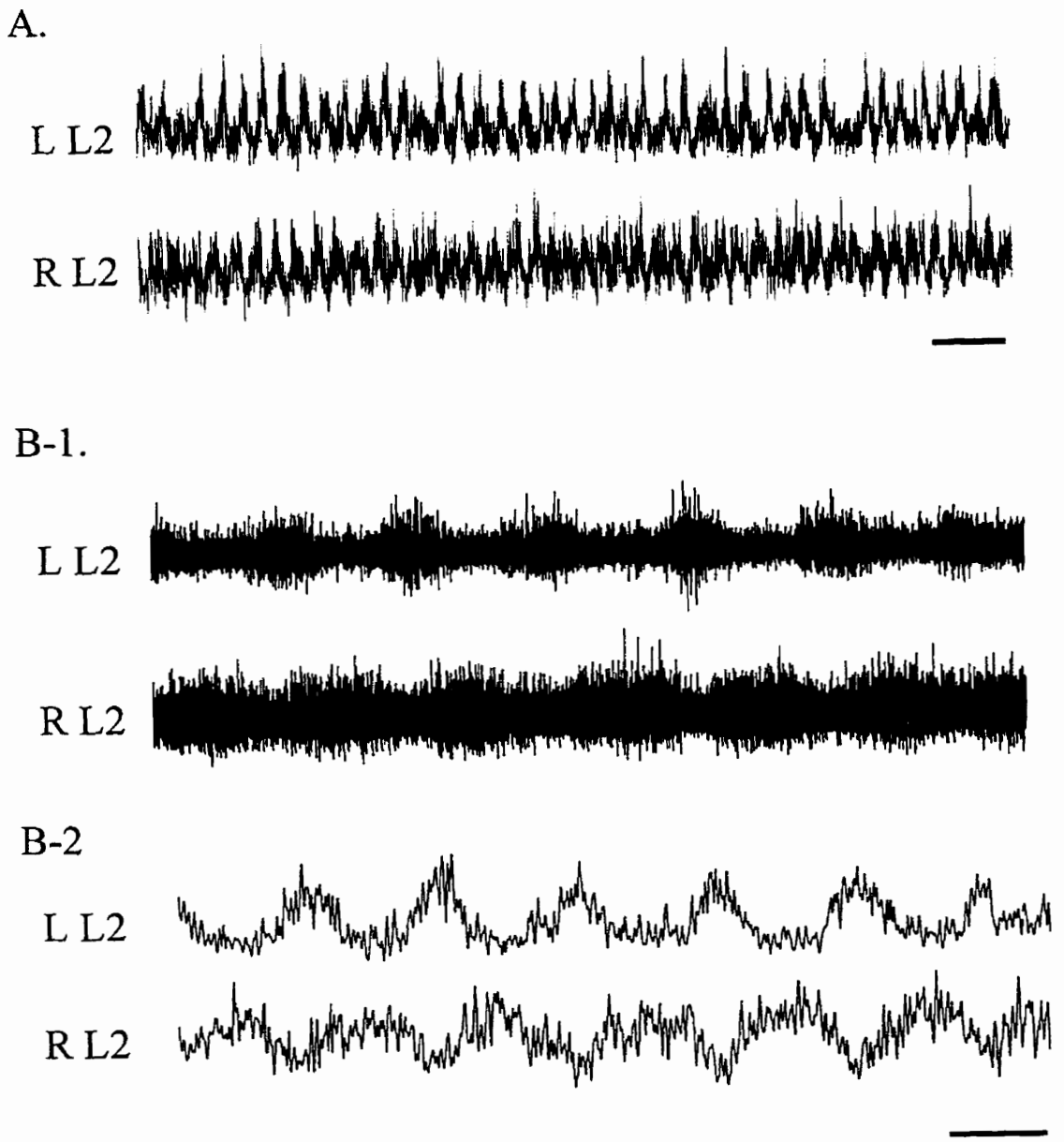


Figure 6

Figure 7. Drug induced activity. Synchronous activity in bilateral L5 ventral roots in a 15 day old mouse after bath application of 10 μM strychnine and 10 μM NMDA. Scale bar is 5 seconds.

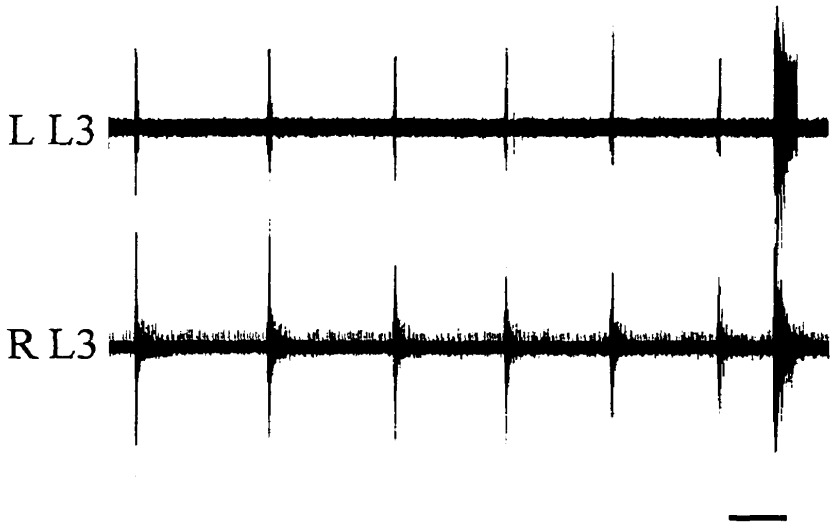


Figure 7

Figure 8. The effect of nifedipine on the strychnine-evoked rhythm recorded in the ventral roots of the in vitro mouse spinal cord at postnatal days 2 (A) and 9 (B). The rhythm was produced by application of strychnine (10 μM) and NMDA (10 μM). Note the lack of effect of L-type calcium channel blockade in the younger animal but significant reversible reduction in ventral root discharges in the older animals. Scale bar: 20 seconds.

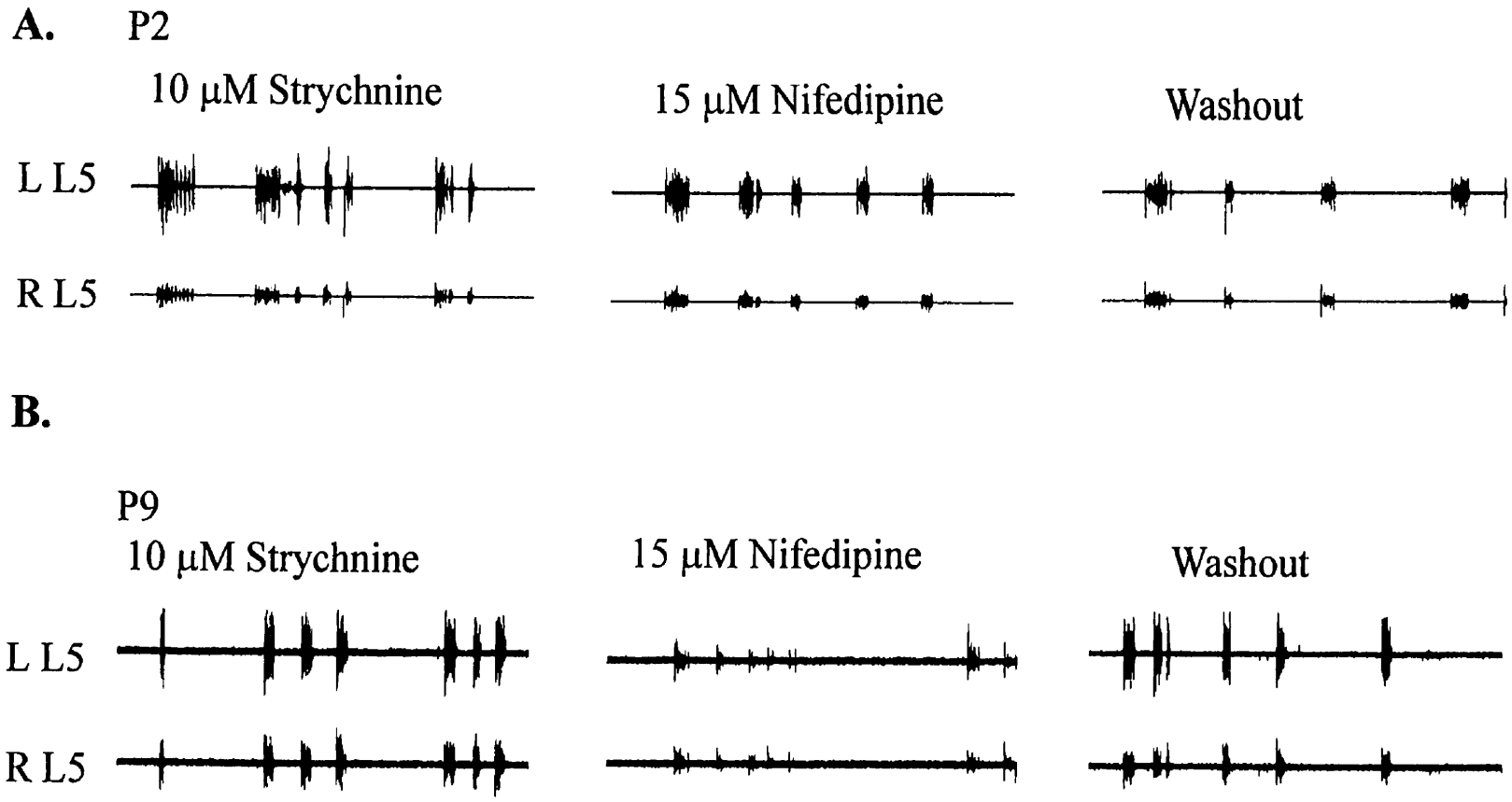

A.
P2
10 μM Strychnine
15 μM Nifedipine
Washout
L L5
R L5
B.
P9
10 μM Strychnine
15 μM Nifedipine
Washout
L L5
R L5

Figure 8

Figure 9. The mean burst area is reduced by nifedipine in animals over 7 days old ($p < 0.01$, indicated by *; Single factor ANOVA). The area was calculated after digital rectification and low-pass filtering (5 Hz), taking the mean of 10 consecutive bursts in each animal. Following washout, there was no significant difference in the mean burst area compared to control.

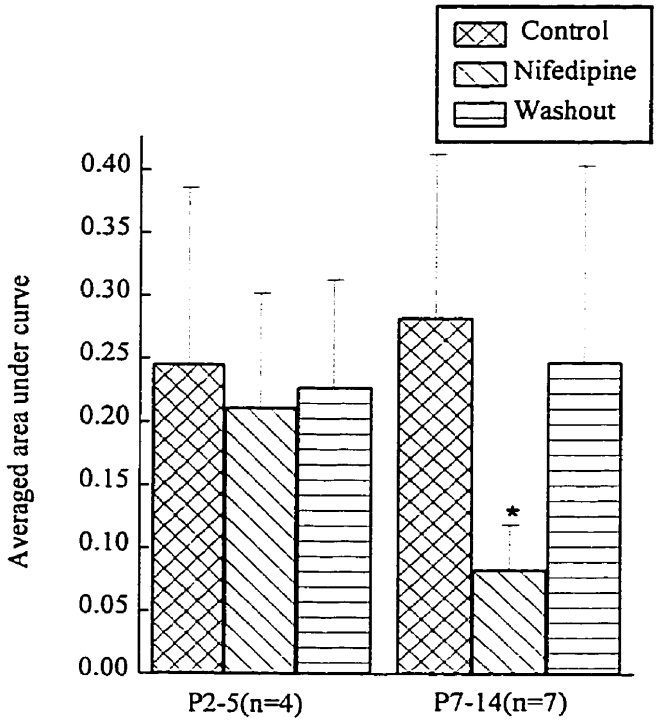

Control
Nifedipine
Washout
Averaged area under curve
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
P2-5(n=4)
P7-14(n=7)

Figure 9

Figure 10. The overlap of bursts before, during and after application of nifedipine. The burst is an average of events over 100 seconds superimposed then rectified and integrated. The scale bar is 0.5 second.

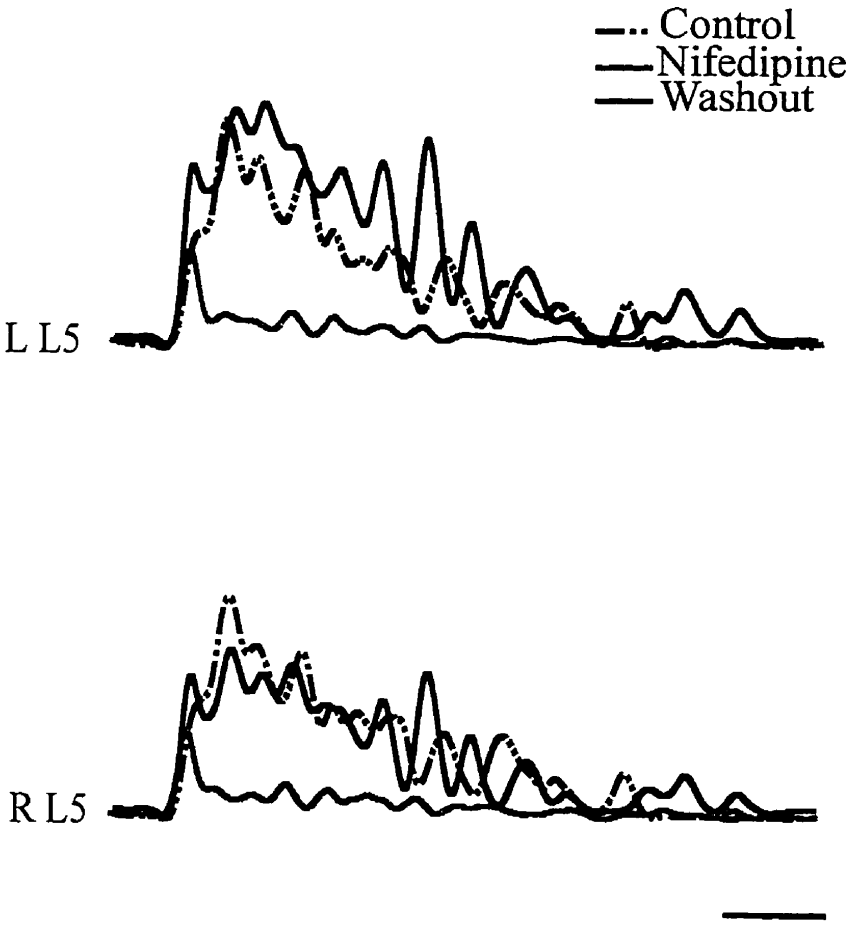


Figure10

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