Cholinergic and Serotonergic Excitation of Ascending Commissural Neurons in the Thoraco-Lumbar Spinal Cord of the Neonatal Mouse

K. P. Carlin, Y. Dai, and L. M. Jordan
Department of Physiology, University of Manitoba, Winnipeg, Manitoba, Canada

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Carlin, K. P., Y. Dai, and L. M. Jordan. Cholinergic and serotonergic excitation of ascending commissural neurons in the thoraco-lumbar spinal cord of the neonatal mouse. J Neurophysiol 95: 1278–1284, 2006. First published October 12, 2005; doi:10.1152/jn.00963.2005. Locomotion requires the coordination of the two sides of the spinal cord—a function fulfilled by commissural neurons. Ascending commissural neurons (aCNs) are known to be rhythmically active during locomotion, and mice lacking a population of aCNs display uncoupling between the left and right hemicords during locomotion. Acetylcholine (ACh) applied to the isolated spinal cord commonly produces left–right alternation, with co-contraction of ipsilateral flexor and extensor motoneuron groups. In this study, aCNs were examined in the neonatal mouse spinal cord after retrograde labeling with a fluorescent dextran. The axons of these cells crossed in the ventral commissure with many crossing in the same transverse plane as the cell body. For cells located in lamina VII and VIII, ACh (10–50 μM) depolarized 92% (13/14) of the cells tested. ACh depolarized and increased the excitability of aCNs in the presence of a decrease in input resistance. ACh was without significant effect on afterhyperpolarization amplitude or voltage threshold of action potential initiation. In those cells sensitive to application of ACh, 90% (9/10 cells) were also depolarized by 5HT (10–50 μM). Application of 5HT significantly increased the input resistance of these cells, and this effect was likely responsible for the observed increase in excitability, because significant effects on the afterhyperpolarization and voltage threshold were again not detected. The high proportion of aCNs excited by both ACh and 5HT suggests that direct activation of aCNs by these two neurotransmitters contributes to the production of a bilaterally coordinated locomotor-like rhythm in the isolated spinal cord.

INTRODUCTION

Motor behaviors often require the coordination of opposite sides of the spinal cord. This coordination is accomplished by commissural neurons—cells that send their axon across the midline of the spinal cord to affect contralateral network components. The axons of the commissural neurons important for one such bilaterally coordinated motor behavior, locomotion, are known to cross in the ventral commissure as lesions to this structure disrupt left–right coupling in the isolated neonatal rat spinal cord (Cowley and Schmidt 1997; Kjaerulff and Kiehn 1996; Nakayama et al. 2002).

In this same preparation, it is well described that coordinated left–right alternating activity can be elicited with bath application of either serotonin (Cazalets et al. 1992; Cowley and Schmidt 1994; Kiehn and Kjaerulff 1996) or acetylcholine (ACh) and/or acetylcholinesterase inhibitors (Cowley and Schmidt 1994; Jordan and McVaugh 2004; Kiehn et al. 1996; Smith and Feldman 1987). These results suggest that commissural cells are directly and/or indirectly activated by these neurotransmitters.

The lower thoracic–upper lumbar region of the spinal cord seems to have special significance with respect to the effect of these neurotransmitters. During ACh-induced rhythmic activity, a discrete lesion of T13 and L1 segments was capable of uncoupling the bilaterally coordinated motor output in the lower lumbar segments (Cowley and Schmidt 1997). Moreover, a series of lesioning experiments during serotonin-induced locomotor-like activity showed the supralumbar region of the cord to be critical for expression of this motor behavior (reviewed in Schmidt and Jordan 2000). These results suggest that commissural neurons in the lower thoracic–upper lumbar region are important for transmitting the locomotor signal across the spinal cord and may be activated by these rhythm-generating neurotransmitters. Because axons of ascending, descending, and bifurcating commissural cells can all be found in the ventral commissure (Stokke et al. 2002) and can produce collaterals in the contralateral hemicord (Bannatyne et al. 2003; Matsuyama et al. 2004), at present it is unclear which population is responsible for these effects.

A number of lines of evidence suggest a role for aCNs in the bilateral coordination of locomotion. First, in the fetal rat, aCNs in an area roughly corresponding to lamina VII and VIII labeled with calcium indicators are rhythmically active during drug-induced rhythmic activity (Nakayama et al. 2004). Second, transcription factor Dbx1 knockout mice display a higher incidence of uncoupled rhythm between the left and right halves of the spinal cord (Lanuza et al. 2004). Dbx1-positive cells have been shown to define a developing population of ventral commissural neurons, a portion of which are ascending (Pierani et al. 2001). Third, in the neonatal rat, electrical stimulation of the ventrolateral funiculus, which contains axons of some ascending commissural neurons (aCNs) (Antonino-Green et al. 2002), can elicit locomotion (Magnuson and Trinder 1997). Furthermore, midsagittal lesions showed the importance of fibers crossing in the lower thoracic and upper lumbar region of the cord for maintenance of rhythmic activity in these experiments. Finally, in the adult cat, rhythmically active locomotor-related aCNs have been identified in the intermediate zone of the cord (Huang et al. 2000; Matsuyama et al. 2004).

In this study, we sought to determine if aCNs in the lower thoracic and upper lumbar segments of the spinal cord were...
responsive to ACh and/or serotonin. To accomplish this, we visually patch clamped retrogradely labeled ascending commissural cells in a mouse spinal cord slice preparation and determined the effects of bath-applied serotonin and ACh.

METHODS

Spinal cord isolation

As described by Jiang et al. (1999), Balb C mice (P1–P8) were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and submerged in ice water for ~2–4 min before being killed by decapitation. During this time, a mixture of 95% O2-5% CO2 was given to the mice because respiration was compromised by the ketamine. After decapitation, mice were eviscerated and pinned ventral side up in a Sylgard-lined petri dish filled with 4°C dissecting artificial cerebrospinal fluid (ACSF). A vertebrectomy was performed, and the spinal cord was removed. Once isolated, the spinal cord was longitudinally hemisected rostral to the lumbar enlargement.

Retrograde labeling

aCNs were labeled using a procedure similar to that described previously (Eide et al. 1999; Nissen et al. 2005; Stokke et al. 2002). In this study, the water-soluble fluorescent tracer–tetramethylrhodamine dextran amine (RDA; 3,000 molecular weight; 25%; lysine fixable; Molecular Probes, Eugene, OR) was used to retrogradely label aCNs for subsequent electrophysiological analysis. To accomplish this, the spinal cord was maneuvered such that one hemicord was isolated from the fluid bathing the rest of the spinal cord by placing it in a small well and creating a petroleum jelly barrier (Fig. 1A). This well was filled with clear Sylgard (184; without hardener added) while the main bath contained regular ACSF. The end of the hemicord in the small well was cut at approximately the midthoracic level. The RDA was injected into the substance of the cut end with the use of a glass micropipette and micromanipulator. In addition to cells with ascending projections alone, this procedure would also label bifurcating cells (having ascending and descending axons; Eide et al. 1999; Stokke et al. 2002). For simplicity, this entire group of cells is referred to as aCNs.

If RDA was seen to escape into the main bath, the preparation was discarded. After allowing between 2.5 and 6 h for retrograde transport, spinal cord slices were prepared (Carlin et al. 2000) for electrophysiological experiments. The hemisected portions of the cord were removed, and the remaining cord was placed in warm 1% agar. The agar was quickly cooled, and the spinal cord was blocked and mounted in a Leica vibratome (VT1000E, Leica, Bannockburn, IL) filled with room temperature regular ACSF. Slices (180–200 μm) were prepared as needed during the experiment to reduce dye leakage.

Confirmation of the method used to identify an aCN in a slice can be seen in Fig. 1D. In this image, a single aCN is shown with its axon crossing in the ventral commissure and turning toward the ventral funiculus (inset). This cell was identified by its RDA labeling and was subsequently patch clamped with Lucifer yellow included in the pipette solution.
Electrophysiology

When viewed with an XF37 cube (Omega Optical), the injected side of the cord could readily be distinguished under low magnification because it contained a much larger number of labeled cells and fibers. The aCNs were identified as fluorescent cells on the side of the cord opposite to that injected. Often axons from these labeled cells could be followed across the ventral commissure (Fig. 1A). To limit phototoxic damage to labeled cells, once a labeled cell was identified, an image was captured (Argus 20 image processor and frame grabber, Hamamatsu Photonics, Bridgewater, NJ), patch clamping was performed under DIC illumination, and the identity of the cell was confirmed by comparison with the stored image (Fig. 1C). Whole cell recordings were made using either an Axopatch 1D or Multiclamp 700 amplifier (Axon Instruments, Union City, CA). Patch pipettes had a resistance between 3 and 5 MΩ when filled with intracellular solution. Series resistance (typically 10-30 MΩ) was balanced in current-clamp mode and adjusted as required throughout the experiment. All experiments were performed at room temperature (~22°C).

A gravity-driven perfusion system was used to both continuously perfuse slices with regular ACSF and apply neurochemicals at concentrations used to elicit locomotor-like activity in in vitro preparations; ACh (15-50 μM; Cowley and Schmidt 1997) and serotonin (SHT; 10–50 μM; Kiehn and Kjaerulff 1996).

Data analysis

Voltage threshold of action potential initiation was determined using the criteria outlined by Brownstone et al. (1992). Briefly, voltage traces from step current injections were chosen from control, test, and if possible, washout conditions. These traces were chosen to as closely as possible match similar initial membrane voltage and firing frequency. This resulted in comparing trains that contained between 3 and 26 action potentials [10.9 ± 6.4 (SD)]. Threshold was defined as the voltage at which the rising phase achieved a rate of ≥10 mV/ms (Brownstone et al. 1992). The first spike in each train was omitted from analysis, with subsequent values being averaged.

In a similar manner, afterhyperpolarization (AHP) amplitude was determined from these same traces that were matched for initial membrane voltage and frequency. AHP amplitude was defined as the difference between the threshold value and the most hyperpolarized potential between spikes.

Unless otherwise noted, statistical significance was determined with a single-tailed paired t-test in the direction of the expected result. In cases where paired t-tests were used, samples were normally distributed (Shapiro-Wilk test; P > 0.33 in all cases), and the variance of the populations was determined not to differ by more than twofold. If these criteria were not met, nonparametric tests were used.

Solutions and chemicals

The dissecting ACSF contained (in mM) 25 NaCl, 188 sucrose, 1.9 KCl, 1.2 NaH₂PO₄, 10 MgSO₄, 26 NaHCO₃, 1.5 kynurenic acid, and 25 glucose. The regular ACSF contained (in mM) 130 NaCl, 4.5 KCl, 1.25 NaH₂PO₄, 2.5 CaCl₂, 1.5 MgCl₂, 10 glucose, and 26 NaHCO₃. The pH of these solutions was ~7.4 when bubbled with carbogen gas.

The intracellular solution contained (in mM) 150 KMeSO₄, (Daniels Fine Chemicals, Edmonton, Canada), 10 NaCl, 10 HEPES, 0.1 EGTA, 3 Mg-ATP, and 0.3 GTP. Lucifer yellow (~1%; Molecular Probes) was added to the intracellular solution in some experiments. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

RESULTS

The labeling method used here, first described by Glover et al. (1986) in the nervous system of the chicken, allowed aCNs to be visualized in live mouse spinal cords without evident phototoxicity. In our hands, this method reliably labeled cells in the T₁₃ to approximately L₃ levels of the spinal cord depending on the length of time that the fluorescent dextran was allowed to travel and the length (age) of the cord. When examining the RDA labeling in 180- to 200-μm transverse sections, the axons of the aCNs could often be seen crossing within the plane of the slice (Fig. 1, B and D). The axons crossed in the ventral commissure and could be seen to make an abrupt turn toward the ventral medial funiculus on the opposite side of the cord. For the most part, the distribution of the aCNs was found to be similar to that described in the postnatal rat (Eide et al. 1999) and embryonic mouse (Nissen et al. 2005).

Electrophysiology/pharmacology

Electrophysiological data were collected from 31 labeled aCNs. Cells were targeted because of their large soma size and ease of identification in both DIC and fluorescent modes. These cells were distributed mainly in lamina VII, but a few in lamina VIII were also sampled because cells in both of these areas have been implicated in the generation of locomotor output (Carr et al. 1995; Dai et al. 2005; Kjaerulff et al. 1994). Because cells in these two lamina showed similar sensitivity to the two neurotransmitters, the data were pooled. Consistent with their large size, the sampled cells had an average whole cell capacitance of 183 ± 125 (SD) pF and input resistance (Rᵢ) of 217 ± 145 MΩ.

Bath application of ACh elicited a depolarization in 92% (13/14) of aCNs. Ten of these cells were also tested for sensitivity to SHT, with 90% (9/10) being depolarized by this neurotransmitter as well. The effects of these two neurotransmitters on membrane properties are discussed in the following sections. The subsample of cells tested for sensitivity to neurotransmitters did not differ significantly from the electrophysiologically sampled population with respect to capacitance or Rᵢ (Kruskal-Wallis; P > 0.9). These values were 190 ± 141 pF and 223 ± 140 MΩ and 207 ± 158 pF and 192 ± 101 MΩ for cells tested for sensitivity to ACh and those sensitive to ACh and SHT, respectively.

ACH

ACh-elicited depolarizations ranged from a few millivolts to ones that depolarized the cell to threshold (Fig. 2A). Twelve cells were examined for changes in synaptic input after application of ACh; the baseline noise was seen to increase in eight of these cells (Fig. 2B). This increased noise could be reduced with application of TTX (Fig. 2B, bottom trace), suggesting that the ACh had effects on cells presynaptic to the aCN being examined. This effect is unlikely to be mediated by release of glutamate because of the presence of the ionotropic glutamate receptor antagonist kynurenic acid (1.5 mM) in the extracellular solution. In the presence of TTX (1 μM; Fig. 2C), ACh was still capable of eliciting a depolarization (7.5 ± 5.8 mV; 3/3 cells), suggesting direct postsynaptic receptor activation.

ACh application led to a decrease in Rᵢ in a majority of cells (8/11). This statistically significant reduction in Rᵢ averaged 57.5 ± 62 MΩ (P = 0.017; paired t-test). In five of six cells, ACh reduced the amplitude of the AHP, but this effect
was small (1.1 ± 1.2 mV) and failed to reach significance compared with control values (*P < 0.05; Wilcoxon rank sum test). Similarly, ACh did not produce significant effects on the voltage threshold. It should be noted that these two later assessments were likely hampered by the small sample size in this study.

Excitability changes were confirmed in these cells by assessing the response of the cells to step current injection. Six of nine cells showed a reduction in the amount of current required to elicit firing and/or an increase in the firing rate in response to a given current stimulus (Fig. 2D). This increase in excitability could be seen as a leftward shift in the frequency-current (F-I) relationship (Fig. 2E).

Although it was not the goal of this study to evaluate active membrane properties, it was noticed that a number of aCNs displayed membrane oscillations during recording. Of the four cells seen to oscillate, three of three cells tested were found to be responsive to ACh. Two cells were capable of oscillations in control solution, whereas oscillations in two other cells were only observed after application of ACh. The cell shown in Fig. 2F was seen to oscillate in the presence of TTX, suggesting an intrinsic mechanism rather than being of synaptic origin. Furthermore, this cell showed an unusual inverse relationship between oscillation frequency and membrane potential.

**5HT effects on ACh-responsive cells**

The effects of 5HT were examined on 10 cells that were depolarized by ACh application. In this subset of cells, 9/10 were also depolarized with the application of 5HT (Fig. 3A). Similar to the response elicited by ACh, baseline noise consisting of small depolarizing potentials was seen to increase (Fig. 3B, inset), again suggesting an action of the 5HT on presynaptic elements in the slice. In the presence of TTX (1 μM; Fig. 3C), 5HT elicited depolarizations in four of four cells (12 ± 14 mV). As with ACh application, this suggests a direct action of 5HT on these cells.

In contrast to the decrease in \( R_i \) seen with ACh application, 5HT significantly increased the \( R_i \) in the majority of cells (6/8; 1 no change) that responded to both neurotransmitters. This
statistically significant increase in $R_i$ averaged 66 ± 81 MΩ ($P = 0.035$; paired $t$-test). Consistent with this increase in $R_i$, the excitability of these cells to current injection was increased (6/7 cells). This was seen as either an increase in the firing frequency for a given current stimulus or by a lowering of the amount of current required to elicit spiking (Fig. 3, D and E). As seen with ACh application, effects on AHP amplitude and voltage threshold with 5HT were not significant ($P > 0.05$; Wilcoxon rank sum test).

Membrane potential oscillations were observed in two cells that were responsive to both ACh and 5HT. One produced oscillations of irregular frequency near threshold for firing after application of ACh, whereas oscillations in the other cell (Fig. 3F) occurred in control solution at more hyperpolarized potentials.

**DISCUSSION**

Previously, studies performed in the adult cat have characterized spinal commissural neurons both anatomically and functionally (Bannatyne et al. 2003; Hammar et al. 2004; Huang et al. 2000; Jankowska et al. 2003; Krutki et al. 2003; Matsuyama et al. 2004). Furthermore, both anatomical (Eide et al. 1999; Nissen et al. 2005; Stokke et al. 2002) and functional (Butt and Kiehn 2003; Butt et al. 2002; Nakayama et al. 2002) characterization has been performed in the neonatal rodent. This study anatomically identified a subpopulation of commissural neurons, those with an ascending projection, and electrophysiologically examined the effects of two neurotransmitters capable of activating locomotor circuitry.

The main finding of this study is that aCNs in the thoracolumbar segments of the mammalian spinal cord are excited by both ACh and 5HT. The ability to see the effects of these neurotransmitters while synaptic input was suppressed with TTX suggests that their action is mediated directly on the postsynaptic membrane of these cells. The mechanism by which these neurotransmitters exert their action is not clear at present, but it is clear that these two neurotransmitters are working through different mechanisms. The cholinergic effect was seen to be dominated by a decrease in input resistance consistent with the opening of membrane channels, whereas 5HT had the opposite effect on input resistance. The observations in this study permit an initial description of a subclass of spinal neuron: a ventrally located interneuron with an ascending projection that is responsive to both ACh and 5HT and whose membrane potential may be capable of oscillating. Cells responsive to 5HT but unresponsive to ACh were observed in this study. The effect of 5HT on these cells was also depolarizing, but effects on the other parameters measured ($R_i$, AHP, and $V_{th}$) were inconsistent.

With respect to ACh application, the identity of the channel type or types mediating the decrease in input resistance is presently not known. The observation of a decrease in resistance alone does not allow either the nicotinic ionotropic or muscarinic metabotropic ACh receptors to be ruled out as possible mediators, because stimulation of both nicotinic (Dajas-Bailador and Wonnacott 2004) and muscarinic receptors (Egorov et al. 2003; Fraser and MacVicar 1996) can activate an inward cation current and subsequent second messenger systems. These observations do, however, discount the inhibition
of an M-type potassium current or closure of other types of potassium channels as the major depolarizing influence.

In spinal neurons, 5HT has been shown to increase excitability through a number of postsynaptic mechanisms. In this study, the 5HT-evoked depolarization was accompanied by an increase in input resistance in the majority of cells. This finding is consistent with the effect of 5HT on spinal motoneurons of the cat (White and Fung 1989), neonatal rat (Elliott and Wallis 1992; Takahashi and Berger 1990; Ziskind-Conhaim et al. 1993), adult turtle (Perrier et al. 2003), and chick (Hayashi et al. 1997).

Because we do not know the termination sites of the labeled cells in this study, these cells may belong to well-defined aCN populations such as cells of the spinothalamic, spinoreticular, or ventral spinocerebellar tracts. For the spinothalamic and ventral spinocerebellar populations, in particular, both anatomical (Hammar and Maxwell 2002) and electrophysiological (Hammar et al. 2002; Jordan et al. 1979) evidence has been provided for serotonergic input. While there exists some evidence for cholinergic input to spinothalamic cells of the dorsal horn (Willecockson et al. 1984), evidence for cholinergic modulation of ventral cells of this population is absent. This work therefore represents the first description of cholinergic modulation of commissural spinal interneurons of the ventral horn.

Although oscillations are not a unique active membrane property, the voltage dependence of the frequency of the oscillations of the cell shown in Fig. 3D is unusual. In this cell, the frequency of the TTX-resistant subthreshold oscillations increased as the membrane was hyperpolarized. This type of inverse relationship between membrane potential and frequency has previously been shown in thalamic cells (Williams et al. 1997) and Hb9-positive interneurons in the spinal cord (Wilson et al. 2005). The observation of this interesting property in aCNs merits further examination.

In summary, this study provides an initial profile of aCNs in the mammalian thoraco-lumbar spinal cord. Given that some aCNs give off the necessary collaterals to affect segmental networks (Matsuyama et al. 2004), the role of the aCNs in pattern generation may be to provide both contralateral coordination as well as to coordinate more rostral segments. The high proportion of aCNs excited by both ACh and 5HT in this study suggests a role for both of these neurotransmitters in these processes as well. These results may partially explain the ability of both of the neurotransmitters to elicit a bilaterally coordinated pattern in the isolated spinal cord. This study also provides preliminary evidence that membrane potential oscillations may be part of the repertoire of aCNs. In other parts of the CNS, subthreshold oscillations are thought to function as a synchronizing or timing device (Lampl and Yarom 1993). If oscillations are a common trait of aCNs, these cells may use this ability to synchronize the left and right halves of the motor circuitry in the spinal cord.

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