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EFFECTS OF CHOLINERGIC AND NORADRENERGIC AGENTS ON LOCOMOTION IN THE MUDPUPPY

by

Mei Fok 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the Degree of Master of Science

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Edmonton, Alberta

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7 Mar 2002

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I wish to dedicate this thesis to my beloved mother and Norbert who care for me so very much and stand by me whenever, whatever and wherever.

Abstract

Some neurotransmitters act consistently on the central pattern generator (CPG) for locomotion in a wide range of vertebrates. In contrast, acetylcholine (ACh) and noradrenaline have various effects on locomotion in different preparations. The roles of ACh and clonidine, an α_2-noradrenergic agonist, have not been studied in amphibian walking, so we examined their effects in an isolated spinal cord preparation of the mudpuppy (*Necturus maculatus*). This preparation contains a central pattern generator that produces locomotor activity when N-methyl D-aspartic acid (NMDA), an excitatory amino acid, is added to the bath. The addition of carbachol, a long acting ACh agonist, to the bath disrupted the walking rhythm induced by NMDA, while not changing the level of activity in flexor and extensor motor neurons. Adding clonidine had no effect on the walking rhythm. Physostigmine, an ACh-esterase inhibitor, disrupted the walking rhythm, presumably by potentiating the effects of endogenously released ACh. Atropine, an ACh antagonist that binds to muscarinic ACh receptors, blocked the effects of carbachol, indicating that the action is mediated by muscarinic receptors. Neither carbachol, nor atropine nor clonidine induced locomotion in a resting spinal cord preparation. We conclude that ACh disrupts locomotion in the mudpuppy by a muscarinic action, but clonidine has no effect. The reasons for the differences in action of ACh and clonidine in this and other preparations are discussed.

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GENERAL INTRODUCTION

INTRODUCTION

The ability to move about with distinctive patterns is one of the characteristics of animals. Fish swim, frogs hop, cats and human beings walk and run. These various forms of locomotion are essential for survival because animals move from one place to the other to seek a better environment, e.g. where food is abundant or oxygen level is appropriate, and to get away from undesirable surroundings, e.g. toxins, predators, etc. What is even more interesting is that there is no need for an animal to have inputs from higher centres whenever a movement occurs, because locomotion is repetitive and rhythmic due to presence of a central pattern generator (CPG). Central pattern generators are neuronal ensembles capable of producing the basic spatiotemporal patterns underlying 'automatic' movements in the absence of peripheral feedback. CPG is involved in locomotion (Nishimaru and Kudo, 2000), chewing (Smith and Denny, 1990, Nakamura and Katakura, 1995), feeding (Straub and Benjamin, 2001), respiration (Smith and Denny, 1990, Gdovin et al.1999), and vomiting (McClellan, 1983, Fukuda et al.1999). These circuits, called CPGs, are believed to receive tonic

input and generate rhythmically alternating sets of commands. CPG can be represented by a neural network model in which there is dependence of some muscles upon sensory feedback and other inputs.

THE ROLE OF CENTRAL PATTERN GENERATOR (CPG) IN LOCOMOTION

Using acute spinal and deafferented cats, Brown (1911, 1914) was the pioneer who put forward the concept of "half-center model" to hypothesize how rhythmicity in locomotion could be produced by neuronal networks in the spinal cord of the cat. He suggested that antagonistic muscle groups (flexors and extensors) were activated by each half-center, which have connections with mutually inhibitory effects to each other. The end result is, when one half-center is active, the other one is not.

The neuronal generation of vertebrate locomotion has been extensively studied in the lamprey. In the lamprey locomotor network (Fig 1-1), the forebrain, brain stem, and spinal cord all contribute to the control of locomotion. The reticulospinal (RS) neurons are glutamatergic and excitatory. They activate all classes of interneurons. The excitatory interneurons (E) can activate all types of neurons in the spinal cord: 1) The inhibitory glycinergic interneurons (I) which inhibit all classes of neurons on

the opposite side of the spinal cord. 2) The lateral neurons (L), which inhibit interneurons on the same side of the spinal cord. 3) The motoneurons (M) which make nicotinic cholinergic connections and activate the muscles of the trunk. The stretch receptor neurons are represented as SR-E (excitatory) and SR-I (inhibitory). In the brain stem, excitatory inputs are sent to the RS neurons via three routes: 1) a cutaneous afferent (Trigem.), 2) mesencephalic locomotor region (MLR), and 3) ventral thalamus (VTH). The basal ganglia also plays a role by sending inputs to VTH (Grillner et al. 1995, 1998).

Roberts et al. (1998) outlined the spinal circuits that control swimming in the young *Xenopus* tadpole (a simple vertebrate). The relative simplicity of the amphibian tadpole nervous system has been utilised as a model for the mechanisms underlying the generation and development of vertebrate locomotion. Their work revealed the glycinergic, glutamatergic, and cholinergic input to spinal neurons during swimming. As shown in figure 1-2, mechanoreceptors (RB) activate sensory pathway interneurons (dl and dlc), which is the equivalent of dorsal horn in higher vertebrates. These will in turn excite the neurons of the CPG bilaterally. Commissural interneurons (c) bring about alternation of activity through glycinergic inhibition on the contralateral side. Decending interneurons (d) excite neurons on the ipsilateral side. Motoneurons (mn) activate the muscles of the

trunk and have central nicotinic cholinergic connections to give positive feedback to the CPG. In addition to this central activation by acetylcholine, glutamate, which is released by premotor excitatory interneurons, exerts its excitatory effect by its action on NMDA and AMPA receptors. Roberts et al. (1998) pointed out that there were areas of uncertainty (denoted as question marks (?) in Fig 1-2) in the spinal circuits to account for: 1) unclear functions of dl interneurons, 2) unclear functions of KA neurons which have GABA activity. For the brain circuit, there are 3 ways of neural projections into the hindbrain to affect operations of the CPG: 1) when the light intensity drops, pineal photoreceptors (p) are activated, and these excite the pineal ganglion cells (pg). These in turn excite diencephalic/mesencephalic (d/md) descending interneurons which have connections to the hindbrain to excite the CPG. 2) Trigeminal (Tt) neurons, which are cutaneous afferents, also have connections to the hindbrain. 3) Central trigeminal (Tn) neurons possibly relay sensation from noxious stimuli from the skin to the hindbrain. When pressure is experienced at the head skin or cement gland, prolonged firing of trigeminal pressure receptors (Tc) occurs. These may excite GABAergic reticulospinal (ri) neurons, which send inputs to the spinal cord to inhibit CPG neurons. Raphe-spinal (R) neurons, by releasing serotonin, are suggested to modulate the CPG (Roberts et al. 1998).

Eide et al. (1999) studied the commissural interneurons (CINs) in the lumbar spinal cord of the neonatal rat. CINs are neurons with axons that extend to the contralateral side of the spinal cord. Therefore they are involved in coordination of left and right limbs which have alternating activities during locomotion. In order to determine the density and distribution patterns of CINs in the lumbar spinal cord, a variety of retrograde and anterograde axonal tracing were performed with fluorescent marker 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate. CINs with ascending and descending fibers were labeled separately, and their locations were different but overlapped. All CIN axons traversed the ventral commissure at right angles to the midline. These CIN axons went up to six or seven segments proximally and distally in the ventral and ventrolateral white matter and gave off collaterals. Eide et al. (1999) concluded that the lumbar spinal cord of the neonatal rat contains large amounts of CINs with axon projections and collateral ranges spanning several segments.

Kjaerulff and Kiehn (1997) investigated the mechanisms responsible for coordination between left and right limbs in locomotion by recording the crossed synaptic input to lumbar motoneurons during contralateral ventral root rhythmicity using *in vitro* neonatal rat spinal cord. The spinal cord was

sectioned and was placed in a split-bath so that while one hemicord was kept in normal solution, the contralateral hemicord was put into a bath where 5-HT and NMDA were added. Kjaerulff and Kiehn found that rhythmic bursting were induced in the ventral roots on the agonist-exposed side, whereas the ventral roots on the agonist-free side remained inactive. Intracellular recordings were made from lumber motoneurons on the silent agonist-free side during rhythmic activity in the contralateral ventral roots. When the membrane potential was at rest, the typical crossed synaptic input was a rhythmic burst of depolarizing inhibitory presynaptic potentials (IPSPs). This input affected the frequency of bursts induced with depolarizing current by inhibiting firing in phase with the contralateral bursts. Kjaerulff and Kiehn suggested that, in contrast to the case of lower vertebrates, the crossed inhibition in neonatal rats appears to have inhibitory premotor neurons located on the same side as the receiving motoneurons. These premotor neurons are rhythmically activated via a crossed pathway that relies on glutamatergic transmission (Kjaerulff and Kiehn, 1997)

The important functions of spinal locomotor central pattern generators (CPGs) are to provide oscillatory motor commands to individual joints or segments and to control the precise timing of those commands across all joints or segments for efficient, coordinated locomotor behavior. Our ability

to understand the neuronal mechanisms underlying intersegmental coordination has been limited by the complexity of propriospinal interconnectivity and the paucity of quantitative data on the magnitude and activity in those connections. Investigators have used theoretical approaches to discover general rules by which CPG-like oscillator systems must be constructed to produce appropriate coordinated locomotor behavior. The locomotor CPG is represented as a network of oscillators, where each oscillator generates local motor output and interoscillator coupling provides intersegmental coordination.

The lamprey is used as a model to examine the cellular bases of vertebrate locomotor behaviour. Via reticulospinal fibers, the forebrain and brainstem cell populations initiate locomotor activity by activating a network comprised of glutamatergic and glycinergic interneurons in the spinal cord. What is more, Grillner has proposed the roles of different channels and neuromodulators. For example, different types of calcium channels (L, P/Q, and N) are present on the neurons in the lamprey spinal cord. By applying antagonists of these calcium channel types, the late afterhyperpolarization caused by small conductance apamin-sensitive calcium-dependent potassium channels (SK_{Ca}) are found to be blocked by N-type channel blockers, but not L-type channel blockers. Since SK_{Ca} largely determines the spike frequency

adaptation, it is important in locomotor pattern generation. In fact, it was found that application of antagonists of N-type calcium channel disrupts the rhythmicity of locomotion. In contrast, antagonists of L-type calcium channel had no effect at all. It was also found that binding of neuropeptide Y to its receptors results in depression of sensory-evoked neurotransmission. By using antagonists of metabotropic glutamate receptors, Grillner et al. (1998) discovered that the reticulospinal excitatory presynaptic potential (EPSP) was reduced by group II and III metabotropic glutamate receptors. Likewise, application of dopamine also results in reticulospinal EPSP depression.

MODULATION OF LOCOMOTOR CPG

Neuromodulation is thought to be responsible for the flexibility of the neural circuits (central pattern generators) that control rhythmic behaviors (Katz, 1998, Morgan et al. 2000). There are two sources of neuromodulation for neuronal circuits: extrinsic inputs and intrinsic components of the circuits themselves. Extrinsic modulators are defined as agents (e.g. neurons, biogenic amines, and neuropeptides) that act from outside CPGs. For example, Jovanovic et al. (1996) demonstrated that 5-HT plays an important role in modulating the locomotor CPG in the mudpuppy (*Necturus*

maculatus) by acting through a well-developed spinal serotonergic system. For this experiment, an *in-vitro* brainstem-spinal cord preparation isolated from the mudpuppy was used. By adding NMDA to the bath, the spinal CPG for locomotion was activated. Although 5-HT alone did not induce locomotion, it exerted a modulatory effect on NMDA-induced locomotion by producing a dose-dependent increase in the walking cycle duration and increased the EMG burst duration (Jovanovic et al. 1996).

In contrast to extrinsic modulators, intrinsic modulators act from within CPGs themselves. Katz and Frost (1995) and Katz et al. (1994) described escape swimming in the mollusc *Tritonia diomedea*, suggesting that the circuit connections are dynamically controlled by the activity of neurons within the circuit itself. The dorsal swim interneurons (DSIs) are sets of serotonergic neurons that participate in the generation of the rhythmic swim motor program. Serotonin released from these CPG neurons functions as a neuromodulator. It enhances the release of neurotransmitter from another CPG neuron, cerebral neuron 2 (C2), and also increases C2 excitability by decreasing spike frequency adaptation. These neuromodulatory actions intrinsic to the CPG may be important for experience-dependent changes in the output such as behavioral sensitization and habituation.

ROLES OF NEUROTRANSMITTERS IN LOCOMOTION

1) Glutamate

Grillner et al. (1981) found that by bath application, NMDA generated locomotor activity underlying swimming in the *in-vitro* preparation of the lamprey spinal cord. The burst frequency of the NMDA-induced activity (dose range 30-5000 μM) is wide and ranges from 0.05-0.1 Hz up to 2.5-4 Hz (Grillner et al. 1981, Brodin et al. 1985).

In *Xenopus* tadpoles, Zhao and Roberts (1998) assessed the excitatory synaptic drive to motoneurons as a result of frequency changes in bursting activities during fictive swimming. What they found was that after applying D-tubocurare, which is a nicotinic antagonist, the glutamatergic excitatory synaptic drive still changed with frequency. In contrast, when glutamate receptors or all chemical transmission was blocked, excitation remained constant even though frequency changed. Due to the fact that most motoneurons fired very reliably at all frequencies, the electrotonic and nicotinic drive from motoneurons remained constant. However, when swimming frequency dropped, the glutamatergic drive also decreased as the number of active premotor interneurons decreases. This result suggested that the frequency of the swimming central pattern generator in tadpoles was

partly caused by the population of glutamatergic premotor interneurons active on each swimming cycle (Zhao and Roberts, 1998).

Using an *in-vitro* brainstem-spinal cord preparation isolated from the mudpuppy, Wheatley and Stein (1992) found that by adding NMDA to the bath, the spinal CPG for locomotion became active. Rhythmical activities were noted when intracellular recordings were made from interneurons and motoneurons. Likewise, simultaneous electromyographic (EMG) recordings made from forelimb muscles also reviewed rhythmical activities were triggered by NMDA (Wheatley and Stein, 1992).

Using a bath containing an isolated brainstem-spinal cord neonatal rat preparation, Cazalets et al. (1992) found that the excitatory amino-acids, glutamate and aspartate triggered a locomotor pattern. This ability of glutamate to trigger locomotion was again reproduced by Cowley and Schmidt (1994). In *in-vitro* neonatal rat spinal cord preparation, NMDA induced rhythmic hindlimb nerve activity in three forms: 1) in about one-third of the cases, there was side-to-side alternation, but co-activated intralimb flexor-extensor pairs; 2) in another one-third of the cases, there was bilateral co-activation of all flexors and extensors; 3) in the remaining one-third of the cases, there was rhythmic but poorly coordinated activity (Cowley and Schmidt, 1994). Nishimaru and Kudo (2000) also used the

neonatal rat spinal cord to study the CPG. When recordings were made in the ventral roots, it was found that bath-application of NMDA caused alternating rhythmic activities in the left and right hind limbs, as well as alternation of activities between flexor and extensor muscles (Nishimaru and Kudo, 2000).

2) Serotonin (5-HT)

In a bath containing an isolated newborn rat brainstem-spinal cord preparation, 5-HT was applied and the activity of fictive locomotion was recorded in the ventral roots. The result was that 5-HT triggered an alternating pattern of right and left action potential bursts (Cazalets et al. 1992). Again, 5-HT was found to induce co-ordinated, alternating flexor-extensor pattern between the right and left limbs in *in-vitro* neonatal rat spinal cord preparations (Cowley and Schmidt, 1994).

Zhang and Grillner (2000) investigated the role of 5-HT in stabilising fictive locomotion in the lamprey (*Petromyzon marinus)* spinal cord. When 5-HT_2 receptors were blocked by spiperone, locomotion became unstable, as shown by the fact that the frequency and the coefficient of variation were increased. However, an addition of 5-HT stabilised the burst generation and as a result, a sustained fictive locomotion was achieved. This implies that there is an endogenous release of 5-HT during fictive locomotion that

contributes to the rhythm generation. 5-HT also plays an important role in modulating the locomotor CPG in other amphibians. As mentioned, Jovanovic et al. (1996) demonstrated that, although 5-HT by itself did not bring about locomotion in the mudpuppy, it exerted a modulatory effect on NMDA-induced locomotion by producing a dose-dependent increase in the walking cycle duration and increased the EMG burst duration (Jovanovic et al. 1996).

Following bath application of NMDA with D-serine, further application of 5-HT to an *in-vitro* brainstem-spinal cord preparation of an adult amphibian urodele, *Pleurodeles waltl*, was found to modulate the neural networks. Recording from ventral roots, Branchereau et al. (2000) found that 5-HT produced an increase in the cycle duration and the duration of rhythmic bursting activity. However, when applied alone, 5-HT did not trigger locomotor activity.

3) Glycine and gamma-aminobutyric acid (GABA)

In a bath containing an isolated brainstem-spinal cord preparation of newborn rat, application of GABA suppressed in a dose-dependent manner the motor activity induced by an excitatory amino acid NMDA (Cazalets et al. 1992).

Bath application of inhibitory neurotransmitters such as glycine and GABA disrupts the rhythmicity of locomotion induced by NMDA in the mudpuppy (Jovanovic et al. 1999). Neither glycine nor GABA induced locomotion when they were added alone. However, when locomotion had already been induced by NMDA, application of glycine and GABA suppressed the rhythmic motor pattern. Moreover, both the $GABA_A$ receptor agonist, muscimol, and $GABA_B$ receptor agonist, baclofen, mimicked the effects of GABA, because they either retarded or stopped the locomotor pattern (Jovanovic et al. 1999).

In the lamprey *in-vitro* spinal cord, Tegner et al. (1993) found that, with recording from the ventral roots or intracellularly from interneurons or motoneurons, GABA influenced the burst frequency and the intersegmental coordination. Bath application of a GABA uptake blocker led to a drop in the burst rate. This effect was counteracted by $GABA_B$ receptor blockade by phaclofen. However, if a $GABA_B$ receptor agonist (baclofen) was added during fictive locomotion, the burst rates lowed down. Thus, $GABA_B$ receptor activation due to an endogenous release of GABA caused a depression of the burst activity (Tegner et al. 1993).

4) Acetylcholine (ACh)

Roberts et al. (1998, 1999) examined the swimming motor pattern of the young *Xenopus* tadpole. Unlike the cases of the neonatal rat and the mudpuppy preparations, motoneurons in the young *Xenopus* tadpole are components of the CPG. The motoneurons make cholinergic synapses within the spinal cord as well as at the neuromuscular junction.

In the lamprey, the reticulospinal (RS) neurons are glutamatergic and excitatory. By activating all classes of interneurons, RS neurons form the main descending drive to the CPG. In 1979, Matthews and Wickelgren found that bath application of a number of neuroactive substances including ACh and noradrenaline have no effect on neurotransmission in RS neurons (Matthews and Wickelgren, 1979). In recent years, LeRay et al. (2000) reported of duel effects of ACh on RS neurons. It was found that local injection of ACh into RS cells caused membrane depolarization. This local injection also changed the response of RS cells to input from the trigeminal neurons, which are the cutaneous afferents. The EPSPs were depressed briefly. This response was mimicked by pilocarpine, but unaffected by D-tubocurarine, and atropine application increased the trigeminal EPSP, suggesting that muscarinic receptors are involved (LeRay et al. 2000).

Atsuta et al. (1991) studied the neonatal rat isolated brainstem-spinal cord preparation. By applying drugs ACh and carbachol, an ACh agonist

which is not hydrolysed by cholinesterase, rhythmic airstepping was seen. The rhythmic airstepping was recorded electromyographically as alternating patterns of activities in agonists and antagonists of the hindlimb muscles. Depending on whether the drug was added to the brainstem bath or to the spinal cord bath, different outcomes were obtained (Atsuta et al. 1991). Introduction of each of these drugs separately to the brainstem bath produced airstepping. However, carbachol only produced locomotor activity in the midpontine preparation that contained the medio-ventral medulla (MED). In contrast, in the midcollicular preparation, which consisted of both the mesencephalic locomotor region (MLR) and the MED, carbachol did not produce airstepping. Adding a drug into the spinal cord bath yielded still a different situation. Addition of NMDA to the spinal cord bath produced rhythmic airstepping, but addition of ACh gave tonic, persistent co-contractions (Atsuta et al. 1991). In another study using the neonatal rat spinal cord nerve preparation, Cowley and Schmidt (1994) found that either ACh or NMDA could induce rhythmic hindlimb movement. For either drug, there was alternating right and left limb movement, but flexor and extensor activity was co-activated in each limb (Cowley et al. 1994). Using whole-cell recordings in neonatal rat isolated spinal cord, Kiehn et al. (1996) found that addition of a glutamate receptor agonist or muscarinic cholinergic agents

had effects on the locomotor pattern. Interneurons from regions of the spinal cord thought to be responsible for generating locomotor rhythm showed rhythmic fluctuations of membrane potentials.

Huang et al. (2000) induced decerebrate cats to produce fictive walking by stimulating the MLR. Using immunohistochemistry, they found a large increase in both the number of c-fos and choline acetyltransferase (ChAT)-labelled neurons in the walking preparations, compared to control animals that had undergone the same surgery but in which fictive walking was not induced. Also, the increase in both the number of c-fos and ChAT-labelled neurons occurred in the intermediate regions of the grey matter in the L3 to S1 spinal cord, while the increase was much smaller in the dorsal and ventral regions. The data suggest that cholinergic interneurons in the cat spinal cord are involved in producing fictive walking (Huang et al. 2000).

In adult turtles, intracellular recordings were made from lumbar motoneurons in slice preparations. Rhythmic activity was induced in motoneurons by the cholinergic drug muscarine and d-tubocurarine. This activity ceased when nifedipine, which blocks the L-type calcium channels selectively, was added (Guertin et al. 1999). Roberts et al. (1998, 1999) examined the swimming motor pattern of the young *Xenopus* tadpole. Unlike the cases of the neonatal rat and the mudpuppy preparations,

motoneurons in the young *Xenopus* tadpole are components of the CPG. The motoneurons make cholinergic synapses within the spinal cord as well as at the neuromuscular junction.

5) Noradrenaline (NE)

Noradrenergic drugs, acting on alpha adrenoceptors (which are of at least two subtypes: alpha1 and alpha2 adrenoceptors), are crucial in both the initiation and modulation of locomotion of spinalized adult cats. Among all the neurotransmitters investigated to date (noradrenergic, dopaminergic, serotonergic, glutamatergic), the noradrenergic system appears to be more effective than others in triggering locomotion in complete spinal cats. Moreover, alpha-2 agonists, e.g. clonidine, was noted to be more potent than the alpha-1 agonist, methoxamine. Clonidine enabled the cats with lesions of the ventral-ventrolateral funiculi to require less support in locomotion (Rossignol et al. 1998).

When adult cats had complete transection at T13, EMG recordings revealed that alpha1 and alpha2 adrenoceptor agonists differ in their actions towards 1) the initiation of locomotion in early spinal cats (i.e., in the first week or so when there is no spontaneous locomotion) and in 2) the modulation of locomotion in the late-spinal cats (i.e., when cats have recovered spontaneous locomotion). It was found that clonidine, both an

alpha2 agonist and an imidazoline receptor agonist, triggered locomotion in early spinal cats. The effect of alpha1 agonist methoxamine was less consistent (it induced short periods of locomotion in three out of five spinal cats but only induced sustained locomotion in one cat). In late spinal cats, clonidine significantly lengthened the cycle duration as well as the flexor muscle burst duration and, at the same time, decreased the extensor activity. By contrast, alpha1 agonist increased the extensor activity of the hindlimbs without altering the step cycle duration (Chau et al. 1998).

Adult cats with ventral and ventrolateral T13 spinal lesion were treated with noradrenergic drugs and their locomotion on treadmill was assessed. Longer duration of locomotion was maintained in cats having norepinephrine (NE) injected in early and plateau periods because the stepping of the hindlimb turned out to be more regular and the interlimb coupling became stabilized. A similar outcome was achieved by using Methoxamine tested only at the plateau period, the alpha1-agonist. By contrast, clonidine (alpha2-agonist) caused deterioration in walking (Brustein and Rossignol, 1999).

In a study using marmoset monkeys (*Callithrix jacchus*) which were decerebrated and acutely spinalized, clonidine elicited rhythmic alternating limb movement. Two kinds of out-of-phase activity were seen. In fifty

percent of the animals, alternating activity was confined to ipsilateral and contralateral flexor nerves, As for the remaining animals, alternating activity existed between ipsilateral flexors and extensors (Fedirchuk et al. 1998).

Clonidine can improve walking in spinal cord injury (SCI) human subjects. This beneficial effect applies especially to the more severely disabled subjects. In paraplegic patients who were unable to walk before the experiment, clonidine application led to increases in maximum speed on a treadmill and a more upright posture (Norman et al. 1998). However, with those subjects who only suffered from paresis and could ambulate before the experiment, clonidine did not cause significant improvement in walking (Norman et al. 1998).

SENSORY MODULATION OF LOCOMOTOR CPG

Passing electrical current to dorsal roots (DR) of the neonatal rat spinal cord *in-vitro* were found to induce fictive locomotor pattern, just like that elicited by bath-applied excitatory transmitter agonists like NMDA. This suggests that sensory inputs from the periphery can arrive at the central pattern generator in the spinal cord and trigger its functioning (Marchetti et al. 2001).

In adult chronic spinal cats which were spinalized at T13, in response to stimulation of the skin of the perineal region, fictive locomotion for hind limb stepping on treadmill was seen (Pearson and Rossignol, 1991).

Mechanoreceptors in the lamprey spinal cord were discovered to send inputs to the CPG for locomotion. Repetitive intracellular stimulation of sensory neurons in the dorsal horn changed the intensities and timing of ventral root bursts. Thus the central pattern generator can be modified by sensory inputs (McClellan and Jang, 1994).

In *Xenopus* embryos with the central nervous system transected distal to the cranial roots, transient mechanical or electrical stimulation of the skin on the trunk can trigger fictive swimming. Thus, the sensory pathway responsible for locomotor patterns must have direct connections with the spinal cord (Soffe, 1991).

SUMMARY OF RELEVENT PREVIOUS WORK

Central pattern generators (CPGs) underlie a wide variety of rhythmic behaviours such as locomotion and respiration in most multi-cellular organisms. The CPGs are capable of generating a patterned output without phasic sensory input. The organization of the CPG is due to both intrinsic properties of the individual neurons and their network interactions. Graham

Brown (1911) first proposed the "half-center model" to illustrate how rhythmicity in locomotion could be produced by mutual inhibition among neuronal networks in the spinal cord of the cat. Since then, investigators have studied the mechanisms underlying the generation of rhythmic output and the mechanisms by which various modulators affect the CPG.

Summing up, several neurotransmitters have shown to have fairly consistent effects on the CPG in a wide range of vertebrates (Grillner et al. 1979). For example, glutamate is excitatory (Grillner et al. 1981, Zhao and Roberts, 1998, Wheatley and Stein, 1992, Cowley and Schmidt, 1994, Rossignol et al. 1998), while glycine and GABA are inhibitory (Cazalets et al. 1992, Tegner et al. 1993, Jovanovic et al. 1999). However, the effects of other neuroactive agents are not so consistent. ACh varies in its effect in different animal preparations. Even in the neonatal rat preparations, different results were obtained, depending on the level of lesion (Atsuta et al. 1991).

THESIS OBJECTIVES AND PREPARATION

The aim of this thesis was to study the effect of neuromodulators on CPG in vertebrate locomotion. To achieve this end, an isolated spinal cord preparation of the mudpuppy (*Necturus maculatus*) was used.

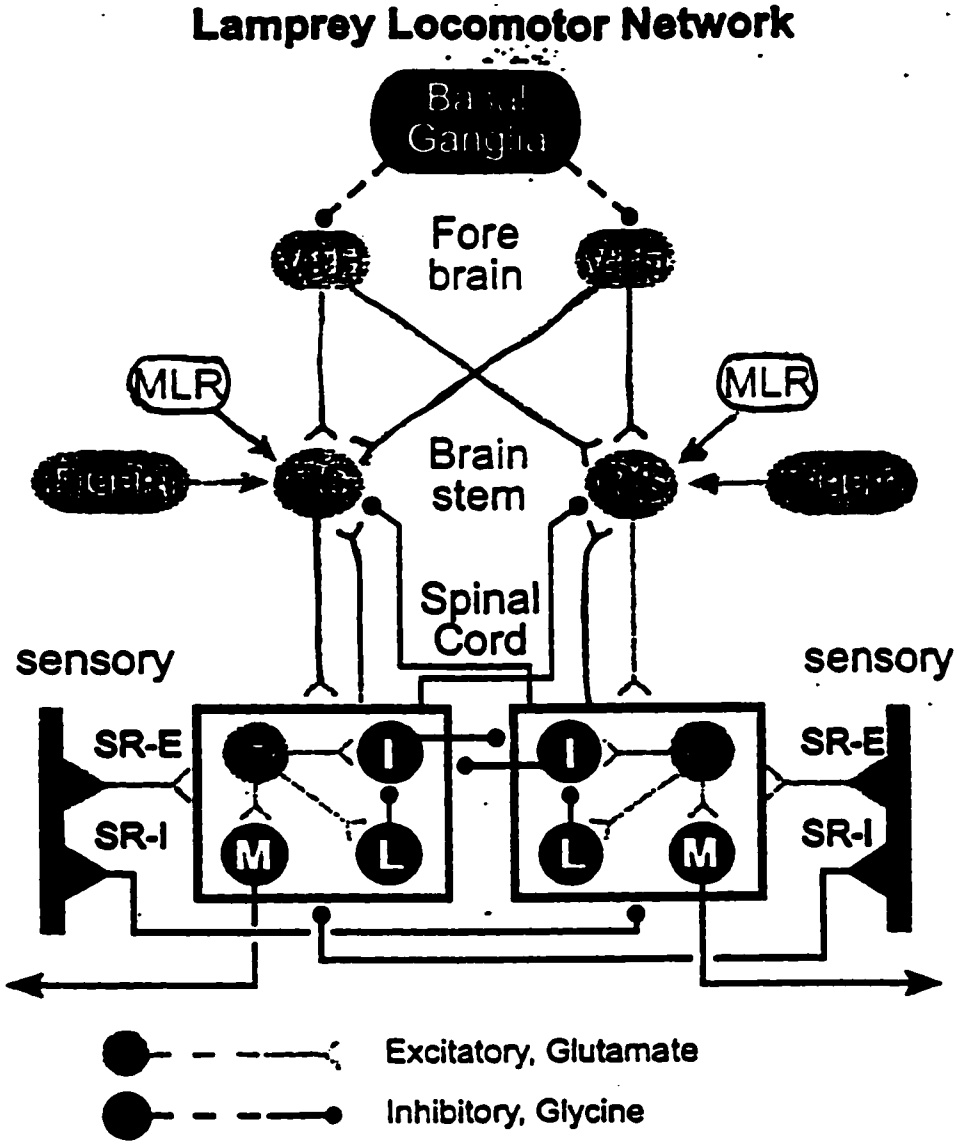
THE MUDPUPPY MODEL

The *in-vitro* preparation consisted of the first five segments of the spinal cord, together with the neurally attached upper limb (Wheatley and Stein, 1992). The mudpuppy is an excellent model for research. It has advantages of *in-vitro* preparations in general, that include 1) better control of external environment, e.g. ionic concentrations, 2) no blood-brain barrier, hence the dosage of applied drug can be precise, 3) better stability for recording of data, e.g. EMG, 4) easier techniques, since most *in-vitro* techniques can be mastered within a short time (Kudo, 1978). The advantages particularly applicable to the mudpuppy *in-vitro* preparations include 1) the mudpuppy walks with a locomotor pattern (an alternating quadrupedal gait) resembling the higher vertebrates (Wheatley et al. 1992), 2) being poikilothermic, the mudpuppy preparations can survive up to two days at reduced temperatures; this longer term viability is more favorable compared to other preparations, e.g. rat preparations which last about 8 hours (Smith and Feldman. 1987); 3) simultaneous stable intracellular and EMG recording during locomotion is possible, 4) compared to the higher vertebrates, the mudpuppy has a simpler nervous system with less neurons in the spinal cord, making circuit analysis easier, 5) unlike other *in-vitro* preparations, movement related afferent feedback is still intact in the

mudpuppy preparation to a great extent (Wheatley and Stein, 1992; Wheatley et al. 1992; Wheatley et al. 1994; Jovanovic et al. 1996).

In order to study the effect of neuromodulators on CPG, two projects were involved. The first project was to study the effect of cholinergic agents in an isolated spinal cord preparation of the mudpuppy (*Necturus maculatus*). In the second project we examined the effect of clonidine, an alpha-2 noradrenergic agonist.

We examined the effect of ACh and clonidine in an isolated spinal cord preparation of the mudpuppy (*Necturus maculatus*). This is of particular interest because vertebrate walking developed in amphibia, and the effect of ACh and clonidine on amphibian walking has never been studied.



glutamatergic and excitatory. They activate all classes of interneurons. The excitatory interneurons (E) can activate all types of neurons in the spinal cord: 1) The inhibitory glycinergic interneurons (I) which inhibit all classes of neurons on the opposite side of the spinal cord. 2) The lateral neurons (L), which inhibit interneurons on the same side of the spinal cord. 3) The motoneurons (M). The stretch receptor neurons are represented as SR-E (excitatory) and SR-I (inhibitory). In the brain stem, excitatory inputs are sent to the RS neurons via three routes: 1) cutaneous afferents (Trigem.), 2) mesencephalic

locomotor region (MLR), and 3) ventral thalamus (VTH). The basal ganglia also plays a role by sending inputs to VTH (Grillner et al. 1998).

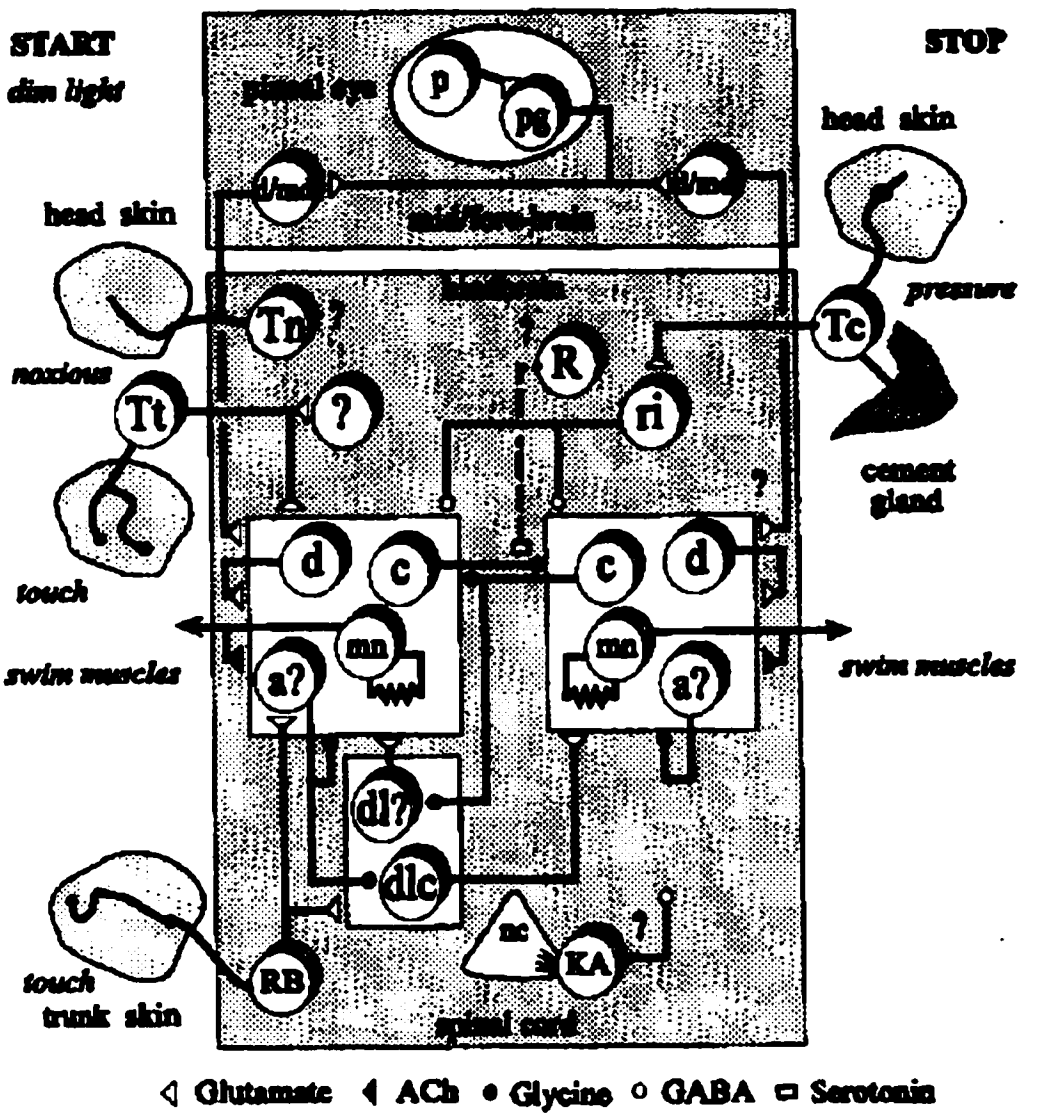


Fig 1-2 The spinal circuits that control swimming in the young *Xenopus* tadpole. Mechanoreceptors (RB) activate sensory pathway interneurons (dl and dlc), which is the equivalent of dorsal horn in higher vertebrates. These will in turn excite the neurons of the CPG bilaterally. Commissural interneurons (c) bring about alternation of activity through glycinergic inhibition on the contralateral side. Decending interneurons (d) excite

neurons on the ipsilateral side. Motoneurons (mn) activate the muscles of the trunk and have central nicotinic cholinergic connections to give positive feedback to the CPG. In addition to this central activation by acetylcholine, glutamate exerts its excitatory effect by its action on NMDA and AMPA receptors. Areas of uncertainty are denoted as question mark (?) in the spinal circuits: 1) unclear functions of dl interneurons, 2) unclear functions of KA neurons which have GABA activity. For the brain circuit, there are 3 ways of neural projections into the hindbrain to excite the CPG. 1) when the light intensity drops, pineal photoreceptors (p) are activated, and these excite the pineal ganglion cells (pg). These in turn excite diencephalic/ mesencephalic (d/md) descending interneurons which make connections to the hindbrain to excite the CPG. 2) Trigeminal (Tt) neurons, which are cutaneous afferents, also have connections to the hindbrain. 3) Central trigeminal (Tn) neurons possibly relay sensation from noxious stimuli from the skin to the hindbrain. When pressure is experienced at the head skin or cement gland, prolonged firing of trigeminal pressure receptors (Tc) occurs. These may excite GABAergic reticulospinal (ri) neurons, which send inputs to the spinal cord to inhibit CPG neurons. Raphe-spinal neurons (R), by releasing serotonin, are suggested to be able to modulate the CPG (Roberts et al. 1998).

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CHAPTER 2

METHODS

Animal preparation

Adult mudpuppies were anesthetized by adding the anesthetic 3-aminobenzoic acid ethyl ester (Sigma) to a water tank containing the animals. The animals were then transferred to a basin containing ice, to maintain a low rate of metabolism. The skin was slit open, epiaxial tissues were dissected out, and a laminectomy was performed. The spinal cord was exposed from the brainstem up to the C5 level. Next, the spinal cord was sectioned right at the caudal end of the medulla oblongata. As a result, the preparation consisted of the first five segments of the spinal cord, together with a neurally attached upper limb (for details see Wheatley and Stein, 1992). The *in-vitro* preparation was put in a recording chamber with Ringer's solution, and oxygen was bubbled into the bath. Bipolar silver wires were inserted into flexor (brachialis) and extensor (extensor ulnae) muscles of the forelimb. The wires were connected to a pre-amplifier and analysed as described under Data Analysis. In each preparation one or more of 2 experimental protocols were used. In each protocol, drugs such as NMDA (Sigma) were applied to the bath. There is a limit on the time for applying drugs since the EMG pattern deteriorates in less than two hours

(Wheatley and Stein, 1992). After each trial, the drugs were washed out by superfusing the preparation with cold Ringer's solution at a rate of 8ml/ min. This rate was selected because higher superfusion rates cause excessive disturbance to the tissues, while lower superfusion rates are ineffective (Wheatley and Stein, 1992). After washing, some preparations were still viable for one or two more experimental protocols, while other preparations could not be used again because the addition of NMDA for a second time did not elicit an alternating rhythmic EMG pattern. A total of 20 animals were used and each of the procedures outlined below was repeated in 4-6 animals. The work conformed to national/local ethics committee guidelines for animal welfare.

First, NMDA was applied to induce locomotion and flexor and extensor EMG were recorded as the control period of walking. In 4 animals, a range of doses of an ACh analogue, carbachol (Sigma), was added to the bath. A range of doses from 1 up to 250μM was administered. For example, if no effect was seen in using a dose of 5μM, a higher dose was used. Any effect of carbachol was noted on the walking pattern previously induced by NMDA, as reflected in the EMG readings. Experiments were repeated with doses of clonidine (Sigma) from 1 up to 320uM was added to the bath and any effect of clonidine was noted in the EMG readings.

In other experiments, the ACh-esterase inhibitor physostigmine (eserine), was used in place of carbachol. The initial concentration of 2μM of physostigmine (Sigma) was used, since there was no effect at this concentration and below. The dose was increased up to 160μM. In a third set of control experiments, locomotion was induced by NMDA, and EMG was recorded before and after the application of an ACh antagonist, atropine (which blocks muscarinic ACh receptors) and carbachol. With a concentration of 100μM of atropine (Sigma), doses of carbachol starting from 8μM increasing up to 64μM were added to the bath.

As controls, either carbachol or atropine or clonidine alone were applied to the bath with a range of concentrations of 1μM, increasing up to 250μM. After 5 minutes of each application, EMG was recorded.

Data Analysis

The recorded EMG was, amplified, filtered (10-300 Hz), rectified, smoothed at 100Hz, and then digitized. The correlation between the flexor and extensor EMG was calculated (Wheatley et al. 1994) using a Matlab program (Math Works, Natick MA). The cross-correlation function (C) was calculated using the formula (Marmarelis and Marmarelis 1978):

$$C(\tau) = \frac{1}{(T-\tau)\sigma_f\sigma_g}\int_0^{T-\tau} f(t)g(t+\tau)dt$$

where t is the time, T is the maximum time, τ is the time lag (between –5 and 5 s), and σ_f and σ_g are the standard deviations of the functions f and g (the rectified and smoothed flexor and extensor EMGs). The cross-correlation has the shape of a damped sinusoidal wave (please refer to Fig 3-2A). The peak of the wave is negative at a time lag of zero, if the flexor and extensor EMGs are out of phase with each other. Conversely, if the flexor and extensor EMGs are in phase, the peak will be positive. The range of possible correlations is ± 1. To determine a dose-response curve, the peak of the cross-correlation was plotted against the dose of the drug. The statistical significance of the dose-response curves (refer to Figs. 3-2, 3-4, 3-5) were tested with a Students t-test and a one-way ANOVA with repeated measures ($p=0.05$).

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CHAPTER 3

RESULTS

Carbachol. As has been reported previously, applying NMDA produces rhythmic alternation of flexor and extensor EMG (Fig. 3-1A). Adding carbachol, a long-lasting ACh agonist, disrupts the rhythm, although both flexor and extensor muscles continue to be active from time to time (Fig. 3-1B) and the limb moved erratically. Applying carabachol (1-250 μM) to a resting preparation had no effect. The limb remained at rest, with occasional movement of one or two fingers (Fig. 3-1C).

The changes can be quantified using cross-correlation analysis (Fig 3-2A). Several points are evident: 1) with NMDA alone (Fig 3-2A), there is a large negative peak (-0.47) near a time lag of zero. This indicates that the flexor and extensor EMGs are out of phase, 2) positive and negative peaks alternate rhythmically with a period of about 1.5 s, which is the duration of the step cycle, 3) after the addition of carbachol, the cross-correlation becomes much smaller and the rhythm is lost (Fig 3-2B). The dose-response relationship for carbachol is shown in Fig 3-2C. Using a one sample Student's t-test, only the values for 0 and 2 μM were significantly different from 0. In other words, the rhythmic alternating pattern was disrupted when higher concentrations of carbachol (≥4 μM) were added. The values became

somewhat positive, indicating a tendency for flexors and extensors to be activated together at 8 and 16 μM, but this tendency was not statistically significant.

Fig 3-2D shows the mean amplitude of the rectified flexor and extensor EMGs respectively. By statistical analysis using an ANOVA with repeated measures, no values were statistically different from one another ($p>0.05$). Thus, adding carbachol did not simply inhibit one or both muscle groups. Instead, it disrupted the walking pattern produced by NMDA without affecting the overall excitation of the motor neurons.

Physostigmine. After NMDA induced locomotion (Fig 3-3A), the cholinesterase inhibitor, physostigmine, was applied to the bath containing the walking preparation. The rhythm of the locomotor pattern was again disrupted (Fig 3-3B). The flexor and extensor EMG bursts were no longer alternating, and the limb moved in an erratic fashion, as when carbachol was added. Analysis by cross-correlation indicated that physostigmine disrupted the out-of-phase locomotor pattern (Fig 3-4 A-B). Fig. 3-4C shows the dose-response relationship. The cross-correlation peaks were only statistically different from 0, when the concentration of physostigmine was below 40 μM (Students t-test, $p<0.05$). In other words, physostigmine ($\geq$40 μM) disrupted the alternating walking pattern. Finally, physostigmine had no effect on the

mean amplitude of the rectified EMG when applied with NMDA and had no effect on a resting preparation when applied without NMDA (not shown). The simplest interpretation of these results is that physostigmine disrupted the walking rhythm by blocking the breakdown of endogenously released ACh.

Atropine. After NMDA induced locomotion, concentrations above 50 μM of the muscarinic antagonist, atropine, prevented carbachol from disrupting the rhythm. The rhythm produced by NMDA (Fig. 3-5A) is not affected by atropine (Fig. 3-5B), but the muscarinic blockade by atropine prevents carbachol from disrupting the rhythm (Fig. 3-5C). In this experiment the activation of the flexor and extensor EMGs by NMDA was somewhat asymmetric, so the negative peak was displaced from 0 s. Atropine and carbachol did not affect the pattern. Note that 32 μM of carbachol was added in Fig. 3-5C, whereas 4 μM was sufficient to disrupt the rhythm in Figs. 3-1 and 3-2. As shown by the dose-response relationship (Fig 3-5D) no points were significantly different from one another (repeated measures ANOVA, $p>0.05$). In summary, addition of atropine prevents the disruption in rhythmicity, suggesting that the effect is muscarinic. Addition of atropine did not trigger locomotion in a resting preparation.

Clonidine. Fig 3-6 shows the effects of clonidine. Applying NMDA at a concentration of 40 μM induced locomotion, as shown in the cross-correlation (Fig 3-6A). A negative peak (-0.26) near a time lag of zero indicates that the flexor and extensor EMG's are out of phase. Positive and negative peaks alternate rhythmically with a period near 1.1 s, the duration of the step cycle. After adding clonidine (80 μM) to the bath, no change was seen (Fig 3-6B). In the dose-response curve (Fig. 3-6C) no values were significantly different from one another (repeated measures ANOVA, $p>0.05$). Clonidine (1-250 μM) did not trigger locomotion in a resting preparation.

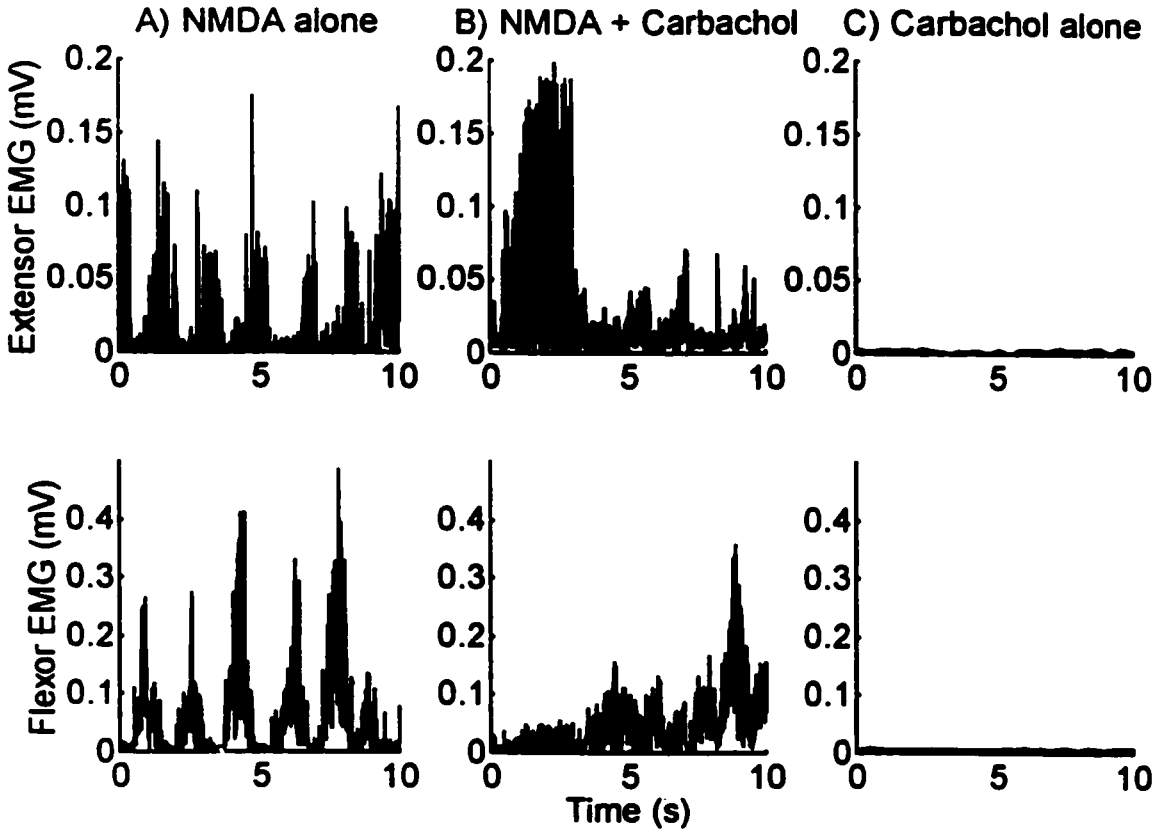

Fig. 3-1 A-C shows the rectified EMG from the extensor and flexor muscles. Applying 80 μM of NMDA produced the control pattern (**A**) characterized by alternating flexor and extensor bursts. The addition of 4 μM of carbachol (a long-acting ACh agonist) to the walking preparation disrupted the fine coordination between the flexor and extensor EMG bursts (**B**). Carbachol alone (80μM) could not trigger locomotion (**C**). The EMG remained flat throughout with few spikes and no burst.

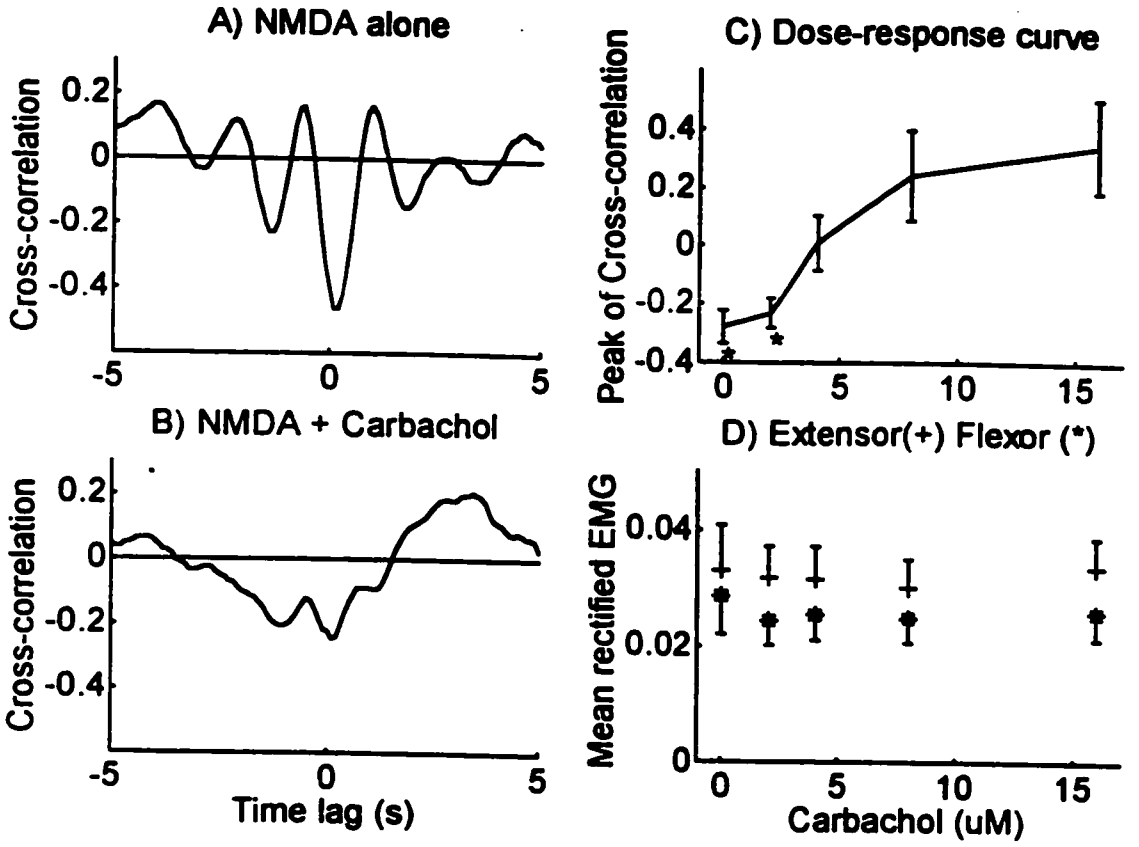

Fig. 3-2A-D. Analysis using cross-correlation of the EMG traces shown in Fig 3-1. After adding carbachol, the rhythmic, out-of-phase locomotor pattern (**A**) becomes much smaller and arhythmic (**B**). Plotting the peak (± S.E.) of the cross-correlation against the concentration (μM) gives a dose-response curve for a group of animals (n=5 trials) (**C**). Values with an asterisk (*) are significantly different from 0 (Students t-test, $p<0.05$). The mean rectified amplitude of the flexor and extensor EMG did not change significantly with the dose of carbachol (**D**). Thus, carbachol disrupted the walking rhythm without affecting the excitation of the motor neurons.

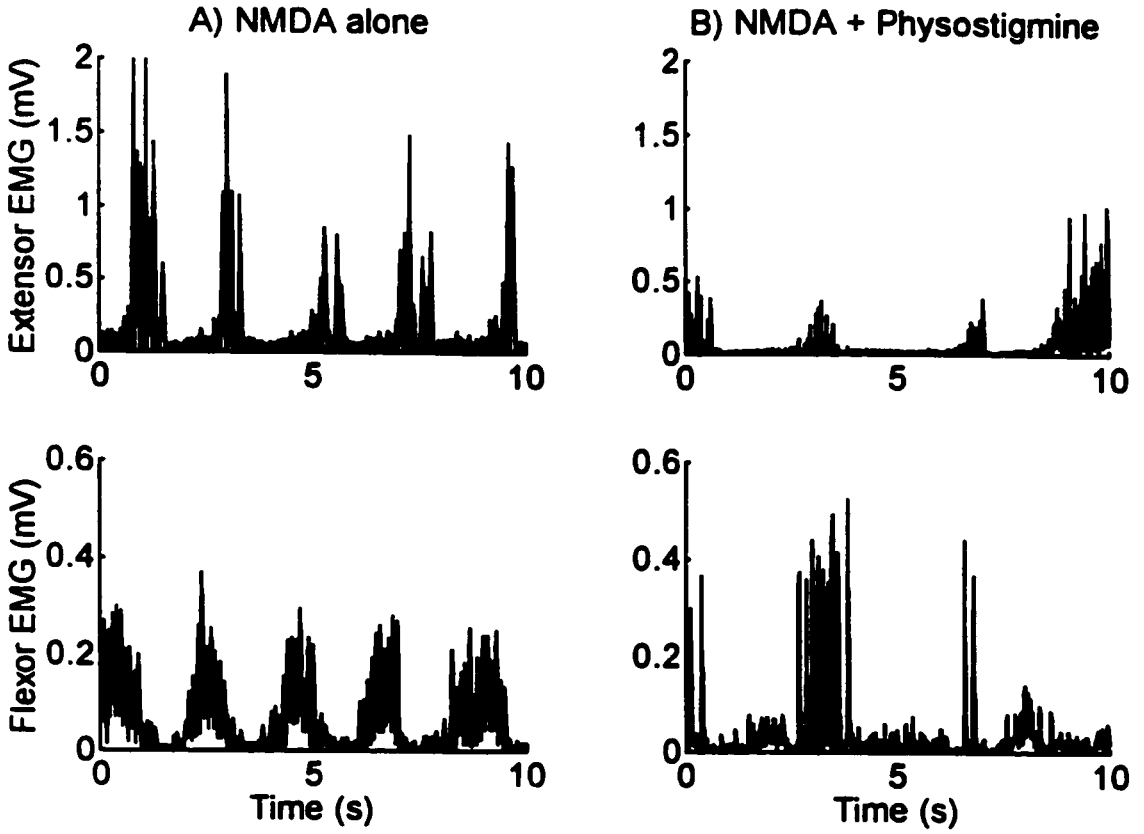

Fig. 3-3 A-B. NMDA (80 μM) induced a typical control locomotor pattern (**A**). Adding 80 μM of physostigmine (a cholinesterase inhibitor) to the walking preparation disrupted the rhythm (**B**). The flexor and extensor EMG bursts were no longer alternating. Presumably, physostigmine disrupted the rhythm by prolonging the action of endogenously released ACh.

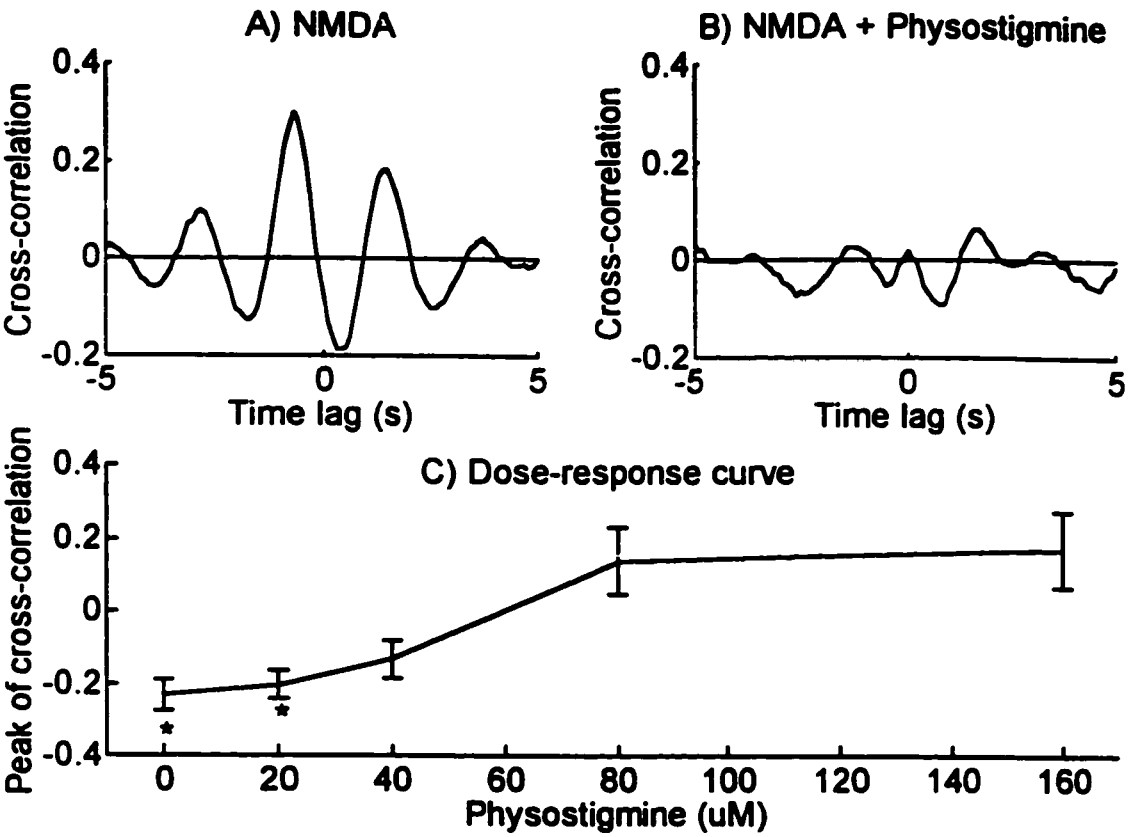

Fig. 3-4A-C. The rhythmic, out-of-phase locomotor pattern produced by NMDA (**A**) becomes much smaller and less rhythmic after adding physostigmine (**B**). The dose-response curve (n=5) for physostigmine (**C**) shows that the peak was only significantly different from 0 for physostigmine concentrations of 0 and 20 μM (*).

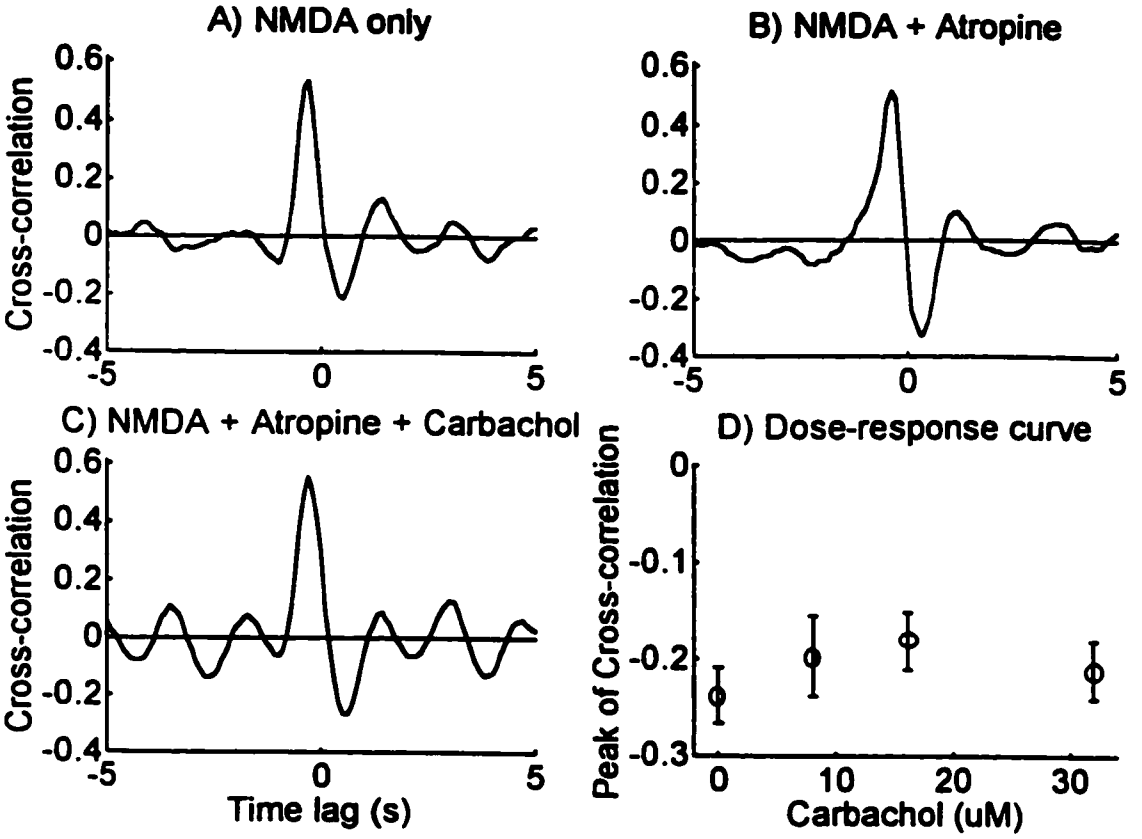

Fig. 3-5 A-D. Atropine blocks the effect of carbachol, indicating that the action is muscarinic. The control locomotor pattern with NMDA alone (80 μM) is shown in (**A**). Adding 50 μM of atropine had no effect on the walking rhythm (**B**). Fig. 3-2 showed that a 4 μM dose of carbachol disrupted the rhythm. However, with 50 μM of atropine in the bath, even 32 μM of carbachol had no effect on the rhythm (**C**). On average (n=7) no change occurred, when any of the tested concentrations of atropine was added to the walking preparation (**D**). This result differs from those obtained by adding either carbachol or physostigmine to a walking preparation (contrast s. 3-5D to 3-2C and 3-4C).

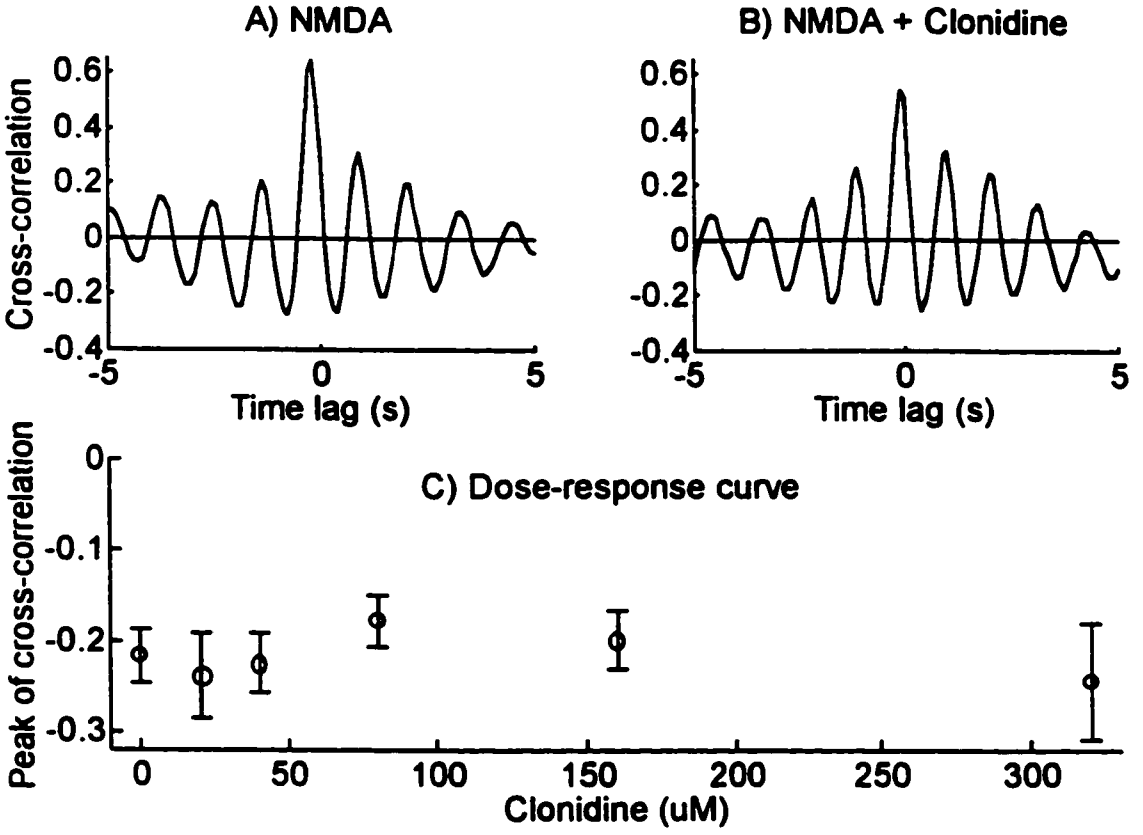

Fig. 3-6 A-C. Cross-correlation analysis shows that adding clonidine (**B,** 80 μM) did not change the rhythm induced by NMDA (**A,** 40 μM). In this preparation the bursts were somewhat asymmetric so there was a positive peak slightly before and a negative peak slightly after a time lag of zero. However, the pattern did not change after the addition of clonidine. No points were significantly different from one another (**C**, n=6, repeated measures ANOVA, $p>0.05$).

CHAPTER 4

GENERAL DISCUSSION

The effect of ACh varies in different animal preparations (see Introduction). In mammals, rhythmic bursts are produced, although generally with co-contraction of flexors and extensors (Atsuta et al. 1991; Cowley and Schmidt 1994). In turtles the cholinergic agent, muscarine, induced rhythmic activity in interneurons (Guertin and Hounsgaard 1999). pACh excited motor neurons that are part of the CPG in Xenopus tadpoles (Roberts et al. 1998; Roberts et al. 1999), but had no direct action on the CPG in the lamprey (LeRay and Dubuc 2000).

In contrast, in the present study ACh agonists disrupted the walking rhythm without changing the net excitation of the flexor or extensor motor neurons. This unique action has not been described before to our knowledge. Although the mechanism of action of ACh is not known from these experiments, Fig. 4-1 shows a simple diagram that is consistent with all our results. The CPG (surrounded by dashed lines) has mutually inhibitory half centers. Excitatory interneurons (E) activate flexor and extensor motor neurons and inhibit (I) the other half center. The excitatory interneurons are normally activated by glutamate that is released by descending and sensory

pathways (not shown). Application of NMDA mimicked the effects of these pathways in our experiments.

The inhibitory interneurons may be glycinergic, since blocking the action of glycine with strychnine changed the normal alternating pattern to a synchronous one (Jovanovic et al. 1999). GABA also had inhibitory effects and the presence of synchronous activity after strychnine or the $GABA_A$ antagonist, bicuculline, suggests that some mutually excitatory connections also exist that are not shown in Fig. 4-1. The action of cholinergic agents suggests that cholinergic interneurons (A) exist that are not part of the CPG, but inhibit both excitatory and inhibitory neurons in the CPG. A balanced action would produce no net excitation of motor neurons (see Results). Although the inhibition is shown as post-synaptic, we can not exclude the possibility of presynaptic actions. With either mechanism blocking the mutually inhibitory neurons that are part of the pattern generator would eliminate the rhythmic alternation.

The model may also explain some differences between the amphibian and mammalian results. If the cholinergic neurons only blocked the inhibitory interneurons (I) and not the excitatory ones in the mammalian CPG, a net excitation (or disinhibition) would result. Thus, cholinergic agents would activate motor neurons, and any mutually excitatory

connections between the flexor and extensor half centers would produce co-activation of extensors and flexors, as has been observed in the neonatal rat (Cowley and Schmidt 1994). Finally, since clonidine has no effect in the mudpuppy, none is included in Fig. 4-1. Perhaps, some of the monoaminergic pathways that modulate the walking rhythm to various degrees in higher animals (Rossignol et al. 1998; Giroux et al. 2001) evolved later and are not present or not as well developed in lower species.

Exogenous application of drugs has potential limitations: 1) addition of drugs cannot mimic the time course of a naturally occurring neurotransmitter that is released and acts on its target receptors, 2) the CPG is a neuronal network that is modulated by many neurotransmitters, so altering the concentration of only one neurotransmitter may be inadequate to activate the neuronal network. Redundancy in neural pathways may render one drug ineffective (Cowley and Schmidt 1994), 3) application of a drug such as NMDA will cause membrane depolarization of all cells with NMDA receptors, not only those in the pattern generator, 4) when we add another neuroactive agent, e.g. carbachol, to a bath that already contains NMDA, some neurons that are excited by both glutamate and ACh may become inactivated due to depolarization block.

Nonetheless, the results of adding drugs to the mudpuppy preparation were clear: 1) only NMDA triggered locomotion in a resting preparation. 2) Carbachol disrupted the walking rhythm induced by NMDA, while clonidine had no effect. 3) Physostigmine disrupted the walking rhythm, probably by potentiating the effects of endogenously released ACh 4) Atropine blocked the effects of the ACh agonist. Thus, all our data indicate that ACh disrupts the walking rhythm in the mudpuppy by acting on muscarinic receptors, while the α_2 noradrenergic agonist, clonidine, has no effect.

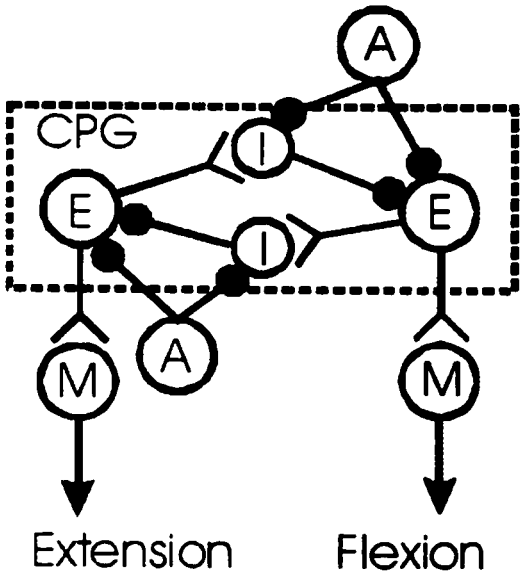

Fig. 4-1. Possible model for the role of ACh in the mudpuppy. The CPG (rectangular box) contains excitatory (E) interneurons connected by mutually inhibitory (I) interneurons. The excitatory interneurons activate the extensor and flexor motor neurons (M). We propose that other interneurons that use ACh as a transmitter (A) produce a balanced inhibition to the excitatory and inhibitory interneurons and thereby disrupt the rhythm without producing a net change in the activity of motor neurons.

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