Adult spinal V2a interneurons show increased excitability and serotonin dependent bistability

Abbreviated title: Adult spinal V2a interneurons

Andreas Husch, Shelby B. Dietz, Diana N. Hong and Ronald M. Harris-Warrick
Department of Neurobiology and Behavior, Cornell University, Ithaca, NY, 14853

Corresponding author:
Ronald M. Harris-Warrick
Department of Neurobiology and Behavior
Seeley Mudd Hall
Cornell University
Ithaca NY 14853
Email: ron.harris-warrick@cornell.edu

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Abstract

In mice, most studies of the organization of the spinal central pattern generator (CPG) for locomotion, and its component neuron classes, have been performed on neonatal (P2-P4) animals. While the neonatal spinal cord can generate a basic locomotor pattern, it is often argued that the CPG network is in an immature form whose detailed properties mature with postnatal development. Here, we compare intrinsic properties and serotonergic modulation of the V2a class of excitatory spinal interneurons in behaviorally mature (>P43) mice to those in neonatal mice. Using perforated patch recordings from genetically tagged V2a interneurons, we revealed an age-dependent increase in excitability. The input resistance increased, the rheobase values decreased and the relation between injected current and firing frequency (f/I plot) showed higher excitability in the adult neurons, with almost all neurons firing tonically during a current step. The adult action potential (AP) properties became narrower and taller, and the AP threshold hyperpolarized. While in neonates the AP afterhyperpolarization was monophasic, most adult V2a interneurons showed a biphasic afterhyperpolarization. Serotonin increased excitability and depolarized most neonatal and adult V2a interneurons. However, in about 30% of adult V2a interneurons, serotonin additionally elicited spontaneous intrinsic membrane potential bistability, resulting in alternations between hyperpolarized and depolarized states with a dramatically decreased membrane input resistance and facilitation of evoked plateau potentials. This was never seen in younger animals. Our findings indicate a significant postnatal development of the properties of locomotor-related V2a interneurons, which could alter their interpretation of synaptic inputs in the locomotor CPG.

Keywords

Locomotion, maturation, neuromodulation, spinal cord
Introduction

The existence of central pattern generator (CPG) neuronal networks in the spinal cord which coordinate locomotion was first demonstrated and studied in adult cats and dogs (Brown 1914; Eccles et al. 1956; Sherrington 1910). Non-mammalian model organisms such as the lamprey and zebrafish have provided important advances in understanding the cellular composition and function of the locomotor CPG (Ausborn et al. 2012; Buschges et al. 2011; Fetcho et al. 2008; Grillner 2003; McLean et al. 2008). The combination of molecular genetics, electrophysiology and imaging techniques in recent years have made the mouse spinal cord an important model system to study the organization of CPGs for mammalian locomotion (Arber 2012; Grossmann et al. 2010; Guertin 2009; Kiehn 2011; 2006).

Several interneuron (IN) classes have been identified by the expression of unique combinations of transcription factors (Goulding 2009; Goulding and Pfaff 2005), allowing scientists to label, record and manipulate the activity of each class during fictive locomotion (Gosgnach et al. 2006; Lanuza et al. 2004; Talpalar et al. 2013; Vallstedt and Kullander 2013). This combined approach has shown that the V2a class of Chx10-expressing ipsilaterally projecting glutamatergic INs plays a number of roles in the spinal cord. They contribute to the descending inputs that normally drive locomotion (Crone et al. 2008; Kimura et al. 2013), and cervical V2a INs help to control skilled reaching (Azim et al. 2014). In the zebrafish, stimulation of V2a INs is sufficient to initiate locomotion in the zebrafish (Ljunggren et al. 2014), and distinct subsets of these neurons are activated with increasing locomotor speed to recruit additional motoneurons for faster swimming (Ampatzis et al. 2014; Fetcho and McLean 2010). In the mouse lumbar spinal cord (where the hindlimb locomotor CPG is located), these neurons are functionally heterogeneous (Dougherty and Kiehn 2010; Zhong et al. 2010); one population shows no locomotor-related activity while other locomotor-related populations synapse on motoneurons or commissural interneurons (Crone et al. 2008; Zhong et al. 2012).
These neurons are increasingly recruited with locomotor speed (Zhong et al. 2011); V2a ablation has little effect on locomotion at low speeds but disrupts locomotion at intermediate speeds and results in left-right synchrony at high speeds (Crone et al. 2009). This suggests that at least a subset of V2a interneurons functions to maintain left-right alternation at high locomotor speeds. (Al-Mosawie et al. 2007; Crone et al. 2008; Crone et al. 2009; Lundfald et al. 2007; Zhong et al. 2011).

The locomotor-related V2a INs receive descending activation from the brain (Crone et al. 2008), including serotonergic innervation. Serotonin (5-HT) plays a key role in spinal cord reflex and locomotor pattern generation (for review see Cazalets et al. 1990; MacLean et al. 1998; Schmidt and Jordan 2000). Distinct components of the motor pattern are controlled by multiple serotonin receptor subtypes (Al-Mosawie et al. 2007; Landry et al. 2006; Liu et al. 2009; Madriaga 2004; Musienko et al. 2011; Pearlstein et al. 2005; Slawinska et al. 2012), which modulate different cellular properties and ionic conductances in locomotor related INs (Abbinanti and Harris-Warrick 2012; Dai and Jordan 2010; Diaz-Rios et al. 2007; Zhong et al. 2006a). In neonatal (P1 - P4) V2a neurons, 5-HT depolarizes and increases neuronal excitability and changes action potential properties (Dietz et al. 2012; Zhong et al. 2010).

To understand the role of 5-HT during rodent locomotion, it is important to determine its effects on adult as well as neonatal spinal neurons. Serotonergic processes reach the lumbar spinal cord by E16, but the innervation pattern is still diffuse after birth, and only achieves a more restricted adult pattern by P21 (Ballion et al. 2002; Rajaofetra et al. 1989). Most studies of spinal locomotor networks have been performed in neonates (typically P0-P5). These studies have furthered our understanding of the neuronal composition of the locomotor CPG, since it is possible to record from identified spinal INs in the isolated intact neonatal spinal cord during fictive locomotion. However, the question remains whether the properties of the neonatal interneurons remain the same as the networks mature to the fully locomoting adult. Postnatal changes in cellular properties during the first 2-3 postnatal weeks, such as narrowing of action
potential shape and emergence of plateau potentials, have been shown in CINs (Abbinanti et al. 2012) and in motoneurons (Carlin et al. 2008; Gao and Ziskind-Conhaim 1998). By developing a method to record electrophysiological activity from spinal INs in adult spinal cord slices of any age (Husch et al. 2011), we can now answer this question more fully. In this paper we demonstrate marked developmental changes in the electrophysiological properties of spinal V2a INs between neonatal (P2 to P4) mice and behaviorally mature adult (>P43) mice.

Materials and Methods

Animals

Experiments were performed using two age groups: neonatal mice (postnatal (P) day P2-P4) and adult, locomotor mature mice (P43–P93). Chx10::CFP heterozygotes, with one recombined Chx10::CFP allele and one wild-type Chx10 allele (Crone et al., 2009) were generated by Drs. Steven Crone and Kamal Sharma at the University of Chicago (Crone et al. 2008). The animal protocol was approved by the Institutional Animal Care and Use Committee at Cornell University and was in accordance with National Institutes of Health guidelines.

Immunocytochemistry and confocal microscopy.

Neonatal and adult mice were anesthetized with ketamine/xylazine IP (150 mg/kg ketamine; 15 mg/kg xylazine), and perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer for 20 minutes. The spinal cord was removed, and the rostral portion of the upper lumbar enlargement postfixed in 4% PFA, 0.1 M phosphate buffer solution for 1.5 hours at 4°C, then transferred to 30% sucrose for 12 hours at 4°C. A cryostat was used to make 15 μm transverse sections of the upper lumbar enlargement. At room temperature, slides were rinsed in phosphate buffered saline (PBS) bath for five minutes. Slides were incubated in 0.2% Triton X-100 for 10 minutes, then rinsed with three changes of PBS over 15 minutes. Slices
were incubated in peroxidase quenching buffer for 60 minutes at room temperature, and then 1% blocking reagent for 60 minutes at room temperature. Slices were incubated with the primary antibody (goat polyclonal to GFP 1:250, Immunostar), for 16 hours at 4°C. Slides were rinsed in 4°C PBS for 15 minutes, then incubated in secondary antibody (donkey polyclonal to goat DyLight 488) for 45 minutes at room temperature, and rinsed in PBS for 15 minutes. Slides were then mounted with Fluor-Gel. A Leica TCS SP2 confocal microscope was used for imaging using the 488 nm laser line. Z-series were taken through the 15 μm tissue in 1 μm steps.

Spinal cord slice procedure in adult mice

The adult mouse spinal cord slice procedure has been described previously in detail (Husch et al. 2011). Animals were deeply anesthetized with ketamine (1.5 mg/10g body weight) and xylazine (0.15 mg/10g body weight) and placed on ice. Pure oxygen was administered via a custom built air mask. The sacral to mid-thoracic spinal cord was exposed by a dorsal laminectomy. Once exposed, the cord was superfused with ice-cold (0-4°C) oxygenated (95% O₂ / 5% CO₂), glycerol-based modified artificial cerebrospinal fluid (GACSF; Ye et al. 2006) which contained (in mM): 222 glycerol, 3.08 KCl, 1.18 KH₂PO₄, 1.25 MgSO₄, 2.52 CaCl₂, 25 NaHCO₃, 11 D-glucose. The ventral and dorsal roots and the meninges were removed. The spinal cord was transferred to low melting point agarose, and 250 μm transverse sections of the upper lumbar cord were made with a vibrating blade microtome (HM-650 V; Thermo Scientific). The slices were immediately transferred for 45 min at 35°C in oxygenated ACSF (in mM: 111 NaCl, 3.08 KCl, 1.18 KH₂PO₄, 1.25 MgSO₄, 2.52 CaCl₂, 25 NaHCO₃, 11 D-glucose. The slices were allowed to recover at room temperature for one hour. Fluorescent INs were visualized with a fixed-stage upright microscope (BX51WI, Olympus) using a 60x water-immersion objective.
Spinal cord slice procedure in neonatal mice

Neonatal (P2-P4) male and female Chx10::CFP mice were euthanized by decapitation and their spinal cords removed by ventral laminectomy (Jiang et al. 1999; Kudo and Yamada 1987) in ice-cold (4°C), oxygenated (95% O₂ / 5% CO₂) DACSF. The meninges were removed and the upper lumbar cord embedded in agarose. Transverse slices (250 µm) were prepared using a vibrating microtome (HM 650 V, Thermo Scientific). The slices were maintained in oxygenated ACSF.

Perforated patch recordings

A detailed description of the perforated patch procedure in adult mouse spinal cord slices has been provided previously (Husch et al. 2011). Slices were continuously superfused with oxygenated ACSF at a flow rate of ~2 ml/min. To block most synaptic input in the slices, neurons were isolated from rapid synaptic inputs with a combination of D,L-2-Amino-5-phosphonopentanoic acid (AP-5, 10 µM) and CNQX disodium salt hydrate (CNQX, 10 µM) to block glutamatergic synapses; picrotoxin (10 µM) to block GABAergic synapses; and strychnine (10 µM) to block glycinergic synapses. Patch pipettes were made with thick wall borosilicate glass (WPI) on a vertical puller (Narishige) with low resistances of 3-5 MΩ. The tip of the pipette was filled with intracellular solution containing (in mM) 135 K-gluconate, 10 KCl, 10 Hepes, 0.1 EGTA, 2 MgCl₂ (adjusted to pH 7.2 with KOH). To prepare for the perforated patch recording procedure (Rae et al. 1991) the pipette was backfilled with a combination of intracellular solution, Amphotericin B (1.2 mg/ml) and pluronic F127 (1 mg/ml). All chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO).
Data acquisition and analysis

Current clamp recordings were made with a Multiclamp 700B amplifier controlled by Clampex (pClamp 9, Molecular Devices, Sunnyvale, CA). Data were sampled at 10 kHz (20 kHz for action potential (AP) shape analysis) and low-pass filtered at 2 kHz. For AP analysis, the membrane potential was adjusted with holding current ($I_{\text{hold}}$) to set the spontaneous firing frequency at 1 Hz or lower to elicit temporally isolated APs. The voltage threshold for AP generation was measured as the peak of the second derivative of voltage with time during the rising phase of the AP. The spike amplitude was measured from the peak of the AP to the peak afterhyperpolarization. The AP half-width was established at the voltage halfway from the spike threshold to the peak of the AP. To measure the membrane input resistance and rheobase, all neurons were held below threshold at -60 mV with $I_{\text{hold}}$. Input resistance was estimated by averaging the responses to small hyperpolarizing current pulses. The minimal amount of current necessary for spike generation from -60 mV was defined as the rheobase. To generate current-frequency (I-V) plots, the neuron was stimulated by a series of increasing current steps; instantaneous firing frequency was the inverse of the first interspike interval, while the average firing frequency was calculated by dividing the number of evoked action potentials by the stimulation duration. To measure the spontaneous firing rate, the mean firing rate of twelve 10 second bins was averaged. To keep our data comparable to previous work on V2a interneurons, we did not correct for the liquid junction potential.

Statistics

All data were analyzed using Excel (Microsoft), Clampfit (Axon Instruments), JMP (SAS), and MATLAB (MathWorks). Wilcoxon rank sum tests were used to compare adult versus neonate parameters, and Wilcoxon rank sum to compare control vs. 5-HT parameters in the same cell. A significance level of 0.05 was accepted for all tests. To compare the response to 5-HT in adult
and neonatal neurons we used regression analysis, with the absolute difference in a parameter under control conditions and during 5-HT as the dependent variable and both age group and initial value of the parameter in control conditions as independent variables. If the initial value variable was not significant, that independent variable was removed and the regression re-run with the age group as the only independent variable. In order to make the regression more correct, data with non-normal distributions were transformed to make the residuals of least-squares best fit normally distributed (Neter 1996). F-I regressions used a random coefficient model to correct for multiple measurements from each cell. In the box plots, the extent of the box indicates the 25th and 75th percentiles, and the central line represents the median. Whiskers extend to maximum and minimum values within $q_3 + w(q_3 - q_1)$ and $q_1 - w(q_3 - q_1)$, where $q_1$ and $q_3$ are the 25th and 75th percentiles, respectively, and $w = 1.5$. Outliers outside these boundaries are plotted as black dots and are included in all analysis. All error bars represent the standard deviation.

Results

Morphological differences between neonatal and adult V2a spinal Interneurons

Mice display dramatic developmental changes in body size, body mass and behavior repertoire over the first 4 weeks of life. They double their length and body weight in the first 4-5 weeks and change their locomotor behavior from nonmoving to normal rapid running. With body growth, the spinal cord increases in diameter (Fig. 1A). While in neonates many CFP-labeled V2a interneurons (INs) (>50) were visible in each lumbar section of the cord, in adults only about 5-10 V2a INs were visible. To confirm and quantify the decrease in the number of visible somata per slice, we conducted immunocytochemistry to amplify fluorescence of eCFP labeled neurons in the lumbar enlargement of the spinal cord (Fig. 1B,C). In neonatal slices we found 30.6 ± 3.7 labeled somata per slice (n = 12), while in adult mice we found 14.5 ± 2.5 somata (n = 12;
p = 0.001; Fig. 1D). The other obvious age-dependent difference in the appearance of the V2a INs was the soma size. Even though the diameter of the neonatal spinal cord was much smaller (Fig. 1A), the neonatal V2a somata appeared to be larger in diameter than the adult somata. This difference was confirmed by measuring the cell capacitance during patch recordings. The neonatal V2a INs showed a significantly higher capacitance compared to adult neurons (neonate $C_M = 17.9 \pm 1.3$ pF, $n = 19$; adult $C_M = 12.2 \pm 0.6$ pF, $n = 24$, $p = 0.002$, Fig. 1E), indicating smaller somata in adults.

### Intrinsic properties of V2a interneurons in neonatal and adult ages

Previous work in neonatal V2a INs using whole cell recordings revealed 3 classes of V2a neurons with distinct firing properties in response to depolarizing square pulse current injections (Zhong et al. 2010). Using perforated patch clamp recordings from neonatal V2a INs, we found a similar distribution of firing types: 45% of the neurons fired tonically during a depolarizing current pulse (tonic class: Fig. 2A), 45% were phasic with an initial burst of action potentials (APs) that terminated during the step (phasic class), and 10% of the cells fired tonically during current injection but with a marked delay of about 200 ms before firing onset (delay class). In contrast to this neonatal distribution, the majority of adult V2a neurons (90%) showed tonic firing with no delay during current steps (Fig. 2A), whereas only 3% were phasic and 7% showed a delayed onset of tonic firing. Since almost all the adult V2a INs were in the tonically firing class, we decided to compare only the tonic firing V2a IN properties between neonatal and adult ages.

Using a series of increasing depolarizing step current injections (5 pA increments, 1 s duration, Fig. 2A), we measured the relation of firing frequency to injected current ($f$-$I$-plot, Fig. 2A). A regression analysis with a random coefficient model to correct for multiple measurements from each cell revealed that the slope of the mean firing rates ($p = 0.0001$, $n = 10-14$; Fig. 2B) and the instantaneous initial firing rates ($p = 0.01$, Fig. 2C) with increasing current were significantly larger in adult V2a cells compared to neonatal neurons. The excitability was
increased in adults, as reflected in lower rheobase values compared to neonatal V2a INs (n = 20-28, p = 0.003, Fig. 2D). When holding the neurons at $V_{\text{hold}} = -60$ mV, the membrane input resistance was significantly higher in adult neurons compared to neonatal neurons (n = 20-25, p = 0.01; Fig. 2E). The neonatal V2a neurons were slightly more depolarized, as seen by significantly greater negative holding current needed to hold them at -60 mV (n = 20-26, p = 0.04, Fig. 2F). This finding was confirmed by measuring the membrane potential of all cells which did not exceed 1 Hz spontaneous firing: neonatal V2a cells were more depolarized (neonate $E_m = -44.4 \pm 5.6$ mV, n = 16) than adult V2a INs (adult $E_m = -52.8 \pm 9.3$ mV, n = 18, p < 0.0001).

One of the most strikingly different features with age was the AP shape. In adult V2a INs, the AP width decreased to half that of neonatal V2a APs (n = 20-22, p < 0.0001, Fig. 3A,B). In addition, in adult V2a INs, the AP threshold was significantly more negative (p = 0.01, Fig. 3C), and the mean AP amplitude was increased by about 10 mV (p = 0.0001, Fig. 3D). While the AP afterhyperpolarization in all neonatal V2a INs was monophasic with a slow afterhyperpolarization peak at about 50 ms after the AP, in most adult V2a INs the post peak kinetics were more complex (Fig. 3E). Most (76 %) adult neurons showed a biphasic afterhyperpolarization (AHP), with a fast AHP that showed a time to peak of about 4 ms after the spike peak (Fig. 3F), followed by a repolarization and a second slower AHP. The time between AP peak and the slow AHP peak was similar to that seen in neonatal V2a INs (p = 0.4, n = 20, Fig. 3G.), and the amplitude of the slow AHP was similar at both ages (p = 0.1, n = 20-21, Fig. 3H). The remaining 24% showed APs with a monophasic slow afterhyperpolarization similar to the neonatal AP AHP. The adult APs showed a higher variability in these parameters reflected by a remarkable increase in the standard deviation (Fig. 3G).
Effects of serotonin on intrinsic firing properties

To compare 5-HT responses in neonatal and adult V2a INs, we applied 10 µM 5-HT in current clamp mode, initially without holding current, and recorded spontaneous membrane potential and firing changes (Fig. 4A,B); this 5-HT concentration evokes a strong response at all ages (Husch et al. 2012). Since many neurons fired tonically before 5-HT application (which hinders an accurate measurement of the membrane potential), we also measured the membrane potential indirectly by monitoring the holding current at -60 mV; this serves as a proxy for the membrane potential in tonically active as well as silent neurons. In a previous study (Dietz et al., 2012), we introduced Amphotericin B perforated patch recordings in neonatal V2a INs and compared whole cell to perforated patch measurements of neuronal activity in the response to 5-HT. Neonatal V2a INs responded to 5-HT with depolarization, as measured by the holding current at -60 mV (n = 20, p < 0.0001, Fig. 4C) and initiation or acceleration of firing (n = 20, p < 0.0001; Fig. 4D). Adult V2a INs showed a similar response to 5-HT, with depolarization (n = 16, p = 0.0004; Fig. 4C) and an increase in firing rate (n = 11, p = 0.03, Fig. 4D). In neonatal V2a INs, input resistance was increased with 5-HT application (n = 20, p =0.002; Fig. 4E). In 8 of 16 adult V2a INs, 5-HT clearly increased the input resistance at -60 mV, often with a clear washout (n = 8, p = 0.001). While most of the remaining cells had a stable input resistance during 5-HT application, one adult cell responded with a decreased membrane resistance on 5-HT application with a partial washout (control: 1.9 ± 0.1 GΩ, 5-HT: 1.6 ± 0.1 GΩ, wash: 1.7 ± 0.1 GΩ). The magnitude of the change in response to 5-HT was larger in neonates for the holding current (p = 0.0001, Fig. 4C) and firing rate (p = 0.03, Fig. 4D); but not the input resistance (Fig. 4E). Both neonatal and adult V2a INs showed significantly increased excitability during 5-HT application. As a first excitability measure, we sought a reduction in the rheobase, calculated as the minimal current injected from -60 mV to reliably elicit an AP. In both neonatal and adult V2a INs, 5-HT reduced the rheobase (neonates: n = 20, p < 0.0001; adults: n = 17, p < 0.0001, Fig.4F). The relative change of the rheobase during 5-HT was not significantly different between
neonates and adults (neonates: $\Delta l_{\text{rheo}} = -28 \pm 24 \%$; adults: $\Delta l_{\text{rheo}} = -34 \pm 21 \%$; $p = 0.46$, Fig. 4F).

We then measured the effect of 5-HT on the shape of spontaneous low-frequency APs (Fig. 4G-K) and compared the magnitude of response between the age groups. In neonatal V2a IN recordings, 5-HT increased the AP amplitude ($n = 20$, $p = 0.049$, Fig. 4G), prolonged the AHP peak time ($n = 20$, $p = 0.0003$, Fig. 4K), and lowered the AP threshold voltage ($n = 20$, $p = 0.0001$, Fig. 4H). There was no effect of 5-HT on AP width (Fig. 4I) or AHP amplitude (Fig. 4J)(Dietz et al. 2012). In adult V2a INs, 5-HT reduced the AP threshold ($n = 11$, $p = 0.001$, Fig. 4H). Interestingly, 5-HT did reduce the amplitude of the slow component of the AHP in adult V2a INs ($n = 11$, $p = 0.02$, Fig. 4J) as has previously been reported in mouse CINs (Zhong et al. 2006b) and cat motoneurons (White and Fung 1989). The AP amplitude, AP width, and AP afterhyperpolarization kinetics were not significantly affected by 5-HT (Fig. 4,G,I,K). For AP amplitude, the magnitude of the change in response to 5-HT was larger in neonates than adults ($p = 0.046$, Fig. 4G); there was no difference in the magnitude of the change in any other measured AP parameter (Fig. 4H-K).

**Effects of serotonin on membrane potential bistability**

In neonatal V2a INs, serotonin (5-HT) did not evoke significant bistable activity (Zhong et al., 2010; Dietz et al., 2012). In marked contrast, in more than 30% of the adult 5-HT sensitive V2a INs, 5-HT introduced periodic membrane potential alterations, causing pronounced irregular firing and shifts in average firing frequency with time (Fig. 5A). Eventually, a neuron would accelerate its firing frequency and depolarize to a state of depolarization block, where no spontaneous AP firing occurred (Fig. 5B,6A). The membrane potential then dropped below AP threshold and firing resumed during subsequent spontaneous depolarizations (Fig. 5B1,B2). In some recordings (2/7), the APs disappeared altogether and it became obvious that the oscillations were not directly mediated by the APs, as they continued in the absence of APs.
(Fig. 5B3). These membrane potential oscillations (0.1 – 0.2 Hz) disappeared upon removal of 5-HT (Fig. 5B4).

During longer 5-HT applications, some adult V2a INs remained in depolarization block for long periods of time (Fig. 6A). With negative current injection, it was possible to restore spontaneous firing of overshooting APs upon post-inhibitory rebound (Fig. 6B, beginning). Releasing the negative holding current elicited a brief bout of high frequency firing that once again resulted in a silent depolarization block. Small hyperpolarizing current pulses revealed a dramatic decrease in membrane input resistance from 1.3 GΩ in the hyperpolarized state to values as low as 0.2 GΩ during the depolarization block (Fig. 6B). In cases where 5-HT established bistable activity with alternating down and up states, the silent depolarization block was typically terminated by a rather sudden repolarization to a nonfiring hyperpolarized state (Fig. 6C). During these prolonged periods of depolarization block (Fig. 6D, grey area), the membrane input resistance increased gradually until the sudden repolarization, where the input resistance reached a peak (Fig. 6D).

In 6 of the V2a INs, which showed 5-HT-induced bistability, 5-HT application facilitated the appearance of plateau potentials upon depolarizing current injections from -60 mV, with accelerating spike frequency that often culminated in depolarization block (Fig. 7). This plateau state outlasted the current step by several seconds before it spontaneously repolarized to a more negative potential than before the current injection (Fig. 7). In neonatal V2a INs we have never been able to elicit plateau potentials with depolarizing current injections. Taken together, these findings suggest that the V2a INs show more complex intrinsic properties in locomotor-mature adults.
Discussion

In the present study, we show that spinal V2a interneurons (INs) undergo extensive maturation in their intrinsic properties from neonatal to adult ages. The results clearly show that the V2a intrinsic properties in neonates differ from those of adults, which may affect their function during locomotion. Until recently, it was very difficult to record for long times from spinal interneurons of adult mice, so estimates of the cellular mechanisms for locomotor generation in adults had to be inferred from studies in neonatal animals. Our recent development of methods to record from adult spinal neurons without age restrictions (Husch et al. 2011) enabled us to directly compare the intrinsic properties as well as the response to locomotor relevant modulatory stimuli such as 5HT in V2a INs from neonatal and adult mice.

Postnatal neuronal differentiation

In transverse lumbar spinal cord slices, the number of detectable CFP-labeled V2a somata per slice decreases significantly with age (Fig. 1). Immunofluorescent amplification of the CFP signal using a GFP antibody revealed additional somata, suggesting that the CFP protein signal is falling with time. CFP synthesis in these transgenic mice occurs only during a limited embryonic time, when the Chx10 promoter is active (Al-Mosawie et al. 2007; Liu et al. 1994); thus, the apparent reduction in V2a neuron number may be an artifact, and many other V2a neurons could be present in adults but with no remaining CFP label. An alternative explanation for the decreased number of adult neurons is V2a cell death during development. Apoptotic cells have been observed in the dorsal and ventral horn from late embryonic development to P12; however, the number of such cells is very low (Lowrie and Lawson 2000). These points raise the question of whether in the adult, we are recording from a subset of the neonatal V2a INs. The only evidence in support of this is the fact that almost all adult V2a INs show tonic firing properties while neonatal neurons show varied tonic, phasic or delayed responses to...
stimulation (Zhong et al., 2010; Dougherty and Kiehn, 2010). However, this could also arise from developmental maturation of the neurons, which eventually mainly become tonic firing neurons, instead of a preferential loss of CFP label in the phasic and delayed firing classes. As described in the introduction, the neonatal V2a class is also functionally heterogeneous, even when restricted to the lumbar spinal cord, only half of V2a interneurons are rhythmically active during fictive locomotion, and these appear to subdivide into classes that synapse onto motoneurons or onto commissural interneurons (Dougherty and Kiehn, 2010, Zhong et al., 2012). Some of the heterogeneity in adult neuron properties (for example, bistability in the presence of 5-HT) may arise from the heterogeneity of the V2a class. In our slice preparations we are unable to distinguish these functional classes.

V2a interneurons show increased excitability in adulthood

With maturation to adult INs, the increase in V2a input resistance leads to a marked increase in membrane excitability, resulting in a steeper I-V-relationship as well as a significantly decreased rheobase in f/I plots (Fig. 2). This increased excitability has functional consequences for the input-output characteristics of the locomotor CPG. Increased V2a excitability and higher input resistance can lead to a lower threshold for activation within the CPG network, because similar synaptic input would have a higher impact on the neurons’ membrane potential. When the synaptic input is mainly excitatory, AP generation is facilitated. In parallel, however, inhibitory input is also amplified by the increased membrane resistance, and might be strong enough to shunt V2a firing activity and affect the neuronal network. This increased responsiveness to all synaptic inputs is appropriate when the locomotor network is actively used for behavior and the neurons must turn on and off at precise and appropriate times.
Action potential properties change dramatically with age

Narrowing of APs during postnatal development (Fig. 3) has been described in a number of other systems. Mouse spinal CINs show significant AP narrowing during the first 2 weeks of life (Abbinanti et al. 2012). Motoneuron APs in developing tadpoles become narrower with age, mainly due to a shift from calcium driven to sodium driven APs (Spitzer and Lamborghini 1976). In rat MNs, spike narrowing with development has been related to changes in the inactivation properties of voltage gated sodium channels (Carlin et al. 2008) and to a change in ionic channel densities of already existing voltage-gated ion channels rather than the expression of new channels (Gao and Ziskind-Conhaim 1998). The faster repolarization in adult APs can contribute to the increased firing rate we saw during depolarizing current injections (Fig. 2). The lower AP threshold probably reflects changes in the set of voltage activated or leak currents expressed with age in these neurons. These could include persistent sodium currents, which influence the AP threshold in CINs (Lamas et al. 2009; Zhong et al. 2007); the onset of bistability often appears to arise from an increase in persistent inward currents (Bouhadfane et al. 2013; see below). All of these properties would contribute to increase V2a spike frequency during locomotion in the adult.

Role of 5-HT-induced bistability for the locomotor controlling network

In the neonatal isolated spinal cord preparation, the V2a INs did not reveal bistable membrane properties (Dougherty and Kiehn 2010; Zhong et al. 2010). In our study on adult V2a INs, we found a subpopulation of V2a INs which shows bistable firing properties, with the potential for rhythmic firing activity, in the presence of 5-HT (Fig. 6). The input resistance is significantly reduced during the plateau state; this suggests activation of tonic inward currents such as the L-type calcium current, which has been shown to underlie the appearance of bistability in older adolescent mouse spinal CINs (Abbinanti et al. 2012). Such bistability could change the
functional significance of synaptic input to the V2a INs with age. In the neonate, the firing pattern of these neurons is shaped directly by the pattern of synaptic input, while in the adult, sufficient initial synaptic input could instead act as a trigger to initiate intrinsically activated firing independent of synaptic excitation, until terminated by sufficiently strong inhibitory synaptic input. Thus, the computational function of synaptic drive to these neurons could change with age. The fact that many of our neurons were sufficiently excited to go into depolarization block may reflect the saturating concentrations of serotonin we used in these experiments. The cells that did not reveal bistability had a 5-HT response similar to that seen in neonatal V2a INs (Dietz et al. 2012), mainly characterized by an increase in excitability and depolarization (Fig. 5).

Bistability is a critical cellular mechanism for the production of stable rhythms in many CPGs (Harris-Warrick 2002; Marder and Bucher 2001). During development, functional adult CPGs are constructed by the establishment of correct synaptic connections simultaneously with the emergence of intrinsic membrane properties and responses to neuromodulation in the CPG INs (Fenelon et al. 1998). In the mouse locomotor network, bistability has been observed to develop in CINs, at P14-16 (Abbinanti et al. 2012), close to the crucial age for the beginning of mature locomotion at P14-16 (Clarac et al. 1998). This study demonstrates that a subset of V2a interneurons also acquires bistability during postnatal development. This property alters the function of synaptic excitation from driving postsynaptic activity to triggering intrinsic postsynaptic activity, which is then regulated by inhibitory input. Such a change would alter the basic rules of network function in the adult locomotor CPG.
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References


Figure Captions

**Figure 1.** Morphological changes of V2a INs in the lumbar spinal cord with age. A Schematic coronal lumbar spinal cord slices at neonate and adult ages indicating the V2a IN somata location. The rectangles indicate the areas shown in B and C. B,C CFP immunoreactivity from neonatal (B) and adult mice (C). D With age there is a significant decrease in the number of immunoreactive somata per slice. E The somata of V2a INs in adult mice have significantly smaller whole cell capacitance than in neonatal mice. ***p ≤ 0.001.

**Figure 2.** V2a INs become more excitable with postnatal maturation. A Responses of neonatal and adult V2a INs to increasing amplitude current pulses from a holding potential of -60 mV. B F/I plots of the average firing frequency during square pulse current injections (grey circles: adult, n = 16; open squares: neonate, n = 10). C F/I plot of the instantaneous firing frequency (inverse of first interspike interval; n same as in B). D Significantly lower rheobase value in adult V2a INs. E The input resistance measured at -60 mV is higher in adult V2a INs. F The holding current (I_{hold}) needed to maintain the membrane potential at -60 mV is smaller in adult V2a spinal INs. *p < 0.05.

**Figure 3.** Action potential (AP) shape narrows dramatically from neonatal to adult age. A Example traces from an adult (grey) and a neonatal (black) V2a IN. AP threshold, width and amplitude measurements are indicated. B The AP width narrows significantly from neonatal to adult ages. C The AP threshold is hyperpolarized in adult V2a INs. D The AP amplitude increases in adult V2a INs. E Example traces (average of 10-12 APs) of a neonatal and an adult AP, showing afterhyperpolarization kinetics. For the adult AP, the fast (AHP_{f}) and slow (AHP_{s}) afterhyperpolarization components are indicated. In neonatal APs there is only a slow AHP. F The AHP_{f} time and the amplitude are measured from the AP threshold to the first peak
of the afterhyperpolarization. G The mean time to the slow AHP peak is not significantly different in neonates and adults. H The amplitude of the slow afterhyperpolarization is similar in neonatal and adult V2a spinal INs.

Figure 4. Comparison of the magnitude of serotonin responses of V2a INs in neonatal and adult mice. A Spontaneous firing activity of a neonatal V2a IN under control conditions, during application of 10 µM 5-HT, and after 30 min washout of 5-HT. B Spontaneous firing activity of an adult V2a IN before, during and after 5-HT application. C-K In the box plots, the extent of the box indicates the 25th and 75th percentiles, and the central line represents the median. Whiskers extend to maximum and minimum values within q3 + w(q3 – q1) and q1 – w(q3 – q1), where q1 and q3 are the 25th and 75th percentiles, respectively, and w = 1.5. Outliers outside these boundaries are plotted as black dots. Significance levels for 5-HT induced changes in neonatal and adult neurons are indicated above the box-plots. Differences in the magnitude of change between neonatal and adult V2a INs are indicated above the thick horizontal lines. C The increase of the holding current with 5-HT at -60 mV, is significant for both neonatal and adult cells, but is larger in neonatal cells (neonate: n = 20; adult: n = 16). D AP firing frequency increases significantly during 5-HT application in neonatal and adult V2a INs (neonate: n = 20; adult: n = 11), with a stronger response in neonatal neurons. E In both age groups, input resistance increases with 5-HT application (neonate: n = 20; adult: n = 8). F The rheobase decreases during 5-HT in neonatal and adult V2a INs (neonate: n = 20; adult: n = 17). G AP amplitude increases in neonatal but not adult neurons upon 5-HT application (neonate: n = 20; adult: n = 11). H AP threshold becomes more negative in neonatal and in adult V2a INs (neonate: n = 20; adult: n = 11). I AP width is not influenced by 5-HT application (neonate: n = 20; adult: n = 11). J Amplitude of the slow afterhyperpolarization is decreased in adult V2a INs (neonate: n = 20; adult: n = 11). K Response of AHP delay increases in neonatal V2a spinal neurons (neonate: n = 20; adult: n = 11). *p < 0.05, **p < 0.01 ***p < 0.001.
**Figure 5.** 5-HT application can evoke membrane potential bistability and oscillation.  
**A** Example of tonically firing V2a IN. 5-HT increases firing frequency, but also induces membrane potential depolarization leading to variable firing frequencies with intermittent depolarization block. Upper trace indicates instantaneous AP-firing frequency.  
**B** Example of a tonically firing adult V2a IN before, during and after 5-HT application. (B1,B2) 5-HT induces membrane potential oscillations marked by accelerating firing frequency to a depolarization block, and a sudden repolarization. (B3) The membrane potential depolarization can occur independently of AP firing. (B4) During 5-HT washout, a sudden transition occurs from oscillations to irregular firing without depolarization events.

**Figure 6.** The depolarization block is characterized by a decrease in membrane input resistance.  
**A** Example trace of a tonically firing adult V2a IN. 5-HT application causes a depolarization and increase in AP frequency resulting in a prolonged depolarization block. Upper trace indicates instantaneous AP-firing frequency.  
**B** The input resistance at -60 mV increases from 0.9 GΩ to 1.3 GΩ with 5-HT application. Release of the negative \( I_{\text{hold}} \) leads to AP firing and a sudden transition into the depolarization block, where the input resistance (0.2 GΩ) is less than 20% of the resistance at -60 mV.  
**C** Another V2a IN that shows irregular oscillations with depolarization block.  
**D** During the depolarization block, the input resistance shifts from very low values to higher values.

**Figure 7.** Serotonin (5-HT) facilitates plateau potentials in adult V2a spinal INs. Square pulse current injections of 10 and 20 pA elicit tonic firing under control conditions. During application of 10 µM 5-HT, accelerating spiking leading to long lasting depolarization block outlasting the current step is seen. This effect is reversible with time.
Figure 1 (1 column)
Figure 2 (1.5 column)
Figure 3 (1 column)
Figure 4 (1.5 columns)
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