Modulation of Intrinsic Spiking in Spinal Cord Neurons

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Czarnecki A, Magloire V, Streit J. Modulation of intrinsic spiking in spinal cord neurons. J Neurophysiol 102: 2441–2452, 2009. First published August 12, 2009; doi:10.1152/jn.00244.2009. The vertebrate spinal cord is equipped with a number of neuronal networks that underlie repetitive patterns of behavior as locomotion. Activity in such networks is mediated not only by intrinsic cellular properties but also by synaptic coupling. In this study, we focused on the modulation of the intrinsic activity of 5-hydroxytryptamine (5-HT, serotonin) and the cholinergic agonist muscarine in spinal cord cultures (embryonic age 14 rats). We investigated these cultures (slices and dissociated cells) at the network level using multielectrode arrays (MEAs) and at the cellular level using whole cell patch clamp. All cultures showed bursting network activity and intrinsic activity when γ-aminobutyric acid, glycine, and glutamate transmission was blocked. Using MEAs, we observed an increase of the intrinsic activity in the ventral part of the slices with 5-HT and muscarine. In single-cell recordings we found that 43 and 35% of the cells that were silent in the absence of fast synaptic activity were transformed into intrinsically spiking cells by 5-HT and muscarine, respectively. We tested the hypothesis that these neuromodulators act via modulation of the persistent sodium currents (I_{NaP}) in these neurons. We found that 5-HT increased threefold the amplitude of I_{NaP} specifically in the nonintrinsically spiking cells, and thus switched these cells into intrinsically spiking cells via activation of 5-HT_2 receptor and the phospholipase C pathway. In contrast, the effect of muscarine on nonintrinsically spiking neurons seems to be independent of I_{NaP}. We conclude from these findings that serotoninergic and cholinergic modulation can turn silent into spontaneously spiking neurons and thus initiate new sources of activity for rhythm generation in spinal networks.

INTRODUCTION

In the spinal cord of vertebrates, local neural networks provide rhythmic output for locomotion (Grillner et al. 1998). These central pattern generators (CPGs) generate the basic pattern of motor outputs in the absence of peripheral feedback. Many studies on vertebrates, using different methods, have shown that the source of activity in the CPGs is due to endogenous oscillators, which drive the whole system. Whether the rhythmic activity is driven by pacemaker neurons or whether the oscillators are based on network properties remains an open question (Del Negro et al. 2005; Pace et al. 2007). A recent study showed that the activity of intrinsically spiking neurons contributes to the regulation of the locomotor activity (Tazerart et al. 2008). In the locomotor system, the intrinsic activity of such neurons can be modulated by command inputs from higher levels of the nervous system. Some neurons express intrinsic activity only under the influence of conditional factors such as command inputs or their neurotransmitters (Arshavsky et al. 2003). Irrespective of whether such neurons can be intrinsically active in the absence of any command inputs and neurotransmitters in vertebrates, the release of neurotransmitters can change membrane properties of such neurons and turn them from a silent to a spiking state and thus be critical for pattern generation. Pilocarpine, an agonist of acetylcholine (ACh) at the command neuron of the anterior pyloric stomatogastric system (Selverston and Moulins 1985), 5-hydroxytryptamine (5-HT, serotonin) on the swimming CPG in the mollusk clione (Arshavsky et al. 1985; Panchin et al. 1996), or N-methyl-D-aspartate, an agonist of glutamate in the reticulospinal system that activates the locomotor CPG in vertebrates (Grillner et al. 1998; Hochman et al. 1994; Kiehn et al. 1996), can all trigger rhythmic activity.

Among various membrane conductances, the persistent sodium current (I_{NaP}) is of particular interest in the generation of rhythmic activity. The activation of such a subthreshold depolarizing conductance is a crucial component in intrinsic spiking. I_{NaP} is involved in rhythmic activity in the hippocampus (Jinno et al. 2003), subthalamic neurons (Beurrier et al. 2000), the neocortex (Van Drongelen et al. 2006), pre-Bötzinger complex inspiratory neurons (Del Negro et al. 2002), and spinal cord neurons (Darbon et al. 2004; Tazerart et al. 2007, 2008; Ziskind-Conhaim et al. 2008). A previous study showed that I_{NaP} is detected in intrinsically spiking cells in spinal cord cultures and underlies their rhythmic properties (Darbon et al. 2004). I_{NaP} can be a crucial target for neurotransmitters that control the excitability of intrinsically spiking neurons in the spinal cord. 5-HT is known to be an essential neurotransmitter that results in locomotor-like activity (Beato and Nistri 1998; Cazalets et al. 1992) and different studies of the ontogeny of descending 5-HT fibers showed that 5-HT innervation is present in the spinal cord of the mouse (Ballion et al. 2002) and in rat (Rajaei et al. 1989) from embryonic age 14.5 (E14.5). In the lamprey and most vertebrates, serotoninergic neurons are turned on during fictive locomotion and are phasically active (Grillner 2003). 5-HT release slows the burst rate and makes the activity more regular and stable. Numerous studies provide evidence that 5-HT_1 and 5-HT_2 receptors are involved in locomotor rhythm generation and modulation. In particular, they show that, although 5-HT_2 receptor agonists promote locomotion, 5-HT_1 receptor agonists have the opposite effect and interfere with the generation of locomotor activity (Beato and Nistri 1998; Landry and Guertin 2004; Perrier and Cotet 2008). Acetylcholine is also an important neuromodulator of CPG activity. Many reports show a cholinergic control of pacemaker frequency. In murine gastric interstitial cells of Cajal (Kim et al. 2003) as well as in pre-Bötzinger complex inspiratory neurons (Shao and Feldman 2000), ACh activates inward current through M3 receptors and accelerates the frequency of pacemaker activity. During development, the cholinergic neurotransmitter pathway is also required to configure...
CPGs. From E12.5 to E14.5, spontaneous motor bursts are dependent on ACh (Myers et al. 2005). Here we studied whether cholinergic and serotoninergic pathways can modulate \( I_{\text{NaP}} \) and intrinsic spiking in spinal cord cultures (organotypic slices and dissociated cell cultures) from E14 rats. Our findings suggest that 5-HT and muscarine recruit new intrinsically spiking neurons. In the case of 5-HT this switch from a silent to a spiking state of spinal neurons is due to an up-regulation of \( I_{\text{NaP}} \) mediated by 5-HT \(_3\) receptors and the phospholipase C (PLC) signaling cascade.

**METHODS**

**Culture preparation and recordings**

All cultures were prepared as described previously (Streit et al. 2001; Tscherter et al. 2001) from spinal cords of rats at embryonic age 14 (E14). The embryos were delivered by caesarian section from deeply anesthetized animals (0.4 ml pentobarbital, administered intramuscularly) and killed by decapitation. Following the delivery of the embryos, the mother was killed by intracardiac injection of pentobarbital. Animal care was in accordance with guidelines approved by Swiss local authorities (Amt für Landwirtschaft und Natur des Kantons Bern, Veterinärdienst, Sekretariat Tierversuche, approbated No. 39/02 and 61/05). The backs of the embryos were isolated from their limbs and viscera and cut into 225-m\(^2\)-thick transverse slices with a tissue chopper.

For the dissociated cultures, slices of all regions of the spinal cord, without dorsal root ganglia, were exposed to a 0.3% trypsin solution for 3 min at 37°C. Then they were mechanically dissociated by forcing them several times through fine-tipped pipettes. The cells were plated on multielectrode arrays (MEAs; Ayanda Biosystems, Landquart, Switzerland) or on glass coverslips at a density of 5,000 and 2,500 cells/mm\(^2\), respectively. MEAs were produced as described previously (Tscherter et al. 2001) and coated for 1 h with diluted (1:50) Matrigel (Falcon/Biocat; Becton Dickinson, Basel, Switzerland) or on glass coverslips at a density of 5,000 and 2,500 cells/mm\(^2\), respectively. MEAs were produced as described previously (Tscherter et al. 2001) and coated for 1 h with diluted (1:50) Matrigel (Falcon/Biocat; Becton Dickinson, Basel, Switzerland). The cells were restricted to an area around the electrodes (\(~20\) mm\(^2\)) using cloning glass cylinders attached to the MEAs or coverslips with silicone sealant. They were maintained in culture dishes containing 150 \( \mu \)l of nutrient medium and incubated in a 5% CO\(_2\)-95% air atmosphere at 36.5°C for \( \leq 12\) wk. The medium consisted of serum-free Neurobasal medium (Gibco BRL, Life Technologies, Basel, Switzerland) supplemented with B27 and Glutamax (both Gibco BRL). Half of the medium was changed weekly.

For organotypic cultures, spinal cord slices with their dorsal root ganglia attached were fixed on MEAs using reconstituted chicken plasma (Cocalico Biologicals, Reamstown, PA and Sigma/Fluka Chemie, Buchs, Switzerland) coagulated by thrombin (Sigma/Fluka Chemie). The cultures were maintained in sterile plastic tubes containing 3.5 ml of nutrient medium and incubated in roller drums rotating at 2 rpm in a 5% CO\(_2\)-containing atmosphere at 36.5°C for \( \leq 6\) wk. The medium was composed of 79% Dulbecco’s modified Eagle’s medium with Glutamax, 10% horse serum (both from Gibco BRL, Basel, Switzerland), 10% \( \text{H}_2\text{O} \), and 5 ng/ml 2.5S nerve growth factor (NGF; Alomone Laboratories, Jerusalem, Israel). During the first week of incubation, a medium with 10 ng/ml NGF was used. Half of the medium was changed weekly.

Recordings were made in a chamber mounted on an inverted microscope (Nikon, Tokyo) from cultures of 4–6 wk in vitro age. The medium was replaced by an extracellular solution containing (in mM): NaCl, 145; KCl, 4; MgCl\(_2\), 1; CaCl\(_2\), 2, HEPES, 5; Na-pyruvate, 2; glucose, 5 (pH 7.4). Recordings were made 5 min after the solution change in the absence of continuous superfusion with solution change every 10–15 min. All recordings were made at room temperature (24 ± 4°C).

**MEA recording and analysis**

MEAs consisted of 68 electrodes laid out in the form of a hexagon. The electrodes of platinum were 40 × 40 \( \mu \)m and were spaced 200 \( \mu \)m apart (center to center, e.g., Fig. 1A). Channels (i.e., electrodes) showing activity (usually 10–60) were selected by eye and their recordings digitized at 6 kHz, visualized, and stored on hard disc using custom-made virtual instruments within LabVIEW (National Instruments Switzerland), as described previously (Streit et al. 2001). Detection of the extracellularly recorded action potentials and further analysis were done off-line with the software package IGOR (WaveMetrics, Lake Oswego, OR) as described previously (Tscherter et al. 2001). The detected signals were fast voltage transients (\(<4\) ms), which correspond to single action potentials in neurons or axons (single-unit activity). These transients often appeared in clusters (multitunit activity) originating from closely timed action potentials of several neurons or axons seen by one electrode. When they appeared at >250 Hz (which is the upper limit of temporal resolution of the detector), they could not be clearly separated from each other and therefore such activity was set by definition to 333 Hz (Tscherter et al. 2001). No attempt was made to sort spikes seen by one electrode. The selectivity of event detection for spiking activity was assessed using recordings obtained in the presence of tetrodotoxin (TTX, 1.5 \( \mu \)M) as a zero reference. The processed data were displayed as activity distribution plots (see Fig. 1A). Activity distribution plots show filled circles whose diameters are proportional to the total activity of the channels (i.e., electrodes) that lie within a given area around a single-unit activity. These transients often appeared in clusters (multitunit activity) originating from closely timed action potentials of several neurons or axons seen by one electrode. When they appeared at >250 Hz (which is the upper limit of temporal resolution of the detector), they could not be clearly separated from each other and therefore such activity was set by definition to 333 Hz (Tscherter et al. 2001). No attempt was made to sort spikes seen by one electrode. The selectivity of event detection for spiking activity was assessed using recordings obtained in the presence of tetrodotoxin (TTX, 1.5 \( \mu \)M) as a zero reference. The processed data were displayed as activity distribution plots (see Fig. 1A). Activity distribution plots show filled circles whose diameters are proportional to the total activity of the channels (i.e., electrodes) that lie within a given area around a single-unit activity.

**FIG. 1.** 5-Hydroxytryptamine (5-HT, serotonin) increases the intrinsic activity in isolated neurons in spinal cord cultures. **A:** activity distribution plot superimposed on a photograph of the slice culture. The size of the white circles is proportional to the activity seen by the electrodes. Intrinsic activity is restricted to the ventral part in control experiments around the central fissure. With 5-HT (20 \( \mu \)M), intrinsic activity is increased and is also found in the dorsal part. **B:** histograms of the activity observed by each of the 39 electrodes recorded in this example. **C:** mean values of intrinsic activity per channel (CI) and number of active electrodes (C2) for organotypic cultures in control and after addition of 5-HT (20–50 \( \mu \)M, \( n = 15 \)). **D:** mean values of intrinsic activity per channel for dissociated cultures in control and after addition of 5-HT (20–50 \( \mu \)M, \( n = 7 \); **= \( p < 0.01 \)).
electrodes projected on a picture of the slice culture. Total activity is measured as the mean of the activity per second detected by each electrode during the whole recording period (usually 10 min).

Whole cell patch-clamp recording and analysis

Intracellular voltage measurements were obtained from individual neurons in cultures on glass coverslips using the whole cell patch-clamp technique (Hamill et al. 1981) with an Axoclamp 2B amplifier (Axon Instruments, Union City, CA). The patch pipettes were filled with a solution containing (in mM): K-glucuronate, 100; HEPES, 10; Mg-ATP, 4; Na₂-GTP, 0.3; Na₂-phosphocreatine, 10 (pH 7.3, with KOH). For the measurement of sodium currents in voltage-clamp, the bath solution was replaced by a 0 Ca solution containing (in mM): NaCl, 145; KCl, 4; MgCl₂, 3; HEPES, 5; Na-pyruvate, 2; glucose, 5 (pH 7.4). The electrodes had a resistance of 4–7 MΩ. No series resistance compensation was applied. Native resting membrane potentials were in the range of −40 to −70 mV. Cells with a potential less negative than −40 mV were discarded. A junction potential of 15 mV was systematically corrected. The recordings were digitized at 6–10 kHz, visualized, and stored on computer using pClamp software (Axon Instruments). The signals were analyzed off-line using custom-made programs in IGOR (WaveMetrics) and Clampfit software (Axon Instruments).

Statistics

Averages are expressed as means ± SE. Differences between groups were evaluated using nonparametric tests (Wilcoxon, Mann–Whitney U, and Kruskal–Wallis ANOVA). Significance was accepted when P < 0.05.

Chemicals

All drugs were bath applied. The following agents were used: D-APV (2-amino-5-phosphonovaleric acid), CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), riluzole [2-amino-6(trifluoromethyl)benzothiazole], strychnine, TTX (tetrodotoxin) (all Sigma–Aldrich, Buchs, Switzerland); and bicuculline [(1S, 9R)–(−)-bicuculline metochloride], α-Met-5-HT (α-methyl-5-hydroxytryptamine), 8-hydroxy-DPAT, and ritanserin from Tocris, Anawa Trading, Wangen, Switzerland. Solutions of riluzole were made up in DMSO (dimethylsulfoxide), final concentration of 0.003%. At the final concentration used in the experiments, DMSO had no significant effect on intrinsic activity and bursting. Solutions of ritanserin were made up in ethanol (final concentration of 0.01%). At the final concentration, ethanol had no significant effect on intrinsic activity.

Results

5-HT increases intrinsic activity in organotypic and dissociated spinal cord cultures

In dissociated and organotypic slice cultures of fetal rat spinal cord, some neurons remain active in the absence of synaptic coupling. These neurons can activate the whole network through recurrent excitation and are present in slices originating from all levels of the spinal cord, from cervical to lumbar. Here we tested the possible modulation of this intrinsic activity by 5-HT. Intrinsic activity was assessed with MEA recordings after full blockade of fast synaptic transmission (with bicuculline, 20 μM; strychnine, 1 μM; APV, 50 μM; and CNQX, 10 μM). As shown previously (Streit et al. 2001), in the absence of synaptic transmission, intrinsic activity still occurred at some electrodes, mainly in the ventral part of organotypic slices (Fig. 1A). In organotypic slices, application of 5-HT increased the intrinsic activity. This increase is located in the ventral part but 5-HT also activates new sources of activity in the dorsal part of the slices (Fig. 1A). Whereas 5-HT had no significant effect at 10 μM (0.55 ± 0.12 vs. 0.34 ± 0.1 events/s, P = 0.4, n = 7), the effects of 5-HT at 20 and 50 μM were comparable (0.94 ± 0.25 vs. 0.28 ± 0.09 events/s, P < 0.01, n = 10, at 20 μM; and 0.65 ± 0.3 vs. 0.24 ± 0.14 events/s, P < 0.05, n = 5, at 50 μM) so we decided to pool both concentrations in one group of data. 5-HT at these concentrations increased the activity per channel by 223% (0.84 ± 0.19 vs. 0.26 ± 0.07 events/s, P < 0.01, n = 15; Fig. 1, B and C) as well as the number of active electrodes (= channels with event frequency >0.1 Hz; see Methods) by 100% (14.1 ± 1.7 vs. 7.1 ± 3, P < 0.01, n = 15; Fig. 1C). In dissociated cultures, this effect of 5-HT at 20–50 μM was less pronounced, in that it increased the activity per channel by only 38% (3.6 ± 1.4 vs. 2.6 ± 1 events/s, P < 0.01, n = 9; Fig. 1D) and was not significant at 10 μM (2.96 ± 0.8 vs. 2.67 ± 0.77 events/s, P = 0.17, n = 6). Because of the high density of intrinsically spiking cells in dissociated cultures, it was not possible to see any change in the number of active electrodes with 5-HT since most of the electrodes are already active in control experiments. Altogether, these results suggest that 5-HT has a modulatory effect on the intrinsic activity in spinal cord cultures. First, it increases the intrinsic activity normally restricted to the ventral part and, second, it recruits new sources of activity in the rest of the slices.

5-HT turns silent into spontaneously spiking neurons

To further investigate the effect of 5-HT on intrinsic activity, we tested its effect at the cellular level in dissociated spinal cord cultures measured by whole cell patch-clamp after full blockade of fast synaptic transmission (with bicuculline, 20 μM; strychnine, 1 μM; APV, 50 μM; and CNQX, 10 μM; see example in Fig. 2, A and B). Under these conditions, intrinsically spiking neurons were difficult to find in organotypic cultures. We therefore investigated these neurons in dissociated cultures where they are more frequent (Darbon et al. 2002). As shown previously (Darbon et al. 2002), in the absence of synaptic transmission some cells still exhibit spontaneous activity. In the present study, on average, 33% of the cells recorded were firing spontaneously, ≤60% in some cultures. In our dissociated spinal cord cultures, motoneurons were absent, as confirmed with choline acetyltransferase labeling (data not shown), suggesting that the neurons we recorded from were spinal interneurons. We called interneurons without intrinsic firing “nonspiking” or “silent” neurons as opposed to interneurons firing intrinsically, called “intrinsically spiking neurons” or spiking cells. The blockade of fast synaptic transmission slightly depolarized on average spiking and nonspiking neurons, but could also hyperpolarize the membrane potential of some neurons as shown on Fig. 2B. However, after full blockade of synaptic transmission, the resting membrane potential of spiking and nonspiking neurons was not different (−52.5 ± 1 mV, n = 64, vs. −51.4 ± 1.2 mV, n = 130). Surprisingly, 5-HT (50 μM) had no effect on the firing frequency of the spiking cells (1.63 ± 0.28 vs. 1.69 ± 0.32 Hz, P = 0.99, n = 46; Fig. 2A), but slightly decreased their membrane potential (−45.1 ± 1.5 vs. −47.8 ± 1.2 mV, P = 0.04, n = 46). We then tested the effect of 5-HT on nonspiking
cells (67% of the cells recorded). Application of 5-HT decreased the membrane potential of nonspiking cells (−44.8 ± 1.3 vs. −48 ± 1.3 mV, \( P < 0.01, n = 74 \)) and switched 43% of these silent cells into spiking neurons (32 of 74 neurons) with a final firing frequency of 1.1 ± 0.24 Hz. This effect was partially reversible (0.62 ± 0.47 vs. 1.15 ± 0.25 Hz, \( P = 0.01, n = 6 \); Fig. 2B). 5-HT had no effect on the input resistance (152 ± 20 vs. 158 ± 20 MΩ, \( P = 0.6 \)) in both group of cells (spiking and nonspiking). 5-HT thus increased the percentage of spiking neurons in the absence of synaptic coupling from 33 to 60%.

5-HT increases the number of intrinsic spiking neurons by facilitating I_{NaP}

As described previously (Darbon et al. 2004), the persistent sodium current (I_{NaP}) is mainly present in intrinsically spiking cells and underlies their activity. We thus hypothesized that 5-HT could activate I_{NaP} in nonspiking neurons. We investigated the presence of I_{NaP} in 119 neurons of dissociated spinal cord cultures. The recorded cells were pharmacologically isolated from the network inputs by using a bath solution containing 0 mM CaCl_2, to suppress both synaptic transmission and voltage-dependent calcium currents. We measured firing frequency and the expression of I_{NaP} in a same neuron. We used a slow ramp current depolarization to prevent the activation of the transient Na current component to reveal I_{NaP} in neurons clamped at −60 mV. Application of a ramp voltage (−80 to 0 mV, at 8 or 4 mV/s) exhibited an inward current activated between −50 and 0 mV (Fig. 3), which was partially blocked by 10 μM riluzole (16.1 ± 3.5 vs. 28.3 ± 6.3 pA, \( P < 0.01, n = 9 \)) and totally blocked by 20 μM riluzole (0 vs. 28 ± 8.8 pA, \( P = 0.04, n = 5 \)) as well as by 1 μM TTX (Fig. 4, A and C). I_{NaP} was found in all spiking neurons with a mean peak amplitude of 35 ± 3.7 pA (\( n = 28 \)). In nonspiking neurons I_{NaP} was present in only 34 of 75 neurons, with an average peak amplitude of 9.9 ± 1.4 pA (\( n = 75 \)). In the nonspiking neurons where I_{NaP} was present, the peak amplitude was smaller than that in spiking neurons (20 ± 1.8 pA, \( n = 34 \)). In these cells, the peak amplitude of I_{NaP} was substantially variable from one culture to another. As shown previously, application of 5-HT (50 μM) on spiking neurons did not change their firing rate (1.23 ± 0.47 vs. 1.61 ± 0.65 Hz, \( P = 0.21, n = 7 \); Fig. 3C). In the same neurons, 5-HT slightly increased the peak amplitude of I_{NaP} by 25% (49.3 ± 6.7 vs. 39.3 ± 10 pA, \( P = 0.14, n = 7 \); Fig. 3B and C) but this increase was not significant. As
shown previously, 5-HT at 50 μM induced intrinsic spiking in 8 of 13 nonspiking neurons (62%) with a final firing rate of 0.67 ± 0.69 Hz (n = 8). On average, the firing rate of previously nonspiking neurons after 5-HT application was 0.41 ± 0.17 versus 0 Hz (P < 0.05, n = 13; Fig. 3C). In the same neurons, the peak amplitude of INaP was increased by 331% (25 ± 4.5 vs. 5.8 ± 3 pA, P < 0.01, n = 13; Fig. 3, A and C). We also saw this effect at lower concentrations. At 20 μM, 5-HT induced intrinsic spiking in 6 of 12 nonspiking neurons (50%) with a final firing rate of 0.25 ± 0.13 Hz (n = 6). On average, the peak amplitude of INaP was increased by 187% (37.5 ± 6.1 vs. 20 ± 6.3 pA, P = 0.01, n = 12). At 10 μM, 5-HT induced intrinsic spiking in 3 of 7 nonspiking neurons (43%) with a final firing rate of 0.06 ± 0.02 Hz (n = 3). On average, the peak amplitude of INaP was increased by 181% (29.3 ± 9.3 vs. 16.4 ± 5.7 pA, P = 0.068, n = 7), although not significant. It has been shown that reducing the extracellular calcium to zero induces an increase of INaP by about 30% (Tazerart et al. 2008). In our study, we systematically recorded INaP after a stabilization period of 30 min in 0 Ca2+ solution. To confirm that the peak amplitude of INaP was stable and that the increase was due to 5-HT and not to the Ca2+ free solution during the time of drug application, we made measurements with a vehicle solution without 5-HT. Our data show no changes in INaP amplitude under such conditions (Fig. 4, B and D; 9 ± 2.7 vs. 13 ± 4.2 pA, P = 0.6, n = 5). Moreover, the percentage of intrinsically spiking cells was similar in 0 Ca2+ solution as in normal extracellular solution containing 2 mM Ca2+ (36 vs. 33%). These findings show that the effects of 5-HT on INaP and on intrinsic spiking were not due to the 0 Ca2+ solution.

5-HT slightly depolarized spiking cells (−49.4 ± 2.2 vs. −52.2 ± 0.5 mV, P = 0.31, n = 7) as well as in nonspiking cells (−46.3 ± 2 vs. −49.5 ± 1.7 mV). However, this depolarization, probably due to the small sample, was not significant (P = 0.08, n = 12). In summary, we found that INaP is present mainly in intrinsically spiking cells, where it is not affected by application of 5-HT, whereas it is up-regulated by 5-HT in nonspiking cells. A possible reason for the activity of intrinsically spiking cells may be an ongoing release of 5-HT that constitutively up-regulates INaP. We measured the intrinsic activity on MEAs with application of ritanserin, a 5-HT2 receptor antagonist. At 1 μM, ritanserin did not change the intrinsic activity (0.49 ± 0.32 vs. 0.48 ± 0.32 events/s, P = 0.89, n = 5). This suggests that the activity and the presence of INaP in intrinsically spiking cells are not due to the release of 5-HT in the slice. These neurons seem to be active independent of 5-HT.

5-HT2 receptor activation increases the intrinsic activity in organotypic and dissociated spinal cord cultures

5-HT is known to exert contrasting effect in the spinal cord. We therefore examined the contribution of two 5-HT receptor subtypes to the increase of the intrinsic activity in the ventral part of the slices and to the increase of the number of sources of activity. We addressed the question of the possible involvement of 5-HT1A and 5-HT2 receptors known to have opposite effects on spinal cord activity (Beato and Nistri 1998; Landry and Guertin 2004; Perrier and Cotol 2008). A specific agonist (8-OH-DPAT) was used to activate 5-HT1A receptors. MEA recordings showed that 8-OH-DPAT (20 μM) had an effect neither on intrinsic activity (1 ± 0.2 vs. 0.96 ± 0.3 events/s, P = 0.6, n = 11) nor on the number of active electrodes (13.9 ± 1.9 vs. 14.5 ± 1.7, P = 0.6, n = 11). Another specific agonist (α-Met-5-HT) was applied to the cultures to activate...
5-HT$_2$ receptor. The effect of α-Met-5-HT (20 μM) was similar to the effect of 5-HT. It increased the intrinsic activity by 117% (1.3 ± 0.28 vs. 0.6 ± 0.17 events/s, P < 0.01, n = 12) and the number of active electrodes by 46% (22 ± 2.9 vs. 15 ± 2.1, P < 0.01, n = 12; Fig. 5). In dissociated cultures a similar result was observed. 8-OH-DPAT had no effect on the intrinsic activity (2.5 ± 1.1 vs. 3 ± 1.6 events/s, P = 0.67, n = 8), whereas α-Met-5-HT increased the intrinsic activity (1.24 ± 0.34 vs. 0.77 ± 0.22 events/s, P < 0.01, n = 9; Fig. 5D).

Augmentation of $I_{\text{NaP}}$ is dependent on the activation of 5-HT$_2$ receptors

We then tested the effects of the 5-HT$_1A$ receptor agonist 8-OH-DPAT and the 5-HT$_2$ receptor agonist α-Met-5-HT at the cellular level to determine the type of 5-HT receptor involved in the up-regulation of $I_{\text{NaP}}$ in nonspiking neurons evoked by 5-HT. As with 5-HT, neither 8-OH-DPAT nor α-Met-5-HT increased the intrinsic activity or $I_{\text{NaP}}$ in spiking neurons (Fig. 6, B and C). In contrast to 5-HT, application of 20 μM 8-OH-DPAT had no significant effect on nonspiking neurons. It turned only 3 of 14 nonspiking neurons (21%) into spiking neurons with a final firing rate of 0.15 ± 0.1 Hz (n = 3). On average, the final firing rate of previously nonspiking neurons after 8-OH-DPAT application was 0.03 ± 0.02 versus 0 Hz (P = 0.16, n = 14). In the same neurons, the peak amplitude of $I_{\text{NaP}}$ was not significantly changed (18.9 ± 4 vs. 9.6 ± 3.9 pA, P = 0.1, n = 14), although there was a tendency of an increase. In contrast, the effect on 5-HT$_2$ receptors was clearly significant. Application of 20 μM α-Met-5-HT turned...
11 of 20 nonspiking neurons (55%) into spiking neurons with a final firing rate of 0.64 ± 0.2 Hz (n = 11). On average, the final firing rate of previously nonspiking neurons after α-Met-5-HT application was significantly increased (0.35 ± 0.13 vs. 0 Hz, P < 0.01, n = 20; Fig. 6, A and C). In the same neurons, the peak amplitude of \( I_{\text{NaP}} \) was increased about 270% (31.5 ± 5.2 vs. 8.5 ± 2.5 pA, \( P < 0.01, n = 20; \) Fig. 6C). In the nonspiking neurons, in which 5-HT or α-Met-5-HT failed to induce intrinsic spiking, \( I_{\text{NaP}} \) was not significantly altered, suggesting a causal relationship between the two phenomena. As with 5-HT, 8-OH-DPAT and α-Met-5-HT slightly depolarized the membrane potential of spiking and nonspiking neurons. Neither 8-OH-DPAT (158.7 ± 10.7 vs. 158 ± 10.5 MΩ, \( P = 0.96) \) nor α-Met-5-HT (158.8 ± 18.3 vs. 159.9 ± 17.1 MΩ, \( P = 0.96 \)) had an effect on the input resistance. We conclude from these findings that the increase of the number of spiking neurons is mainly due to an activation of 5-HT\(_2\) receptors. Our results suggest that 5-HT increases \( I_{\text{NaP}} \) and thereby activates silent neurons through 5-HT\(_2\) receptors.

**5-HT\(_2\)** receptor-mediated increase of \( I_{\text{NaP}} \) depends on the activation of phospholipase C (PLC)

In the spinal cord, 5-HT receptors have been less extensively studied than in the rest of the nervous system with respect to the analysis of the transduction mechanisms. Pharmacological studies showed that 5-HT\(_2\) receptors are coupled to specific G(alpha)-subunits such as G(alpha q) (Roth et al. 1998) with activation of phospholipase C (PLC) (Noda et al. 2004), whereas activation of the 5-HT\(_1\) receptor subtype is negatively coupled to adenylate cyclase (Hoyer et al. 1994). We examined whether in our cells in the spinal cord, the 5-HT\(_2\)-mediated activation of \( I_{\text{NaP}} \) was PLC dependent. A new series of experiments with the 5-HT\(_2\) agonist α-Met-5-HT was carried out with the PLC inhibitor O-(octahydro-4,7-methano-1H-inden-5-yl) carbonapotassium dithioate (D609, 30 μM) in the pipette. With D609, α-Met-5-HT recruited only one of eight nonspiking neurons (12%) instead of 55% without the PLC inhibitor. In the presence of D609, α-Met-5-HT no longer had an effect on the peak amplitude of \( I_{\text{NaP}} \) (6.9 ± 5.1 vs. 8.7 ± 5.9 pA, \( P = 0.7, n = 8; \) Fig. 7A). Figure 7B shows the changes in \( I_{\text{NaP}} \) in the presence of the different drugs used. It is evident that the 5-HT\(_2\) agonist reproduces the effect of 5-HT, whereas the effect of the 5-HT\(_1\) agonist is much smaller and not statistically significant. The dependence of the up-regulation of \( I_{\text{NaP}} \) by α-Met-5-HT on an intact PLC cascade shows that the PLC pathway is involved in the serotonergic modulation of \( I_{\text{NaP}} \).

**Muscarine turns silent into spontaneously spiking neurons**

We investigated a possible cholinergic neuromodulation of the intrinsic activity after full blockade of fast synaptic transmission. We tested the effect of the cholinergic agonist muscarine (20 μM) on intrinsic activity in organotypic and dissociated cultures. The recordings obtained with MEA show that, as with 5-HT, muscarine increased the intrinsic activity in the
ventral part and activated new sources of activity in the dorsal part of the slices. In organotypic slices, it increased the activity per channel by 40% (0.94 ± 0.27 vs. 0.67 ± 0.24 events/s, P = 0.02, n = 9; Fig. 8A) as well as the number of active electrodes by 30% (3.2 ± 0.9 vs. 2.1 ± 0.8, events/s, P = 0.02, n = 9; Fig. 8B). In dissociated cultures, muscarine increased the activity per channel by 52% (3.2 ± 0.9 vs. 2.1 ± 0.8, events/s, P = 0.02, n = 9; Fig. 8C). We then measured the effect of muscarine on the intrinsic activity and $I_{\text{NaP}}$ in spiking and nonspiking neurons. Muscarine switched 9 of 26 nonspiking neurons in spiking neurons (35%) with a final firing rate of 0.52 ± 0.05 Hz (n = 9). On average, the final firing rate of previously nonspiking neurons after muscarine application was 0.18 ± 0.06 versus 0 Hz (P < 0.01, n = 26; Fig. 8D). However, in the neurons in which muscarine induced spiking, it had no effect on the peak amplitude of $I_{\text{NaP}}$ (14.4 ± 5.4 vs. 12.5 ± 4.3 pA, P = 0.68, n = 9; Fig. 8D). As with 5-HT, muscarine did not change the firing rate (1.8 ± 0.6 vs. 2.4 ± 0.5, Hz, P = 0.09, n = 14) or the peak amplitude of $I_{\text{NaP}}$ (35 ± 8.7 vs. 34.3 ± 8.3 pA, P = 0.9, n = 7) in spiking neurons. It also depolarized the neurons (−50.3 ± 2.5 vs. −56.5 ± 1.8 mV, P = 0.01, n = 26). In summary, muscarine-induced, as did 5-HT, intrinsic spiking in previously silent neurons in spinal cord cultures but this effect was not mediated by an up-regulation of $I_{\text{NaP}}$.

**DISCUSSION**

The present experiments demonstrate that, first, intrinsic activity is effectively modulated by 5-HT in spinal cord cultures. Second, we show that 5-HT increases the number of intrinsically spiking cells that constitute the source of the activity in spinal cord networks. Third, we show that the 5-HT-mediated increase of the number of intrinsically spiking cells is due to an activation of 5-HT$_2$ receptors, which in-

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**FIG. 7.** 5-HT$_3$ receptor-mediated increase of $I_{\text{NaP}}$ expression depends on the activation of phospholipase C (PLC). A: mean values of the amplitude of $I_{\text{NaP}}$ and the firing frequency recorded in the same cell for nonspiking neurons under α-Met-5-HT with D609 (30 μM; PLC inhibitor) in the pipette. B: difference of $I_{\text{NaP}}$ from control under different conditions. Note that only 5-HT at 20 and 50 μM and α-Met-5-HT has a significant effect on the current and that D609 totally blocks its effect (*P < 0.05).

**FIG. 8.** Muscarine (20 μM) increases the intrinsic activity (bicuculline, 20 μM; strychnine, 1 μM; APV, 50 μM; and CNQX, 10 μM) in spinal cord cultures. A: mean values of intrinsic activity per channel for organotypic cultures in control and after addition of muscarine (20 μM, n = 9). B: mean values of the number of active electrodes for organotypic cultures in control and after addition of muscarine (20 μM, n = 9). C: mean values of intrinsic activity per channel for dissociated cultures in control and after addition of muscarine (20 μM, n = 9). D: mean values of the amplitude of $I_{\text{NaP}}$ and the firing frequency recorded in the same cell from dissociated cultures for nonspiking neurons (in light gray, n = 26) (**P < 0.01) and for spiking neurons (in dark gray, n = 7).
creases $I_{\text{NaP}}$ through activation of the PLC–PKC signaling cascade. We also show that muscarine, like 5-HT, increases the number of intrinsically spiking cells but does not modulate $I_{\text{NaP}}$. The mechanism by which muscarine turns silent neurons into spiking neurons remains to be explored.

5-HT and muscarine increase the intrinsic activity and the number of intrinsically spiking cells in spinal cord cultures

It has been shown previously that rhythm generation in spinal cord networks in culture is based on intrinsic spiking in some neurons and subsequent network recruitment by recurrent excitation through glutamatergic synaptic transmission and a network refractory period following the bursts (Czarniecki et al. 2008; Darbon et al. 2002, 2004). Such intrinsic spiking neurons thus play a key role in the generation of rhythmic activity in spinal cord networks. Intrinsic activity is mainly located in the ventral part of the slice and is based on the subthreshold persistent sodium current ($I_{\text{NaP}}$) (Darbon et al. 2004). This intrinsic activity usually consists of tonic activity in individual channels with no correlation between channels. We found that both serotonergic and cholinergic neuromodulation increase intrinsic activity, whereas catecholamines have no effect (results not shown). Intrinsic activity is mainly increased in the ventral part of the spinal slices. Different types of interneurons (INs) are localized in this area in the intact spinal cord such as EphA4-positive INs (Butt et al. 2005), Hb9 INs identified as putative CPG interneurons (Hinckley et al. 2005), and 5-HT–sensitive commissural interneurons (CINs) (Diaz-Rios et al. 2007; Zhong et al. 2006). Some of these interneurons such as Hb9 INs have intrinsic bursting properties and exhibit a pacemaker-like behavior (Tazerart et al. 2008; Ziskind-Conhaim et al. 2008). Which of these types contribute to the intrinsic activity seen in culture remains to be shown. The increase in intrinsic activity is attributed to an increase in the percentage of intrinsically firing neurons. It therefore leads not only to an increase in the activity of individual channels (which record activity from several cells) but also to an increase in the number of electrodes that record intrinsic activity. Some of these new sources of activity were even located in the dorsal parts of the slices, suggesting that 5-HT may influence sensory circuits in the dorsal parts of the spinal cord as well. It is indeed well known that 5-HT can control pain-processing circuits and has antinociceptive effects (Grudt et al. 1995; Hori et al. 1996; Yoshimura and Furue 2006). In dissociated cultures, the effect of 5-HT was similar but less intense. Since intrinsic activity was much higher in dissociated compared with slice cultures, it covered most of the electrodes already in control experiments. A spread of intrinsic activity onto more electrodes by 5-HT or muscarine could therefore not be seen in MEA recordings, although more intrinsically spiking neurons were present. The higher intrinsic activity in dissociated compared with slice cultures has been described previously (Yvon et al. 2007) and is probably related to the fact that neurons in dissociated cultures are in general more depolarized than those in slice cultures (results not shown). A substantial percentage of the interneurons (30–60%) was already spiking before the application of 5-HT. They may be under the influence of intrinsic release of 5-HT in the slice due to possible intraspinal 5-HT neurons (Ballion et al. 2002; Newton et al. 1986). Experiments with the 5-HT$_2$ receptor antagonist ritanserin ruled out this possibility. These results thus suggest that mechanisms for genuine intrinsic spiking are present in a substantial part of the interneurons. A possible role of such mechanisms for rhythm generation in vivo remains to be shown.

5-HT plays important roles in controlling rhythmic behaviors including locomotion or whisker movements (Cramer et al. 2007; Hattox et al. 2003; Houssgaard and Kiehn 1989). In spinal cord, 5-HT has complex effects on rhythm generation depending on the type of receptor activated. Many studies showed that 5-HT promotes rhythmic activity and locomotion through activation of 5-HT$_2$ receptors (Cazalets et al. 1992; Landry and Guertin 2004; Madriaga et al. 2004). In contrast, 5-HT$_{1A}$ receptor activation tends to oppose the excitatory action of 5-HT on 5-HT$_2$ receptors (Beato and Nistri 1998). In line with these findings we found that 5-HT promotes intrinsic spiking in spinal neurons through 5-HT$_2$ receptors, whereas agonists to 5-HT$_{1A}$ receptors are not effective.

5-HT induces intrinsic spiking by up-regulating $I_{\text{NaP}}$ through 5-HT$_2$ receptors and the PLC cascade

It is known that 5-HT can initiate cellular signaling cascades and change membrane properties to activate intrinsic activity in different types of cells (Cramer et al. 2007; Harvey et al. 2006). Other studies showed that intrinsic firing is associated with a high $I_{\text{NaP}}$ in spinal cord neurons (Darbon et al. 2004; Kuo et al. 2006) as well as in Purkinje cells and subthalamic neurons (Do and Bean 2003; Kay et al. 1998). We show here that $I_{\text{NaP}}$ is present in all spiking neurons, whereas it is almost absent in nonspiking neurons. $I_{\text{NaP}}$ seems to be a sort of signature of spiking neurons. $I_{\text{NaP}}$ is implicated in a number of processes such as fine-tuning of intrinsic firing patterns (Crill 1996), generation of membrane oscillations (Alonso and Llinás 1989), and the amplification of excitatory synaptic input in different neuronal compartments (Stuart and Sakmann 1995). $I_{\text{NaP}}$ is a voltage-dependant membrane current that resists inactivation and is involved in many types of neuronal rhythm generation. It enhances excitability and promotes rhythmic activity in neurons in the pre-Bötzinger respiratory complex (Del Negro et al. 2005; Pace et al. 2007). In the neocortex, $I_{\text{NaP}}$ is found in a subtype of persistently active neurons in layers 2/3 and 5 (Aracri et al. 2006). In these cells, $I_{\text{NaP}}$ is necessary for the generation of “pacemaker-like activity” (Le Bon-Jego and Yuste 2007) and enables cortical neurons to generate network bursts (van Drongelen et al. 2006). In the spinal cord, many studies show a contribution of $I_{\text{NaP}}$ to the enhancement of neuronal activity and the promotion of rhythm generation (Theiss et al. 2007; Zhong et al. 2007). Recent studies show that an up-regulation of $I_{\text{NaP}}$ slows the locomotor rhythm and provides bursting discharge properties to putative pacemaker neurons such as Hb9 or commissural interneurons in isolated spinal cord (Tazerart et al. 2008; Ziskind-Conhaim et al. 2008). In line with these findings, our experiments in cultures of dissociated spinal cord neurons suggest that $I_{\text{NaP}}$ promotes intrinsic spiking in previously silent neurons.

There is growing evidence that $I_{\text{NaP}}$ is modulated by 5-HT. 5-HT facilitates persistent inward current in spinal cord and vibrissa motoneurons (Cramer et al. 2007; Li et al. 2007) and it facilitates $I_{\text{NaP}}$ in mesencephalic and spinal motoneurons (Harvey et al. 2006; Tanaka and Chandler 2006). In line with these reports we found that the induction of intrinsic spiking in
spinal neurons by 5-HT goes in parallel with an increase in \( I_{\text{NaP}} \). This suggests that 5-HT contributes to depolarize the neuron to its threshold for action potential generation and spontaneous firing (Kuo et al. 2006). A depolarization of the membrane may thus be important and explain the activation of nonspiking cells by 5-HT. It has been shown that the firing rate of spiking cells depends strongly on the membrane potential (Darbon et al. 2002) and that the blockade of \( I_{\text{NaP}} \) in the absence of synaptic transmission hyperpolarizes the intrinsic spiking cells by about −4 mV (Darbon et al. 2004). In the present study we found that 5-HT slightly depolarized both intrinsically spiking and nonspiking neurons. However, we found no significant difference in membrane potential between spiking and nonspiking neurons. Therefore intrinsic spiking cannot be based solely on neuronal depolarization. A similar conclusion was reached by Harvey et al. (2006).

We could confirm in our study that 5-HT augments \( I_{\text{NaP}} \) and induces intrinsic spiking through 5-HT2 receptors and the PLC cascade. Several studies have investigated the PLC–PKC pathway activated by 5-HT2 receptor (Raymond et al. 2001). Even if most studies have demonstrated that 5-HT2 receptors coupled to the PLC–PKC signaling cascade increase excitability (Miller et al. 1996; Shay et al. 2005; Yamazaki et al. 1992) and facilitates \( I_{\text{NaP}} \) (Cramer et al. 2007; Harvey et al. 2006), it is important to notice that activation of 5-HT2 receptors can also have inhibitory effects in the cerebral cortex (Brett et al. 1988; Carr et al. 2002). In contrast to our study, Dai et al. (2009) reported that PKC can reduce the amplitude of the fast and persistent inward current in spinal ventral neurons. The bufferring of intracellular calcium may contribute to this discrepancy (Harvey et al. 2006).

5-HT has also been shown to promote the recovery of locomotor functions after lesions (Fong et al. 2005; Landry and Guertin 2004; McEwen et al. 1997). The 5-HT2 receptor agonist quipazine enhances locomotor function in rats that received neural transplants after spinal transection (Kim et al. 2000) and promotes recovery of hindlimb movement after spinal cord injury (Antri et al. 2005; Ung et al. 2008). Our finding of an activation of silent neurons with 5-HT through 5-HT2 receptors may help to explain how quipazine can promote recovery and functional recovery of locomotion.

**Muscarine induces intrinsic spiking through a mechanism that is independent of \( I_{\text{NaP}} \)**

We also investigated the effect of muscarine as an agonist of the cholinergic system that plays an important role in the spinal cord. The cholinergic pathway is required for proper development of locomotor circuits during a transient period of embryonic development (Myers et al. 2005). Whereas some studies show that ACh, through activation of muscarinic receptors, can control the excitability of motoneurons (Chevallier et al. 2006; Miles et al. 2007) and affects repetitive firing by modulating the medium afterhyperpolarization (mAHP) (Lape and Nistri 2000), little is known about its effect on interneurons in the spinal cord on the cellular level. In this study, muscarine has the same positive effect as 5-HT on intrinsic activity since it evoked intrinsic activity in previously silent neurons. Nevertheless, our data suggest that this effect is not dependent on the activation of \( I_{\text{NaP}} \). In contrast, muscarine has been shown to modulate \( I_{\text{NaP}} \) in neocortical pyramidal neurons (Mittmann and Alzheimer 1998). Further investigations must investigate the possible modulation by muscarine of other currents, such as the G-protein–coupled inward rectifying potassium currents underlying the mAHP in motoneurons (Chevallier et al. 2006) or like cation currents in pre-Bötzinger inspiratory neurons (Shao and Feldman 2000).

Taken together, our results show that, although 5-HT and muscarine have similar effects on the activation of spinal cord neurons, only for 5-HT is this effect based on the up-regulation of \( I_{\text{NaP}} \).

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