Endogenous Monoamine Receptor Activation Is Essential for Enabling Persistent Sodium Currents and Repetitive Firing in Rat Spinal Motoneurons

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Harvey, P. J., X. Li, Y. Li, and D. J. Bennett. Endogenous monoamine receptor activation is essential for enabling persistent sodium currents and repetitive firing in rat spinal motoneurons. J Neurophysiol 96: 1171–1186, 2006. First published June 7, 2006; doi:10.1152/jn.00341.2006. The spinal cord and spinal motoneurons are densely innervated by terminals of serotonin (5-HT) and norepinephrine (NE) neurons arising mostly from the brain stem, but also from intrinsic spinal neurons. Even after long-term spinal transaction (chronic spinal), significant amounts (10%) of 5-HT and NE (monoamines) remain caudal to the injury. To determine the role of such endogenous monoamines, we blocked their action with monoamine receptor antagonists and measured changes in the sodium currents and firing in motoneurons. We focused on persistent sodium currents (Na PIC) and sodium spike properties because they are critical for enabling repetitive firing in motoneurons and are facilitated by monoamines. Intracellular recordings were made from motoneurons in the sacrocaudal spinal cord of normal and chronic spinal rats (2 mo postsacral transection) with the whole sacrocaudal cord acutely removed and maintained in vitro (cords from normal rats termed acute spinal). Acute and chronic spinal rats had TTX-sensitive Na PICs that were respectively 0.62 ± 0.76 and 1.60 ± 1.04 nA, with mean onset voltages of −63.0 ± 5.6 and −64.1 ± 5.4 mV, measured with slow voltage ramps. Application of 5-HT2A, 5-HT2C, and α1-NE receptor antagonists (ketanserin, RS 102221, and WB 4101, respectively) significantly reduced the Na PICs, and a combined application of these three monoamine antagonists completely eliminated the Na PIC, in both acute and chronic spinal rats. Likewise, reduction of presynaptic transmitter release (including 5-HT and NE) with long-term application of cadmium also eliminated the Na PIC. Associated with the elimination of the Na PIC in monoamine antagonists, the motoneurons lost their ability to fire during slow current ramps. At this point, the spike evoked by antidromic stimulation was not affected, suggesting that activation of the transient sodium channel current was not impaired. However, the spike evoked after a slow ramp depolarization was slightly reduced in height and rate-of-rise, suggesting decreased sodium channel availability as a result of increased channel inactivation. These results suggest that endogenous monoamine receptor activation is critical for enabling the Na PIC and decreasing sodium channel inactivation, ultimately enabling steady repetitive firing in both normal and chronic spinal rats.

INTRODUCTION

Motoneurons possess voltage-gated persistent inward currents (PICs), composed of a persistent sodium current (Na PIC) and a persistent calcium current (Ca PIC) (Carlin et al. 2000; Hounsgaard and Kiehn 1985; Hsiao et al. 1998; Li and Bennett 2003). The Na PIC has been shown to be absolutely essential for normal, steady repetitive firing in motoneurons (Harvey et al. 2006b; Lee and Heckman 2001; Miles et al. 2005) and to play an important role in the firing of many other neurons (French et al. 1990; Jahnsen and Llinares 1984; Stafstrom et al. 1982; Taddese and Bean 2002; Urbani and Belluzzi 2000). The Na PIC is not a large current compared with the transient sodium current underlying the spike, but it is rapidly activated just subthreshold to the spike (fast and persistent), and thus plays a critical role in ensuring a sufficiently rapid depolarization to secure activation spikes (Crill 1996; Lee and Heckman 2001). That is, the transient sodium current inactivates relatively quickly on depolarization (Hodgkin and Huxley 1952); thus spike generation requires the fairly rapid subthreshold depolarization from the Na PIC to ensure that transient sodium channel activation keeps pace with the transient sodium channel inactivation (Lee and Heckman 2001). With no Na PIC present, spikes can be initiated only with rapid-onset stimulations (e.g., current steps) that replace the rapid depolarization of the Na PIC, and steady repetitive firing during a constant depolarizing current is generally absent (except at high firing rates; Harvey et al. 2006b; Zeng et al. 2005).

Motoneurons in the spinal cord are densely innervated by monoaminergic [serotonin (5-HT) and norepinephrine (NE)] axons arising from the brain stem (Alvarez et al. 1998; Schroder and Skagerberg 1985), and there are even intrinsic spinal monoaminergic neurons (Cassam et al. 1997; Newton et al. 1986). Exogenous application of the monoamine 5-HT facilitates a Na PIC in motoneurons (Harvey et al. 2006a; Hsiao et al. 1998) and, accordingly, enhances repetitive firing ability (Harvey et al. 2006a). Furthermore, PICs in general (Na and Ca PICs) are enhanced by both 5-HT and NE in motoneurons (Conway et al. 1988; Hounsgaard et al. 1988; Lee and Heckman 1999). Thus motoneurons should be affected by endogenous sources of 5-HT, or perhaps even by spontaneous activity of 5-HT receptors without 5-HT present (constitutively active receptors; see Egan et al. 1998). However, this has not been directly investigated before, with the exception of the recent studies of Perrier and Delgado-Lezama (2005) that demonstrated that endogenous 5-HT3 receptor activation facilitates the Ca PIC. Thus the purpose of this paper was to examine the importance of the endogenous 5-HT and NE receptor activation in regulating the Na PIC and normal firing behavior.

Immediately after spinal transection (acute spinal), PICs and associated plateau properties are dramatically reduced, consistent with a loss of brain stem–induced release of monoamines (Conway et al. 1988; Hounsgaard et al. 1988). However, the reduction in PICs with spinal injury might just as well be...
accounted for by a loss of other nonmonoamine neuromodulators of the Na PIC derived from supraspinal sources (e.g., glutamate; Delgado-Lezama et al. 1997). As a result, spinal transection experiments do not by themselves give definitive information on the role of monoamines in regulating PICs. Furthermore, although PICs are reduced, they are not completely lost with acute spinal transection (Harvey et al. 2006b). Nor does acute spinal transection lead to a complete loss of monoamines because the terminals of brain stem axons take several weeks to completely degenerate (Haggendal and Dahlstrom 1973; Newton et al. 1986), and nonspike-mediated leakage of monoamines from these terminals is likely, especially because the axons are injured (see Discussion and Anden 1977; Angleson and Betz 2001). Thus the small but significant Na PIC remaining in acute spinal animals might arise from such a leak of monoamines (or from constitutively active receptors). To test the hypothesis that such endogenous monoamine receptor activation facilitates the Na PIC in acute spinal rats, a definitive experiment is to block the action of these monoamines with monoamine receptor antagonists. This was the first goal of the present report and we used antagonists to the major receptors known to facilitate PICs in motoneurons: the 5-HT$_2$A, 5-HT$_2$C, and 5-1-NE receptors (Harvey et al. 2006a; Lee and Heckman 1998; Perrier and Hounsgaard 2003).

Another approach to examining the importance of endogenous monoamines in regulating the Na PIC is to wait for the descending monoaminergic axons to degenerate with long-term transection (chronic spinal state). The obvious advantage of this approach is that the potential for monoamine leakage from acutely injured axons is avoided. However, the chronic spinal state is not simply characterized by a complete loss of monoamines and the Na PIC. That is, whereas all descending monoaminergic axons degenerate, about 2–15% of the normal levels of monoamines still remain (Schmidt and Jordan 2000), arising from the intrinsic spinal 5-HT and NE neurons (Cassam et al. 1997; Newton et al. 1986) and possibly other sources of 5-HT in the spinal cord (e.g., sympathetic efferents; McNicholas et al. 1980). To further complicate matters, chronic spinal rat motoneurons, and their associated reflexes, become supersensitive to 5-HT and NE (Barbeau and Bedard 1981; Harvey et al. 2006a; Li et al. 2004b; Nozaki et al. 1977). Specifically, the Na PIC is enhanced in motoneurons of chronic spinal rats by concentrations of 5-HT that are about only 1/30 (3%) of the normal concentration needed to enhance the Na PIC in normal motoneurons (Harvey et al. 2006a), indicating a 30-fold supersensitivity. Taken together with the 2–15% residual monoamines after chronic injury, this 30-fold supersensitivity suggests that the Na PIC may be facilitated at levels near, or even well in excess of, normal (30 × 2 to 15% = 60 to 450%), consistent with the large Na PIC seen in chronic spinal rats (Harvey et al. 2006b; Li and Bennett 2003). Thus our second goal was to test the hypothesis that these residual monoamines in the spinal cord do indeed produce the large Na PIC seen in chronic spinal rats (by action on supersensitive receptors). To do this we again used a monoamine receptor blockade (with antagonists) to stop the action of endogenous monoamines on the PIC in motoneurons in chronic spinal rats. Surprisingly, we found that this monoamine blockade was so effective that it eliminated the Na PIC and this severely impaired normal repetitive firing. Thus we also quantified the detailed changes in the firing that occurred. These antagonist experiments cannot by themselves rule out the possibility that the Na PIC was in part produced by constitutively active monoamine receptors, especially considering that many monoamine receptor antagonists (including the ones we used) block constitutively active receptors, as well as receptors activated directly by monoamines (Rossier et al. 1999; Vanover et al. 2006; Weiner et al. 2001). Thus we also examined blocking presynaptic transmitter release (including monoamine release) to further confirm the role of endogenous monoamine release in facilitating the Na PIC in chronic spinal rats. Parts of this paper were previously published in abstract form (Harvey et al. 2004).

**METHODS**

Intracellular recordings were made from motoneurons in the in vitro sacrocaudal spinal cord of adult female Spraque-Dawley rats. Both normal adult rats (2–4 mo old) and spastic adult rats with chronic spinal injury (2–4 mo old) were included. The spinal cord of normal rats was transected at the S2 level at the time of removal of the sacrocaudal cord for recording in vitro (acute spinal rats). Chronic spinal rats had a complete transection at the S2 spinal level at age 50–55 days (adult) and developed spasticity in the tail muscles within 1 mo (Bennett et al. 1999, 2001b). Only rats >1.5 mo postinjury with clear spasticity were used for in vitro recording (Bennett et al. 2001c; Li and Bennett 2003). All experimental procedures were conducted in accordance with guidelines for the ethical treatment of animals issued by the Canadian Council on Animal Care and approved by the University of Alberta Health Sciences Animal Policy and Welfare committee.

**In vitro preparation**

Details of the in vitro procedures were described at length in a companion paper (Harvey et al. 2006b). Briefly, normal and chronic spinal rats were anesthetized with urethane (0.18 g/100 g; maximum of 0.45 g per rat for animals >250 g) and the whole sacrocaudal cord was removed to a dissection chamber containing modified artificial cerebrospinal fluid (mACSF). After 1.5 h in mACSF, the cord was transferred to a recording chamber containing normal artificial cerebrospinal fluid (nACSF). Usually, from the outset nimodipine was added to the nACSF to block the Ca PIC, and AP5, CNQX, strychnine, and picrotoxin were added to block fast synaptic transmission (control condition; see details below).

**Drugs and solutions**

Two kinds of ACSF were used in these experiments: a modified ACSF (mACSF) was used in the dissection chamber before recording and a normal ACSF (nACSF) in the recording chamber. Composition of the mACSF was (in mM) 118 NaCl, 24 NaHCO$_3$, 1.5 CaCl$_2$, 3 KCl, 5 MgCl$_2$, 1.4 NaH$_2$PO$_4$, 1.3 MgSO$_4$, 25 d-glucose, and 1 kynurenic acid. The nACSF was composed of (in mM) 122 NaCl, 24 NaHCO$_3$, 2.5 CaCl$_2$, 3 KCl, 1 MgCl$_2$, and 12 d-glucose. Both types of ACSF were saturated with 95% O$_2$–5% CO$_2$ and maintained at pH 7.4. The synaptic transmission blocking cocktail added to the nACSF contained 50 µM t-(-)-2-amino-5-phosphonopentanoic acid (AP5, Tocris Cookson, Ellisville, MO), 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris Cookson), 1 µM strychnine (Sigma, St. Louis, MO), and 100 µM picrotoxin (Tocris Cookson) to block N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA)/kainate, glycine, and γ-aminobutyric acid type A (GABA$_A$) receptors, respectively. The monoamine receptor blockade consisted of a combination of the monoamine receptor antagonists WB 4101 (4 µM; Tocris Cookson), RS 102221 (2 µM; Tocris Cookson), and ketanserin (5–10 µM; Tocris Cookson) to block activity at α1-norepinephrine (α1-NE), 5-HT$_{2C}$, and 5-HT$_{2A}$ recep-
tors, respectively. The antagonists ketanserin and WB4101 block classic receptor activation produced directly by monoamines, as well as constitutive receptor activation (without monoamines, inverse agonists; Rossier et al. 1999; Vanover et al. 2006; Weiner et al. 2001). In some cases these drugs were also added individually or in combinations of two out of three antagonists. Additional drugs were added as required, including 15 μM nimodipine (Tocris Cookson) to block L-type Ca channels mediating the Ca PIC, 50 μM 5-hydroxytryptamine (5-HT; Sigma), 30 μM of the 5-HT2 receptor agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI; Sigma), and 100 μM of the α1-NE receptor agonist methoxamine (Sigma). It should be noted that the whole sacral spinal cord is a large piece of tissue, with overlying white matter and pia, and thus effective drug concentrations are orders of magnitude higher than that of those in thin slice preparations.

Voltage- and current-clamp recordings

Intracellular recordings were made from motoneurons as also detailed in Harvey et al. (2006b). Briefly, motoneurons were identified by antidromic stimulation of the ventral roots. Slow triangular current ramps (0.4 nA/s) in discontinuous current clamp (DCC) were used to measure firing, frequency–current (F–I) relations, and self-sustained firing (∆f) (Harvey et al. 2006b; Li and Bennett 2003). Other cell properties, such as input resistance (Rin), spike threshold (Vth), spike overshoot, and spike maximum rate-of-rise (dV/dtmax) were determined from the current-clamp ramps as described in detail in Harvey et al. (2006a). Slow voltage ramps (3.5 mV/s) between −80 and −40 mV, in discontinuous single-electrode voltage clamp (SEVC) mode, were used to measure the PICs, as described in detail previously (Harvey et al. 2006b; Li and Bennett 2003). The PIC was quantified as the downward deviation in the current response relative to the leak current (extrapolated linear subthreshold current response).

Data analysis

Data were analyzed in Clampfit 9.0 (Axon Instruments, Union City, CA) and figures were made in Sigmaplot 8.0 (Jandel Scientific, San Rafael, CA). Data are shown as mean ± SD. Unless otherwise specified, an unpaired Student’s t-test was used to test for significant differences compared with control groups, with a significance level of P < 0.05. As required for the t-test, the data were verified to be normal, using a Kolmogorov–Smirnov test for normality, with a P < 0.05 level set for significance. In a few cases, data sets were not normal and thus instead of a t-test, the nonparametric Mann–Whitney U test was used, which does not depend on the normality of the data (denoted U test in RESULTS, tested with the usual P < 0.05 significance level).

For each data set the number of motoneurons n is indicated, and this usually corresponded to the number of rats, with the exceptions detailed next and in the RESULTS. The main exception occurred under control conditions. That is, before monoamine drug applications we usually recorded one to three cells per rat; this gave a total of 27 cells from 13 acute spinal rats and 12 cells from 10 chronic spinal rats under control conditions. Such data with multiple cells per rat were treated in two ways. First, we computed the average control response for each rat (e.g., average Na PIC from the one to three cells), and performed statistical comparisons with n = the number of rats. Second, we treated all cells as statistically independent and performed statistical comparisons with n = number of cells, ignoring the number of rats. Both methods yielded the same results in terms of whether group comparisons were significantly different (and the means were not different) because the numbers of cells and rats were sufficiently large (Gardiner and Seburn 1997); for simplicity only the mean ± SD from the second method are reported in the RESULTS. This result is consistent with the idea that multiple cells recorded in the same animal are statistically independent and can be grouped with cells from other animals, as has been extensively verified for rat motoneurons (Gardiner and Seburn 1997), and is common practice in large animal studies (Prut and Fetz 1999). Indeed, the two to three control cells that we recorded in a given acute spinal animal were recorded spatially far apart in the whole sacral spinal cord (and often on the contralateral side), and thus the electrode tract damage from one recording had no effect on the others, making these recordings independent. Also, after considerable practice, our in vitro preparations were consistently of good quality, making animal-to-animal variability low.

RESULTS

Initially, motoneurons were recorded under control conditions in which fast synaptic transmission was blocked to eliminate circuit activity in the spinal cord (with AP5, CNQX, strychnine, and picrotoxin; NMDA, AMPA/kainate, glycine, and GABA receptor antagonists; see METHODS), and nimodipine was used to block the L-type calcium channels carrying the Ca PIC. Under these conditions, during a slow voltage ramp, there was a PIC that was tetrodotoxin (TTX) sensitive, and thus resulted from a TTX-sensitive Na PIC, as described in a companion paper (Harvey et al. 2006b). In motoneurons (n = 12) of chronic spinal rats, the Na PIC amplitude was 1.60 ± 1.03 nA, with a voltage onset (Vstart) of −64.1 ± 5.4 mV. Also, the average resting membrane potential (Vm) was −76.0 ± 8.4 mV, the input resistance (Rin) was 7.4 ± 4.3 MΩ, the spike voltage threshold (Vth) was −56.9 ± 4.2 mV, and the spike height (triggered by antidromic stimulation from rest) was 89.6 ± 11.1 mV. In these motoneurons, the Na PIC amplitude was much larger on average than that in motoneurons of acute spinal rats (more than double; compare Fig. 1, A and D to Fig. 2, A and D), as previously reported (Harvey et al. 2006b; Li and Bennett 2003). The average Na PIC amplitude in motoneurons (n = 27) of acute spinal rats was 0.62 ± 0.76 nA with a voltage onset of −63.0 ± 5.6 mV. The average resting membrane potential (−75.7 ± 6.2 mV), spike voltage threshold (−56.0 ± 4.9 mV), and spike height (85.7 ± 10.0 mV) were not significantly different from corresponding values in motoneurons of chronic spinal rats (unpaired t-test, P < 0.05); however, membrane input resistance was significantly lower (4.8 ± 3.1 MΩ, U test). The current threshold for spike activation during slow current ramps was significantly lower in motoneurons of chronic spinal rats (2.00 ± 1.14 nA) than in motoneurons of acute spinal rats (4.02 ± 2.09 nA, unpaired t-test); as such, motoneurons were easier to activate after long-term spinal transection than immediately after injury, as previously described (Harvey et al. 2006b). Because motoneurons of chronic spinal rats, compared with acute spinal rats, have larger more robust PICs and are easier to depolarize (Rin higher and voltage clamp better) we focus on these cells first (Fig. 1).

Endogenous monoamine receptor activation facilitates the Na PIC after chronic spinal transection

To directly test whether endogenous activation of monoamine receptors (by endogenous spinal sources of monoamines or by constitutive activity) facilitates the Na PIC in chronic spinal rats, we applied antagonists to the major monoamine receptors (5-HT2A, 5-HT2C, and α1-NE; all Gq-protein–coupled receptors) known to facilitate PICs in vivo (Hounsgaard et
and WB 4101 (a relatively linear current–voltage (Na PIC so that all motoneurons recorded in this blockade had ably, the monoamine receptor blockade entirely eliminated the $5–10$)

At Na PIC onset (monoamine receptor blockade).

triple combination of ketanserin (5-HT2A receptor antagonist, 2006a; Perrier and Hounsgaard 2003). That is, we applied a (antagonists). Note the absence of negative deviation from leak current,

tion, gray line). Note slow reduction in Na PIC amplitude with time.

antagonists (black bar). Na PIC was significantly reduced in antagonists, and average Na PIC amplitude measured in motoneurons of chronic spinal rats (Fig. 1

tions). At Na PIC onset ($V_{START}$), the current (bottom trace) deviated negatively from the extrapolated leak current (thin line), resulting in a negativeslope region (NSR). Na PIC amplitude was estimated from maximum difference between leak current and actual current, shown with down arrow. $B$: same cell as in $A$, 50 min after addition of ketanserin, RS 102221, and WB 4101 (antagonists). Note the absence of negative deviation from leak current, indicating Na PIC was eliminated. $C$: raw currents from $A$ (Control) and $B$ (50 min) were filtered and plotted against voltage. Also shown is the current–voltage ($I–V$) relation during transition period (30-min postantagonist application, gray line). Note slow reduction in Na PIC amplitude with time. $D$: average Na PIC amplitude measured in motoneurons of chronic spinal rats under control conditions (white bar) and after addition of monoamine receptor antagonists (black bar). Na PIC was significantly reduced in antagonists, and not significantly different from zero.

al. 1988; Lee and Heckman 1999) and in vitro (Harvey et al. 2006a; Perrier and Hounsgaard 2003). That is, we applied a triple combination of ketanserin (5-HT$_{2A}$ receptor antagonist, 5–10 $\mu$M), RS 102221 (5-HT$_{3C}$ receptor antagonist, 2 $\mu$M), and WB 4101 (5-HT$_{1-\alpha}$ receptor antagonist, 4 $\mu$M), which for brevity we labeled the monoamine receptor blockade. Remarkably, the monoamine receptor blockade entirely eliminated the Na PIC so that all motoneurons recorded in this blockade had a relatively linear current–voltage ($I–V$) relation ($n = 7/7$; Fig. 1, $B$ and $C$). On average, motoneurons recorded in the monoamine receptor blockade had a Na PIC of $-0.09 \pm 0.40$ nA (Fig. 1$D$, black bar; $n = 7$ cells; four cells of which were recorded throughout the antagonist application in four rats, as in Fig. 1C, black bar; the remaining three cells were obtained from two additional rats after the antagonists had taken effect; see following text), not significantly different from zero and significantly less than the large Na PIC in motoneurons of chronic spinal rats under control conditions (1.60 $\pm 0.63$ nA,

$\gamma$-aminobutyric acid (GABA$_{A}$), glycine, and glutamate (Glu) receptors (Hounsgaard and Belford 1982; Harvey et al. 1984; Harvey et al. 1988; Machacek et al. 2001). That is, we applied a component of the Na PIC that was eliminated by the monoamine receptor blockade, showing complete absence of Na PIC, $I–V$ plot for motoneuron held through monoamine receptor blockade (filtered). Small Na PIC in control was eliminated 55 min after addition of antagonists (linear $I–V$ relation). $D$: group data for motoneurons of acute spinal rats, as in Fig. 1$D$. Na PIC amplitude significantly reduced in antagonists (black bar) compared with control (white bar), and not significantly different from zero.

FIG. 1. In motoneurons of chronic spinal rats the persistent inward sodium current (Na PIC) is blocked by antagonists acting on 5-hydroxytryptamine2A and -2C (5-HT$_{2A}$ and 5-HT$_{2C}$) and $\alpha$-1-norepinephrine ($\alpha$1-NE) receptors (monoamine receptor blockade). $A$: intracellular recording from motoneuron of chronic spinal rat during slow triangular voltage ramp (3.5 nA/s from $–80$ to $–40$ mV and back to $–80$ mV; top trace) in single-electrode voltage clamp (SEVC) mode, performed during pharmacological block of fast synaptic transmission [using $(-)-2$-amino-5-phosphopentanoic acid (AP5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), strychnine, and picrotoxin] and the Ca PIC (using nimodipine; see METHODS) to isolate the Na PIC (control conditions). At Na PIC onset ($V_{START}$), the current (bottom trace) deviated negatively from the extrapolated leak current (thin line), resulting in a negativeslope region (NSR). Na PIC amplitude was estimated from maximum difference between leak current and actual current, shown with down arrow. $B$: same cell as in $A$, 50 min after addition of ketanserin, RS 102221, and WB 4101 (antagonists). Note the absence of negative deviation from leak current, indicating Na PIC was eliminated. $C$: raw currents from $A$ (Control) and $B$ (50 min) were filtered and plotted against voltage. Also shown is the current–voltage ($I–V$) relation during transition period (30-min postantagonist application, gray line). Note slow reduction in Na PIC amplitude with time. $D$: average Na PIC amplitude measured in motoneurons of chronic spinal rats under control conditions (white bar) and after addition of monoamine receptor antagonists (black bar). Na PIC was significantly reduced in antagonists, and not significantly different from zero.

Motoneurons of chronic spinal rats recorded in the monoamine receptor blockade were considered healthy, with an average resting membrane potential of $-73.2 \pm 8.0$ mV ($n = 7$), input resistance of $5.4 \pm 1.6$ M$\Omega$ ($n = 7$; $U$ test), and antidromic spike height of $87.3 \pm 8.4$ mV ($n = 7$; evoked from rest; see inset of Fig. 4$E$), all not significantly different from control. Thus the loss of the Na PIC was again not a result of cells deteriorating.
Endogenous monoamine receptor activation facilitates the Na PIC in motoneurons of acute spinal rats

In acute spinal rats, the potential source of monoamines is larger than that in chronic spinal rats because the terminals of the axotomized brain stem monoamine neurons are not degenerated and may leak their monoamines (see DISCUSSION). However, the potentially higher concentrations of available endogenous monoamines act on receptors that are not supersensitive to monoamines (Harvey et al. 2006a), and thus may produce only the small and variable Na PIC observed after acute injury (Harvey et al. 2006b). To test whether endogenous monoamines are responsible for the small Na PIC in motoneurons of acute spinal rats, we again applied the monoamine receptor blockade of ketanserin, RS 102221, and WB 4101 (Fig. 2).

Without exception, all motoneurons of acute spinal rats recorded in this monoamine receptor blockade (n = 6/6) had a relatively linear I–V relation (or net outward current; Fig. 2, B and C), and thus had little or no detectable Na PIC. On average, the Na PIC amplitude was −0.07 ± 0.24 nA (Fig. 2D, black bar), which was not significantly different from zero and significantly less than the Na PIC in control conditions (0.62 ± 0.76 nA, n = 27; Fig. 2D, white bar). In the monoamine receptor blockade, these motoneurons were again all characterized as viable cells, in that the resting potential was on average Vm = −70.3 ± 4.2 mV and spike height measured by antidromic stimulation from rest was 87.6 ± 14.8 mV (n = 6; see inset of Fig. 4B). So, again, the monoamine receptor blockade selectively eliminated the Na PIC, consistent with the idea that endogenous monoamines (and/or constitutively active monoamine receptors) play a central role in facilitating the Na PIC in acute spinal rats, as in chronic spinal rats.

5-HT2A, 5-HT2C, and α1-NE receptors are all involved in facilitating the Na PIC

When just the 5-HT2 receptor antagonists (combination of RS 102221 for 5-HT2C and ketanserin for 5-HT2A) were applied alone (without the α1-NE antagonist WB 4101), the Na PIC was significantly reduced to 37.7% of the control values, although there was a significant Na PIC remaining (0.57 ± 0.57 nA, n = 9; compared with 1.60 ± 1.03 nA, n = 12 in control conditions; only chronic spinal animals tested). Thus endogenous activation of 5-HT2 receptors partly, but not completely, controls the Na PIC, consistent with the previously established action of 5-HT2 receptors in facilitating the Na PIC (Harvey et al. 2006a). The remaining portion of the Na PIC was attributed to the action of endogenous NE because addition of WB 4101 together with the 5-HT2 receptor antagonists completely eliminated the Na PIC, as discussed above (n = 13, with six acute and seven chronic, described earlier). Application of the NE antagonist WB 4101 alone significantly reduced the Na PIC to 21.9% of the Na PIC amplitude in control conditions (Na PIC was 0.35 ± 0.32 nA in WB 4101; n = 7 cells from five rats; see example in Fig. 3 where, as usual, Na PIC amplitude is denoted by length of arrows; chronic spinal), but was insufficient to eliminate it completely. Again, the Na PIC was eliminated by ketanserin and RS 102221 in addition to WB 4101 (n = 13). The reduction of the Na PIC by either the α1-NE receptor antagonist (WB 4101) or 5-HT2 receptor antagonists (ketanserin and RS 102221) clearly did not sum linearly (each caused a reduction >50%), suggesting that perhaps there is a threshold for readily facilitating the Na PIC (see DISCUSSION), below which the Na PIC is harder to modulate.

Because of the slow action of these antagonists (see above), care was taken to avoid overlap in the action of sequentially applied antagonists. That is, the response for the first antagonist (e.g., WB 4101) was used only after a steady state had been reached in the recorded PIC, usually >1 h after drug(s) application. The next antagonist (such as 5-HT2 antagonist) was added so that its action was after this steady state was reached in the first antagonist. Thus there were no interactions between drugs, and the nonlinear summation was not a trivial result of overlap in the action of these drugs. Often this second drug response required recording from another cell in the same rat (or another rat) because cells could rarely be held longer than 2–3 h, and the advantage of this was that the response was recorded hours after the first antagonist application.

Ketanserin is reported to block not only the 5-HT2A receptors but also the 5-HT2C receptors (Bonhaus et al. 1997). However, it has a lower affinity for 5-HT2C receptors (Bonhaus et al. 1997), and we did not find it to be an effective 5-HT2C receptor antagonist (even though we used a high dose; 10 μM). That is, a combination of just ketanserin and WB 4101 did not eliminate the Na PIC (unlike when RS 102221 was also present) and left a significant Na PIC of 0.20 ± 0.19 nA (n = 8, with five chronic and three acute combined), suggesting that 5-HT2C receptors were not blocked by ketanserin. Thus elimination of the Na PIC required the selective 5-HT2C receptor antagonist RS 102221.

Endogenous monoamine receptor activation is critical for repetitive firing in motoneurons

Previously, it was reported that the Na PIC is critical for initiation of repetitive firing because, for example, a direct block of the Na PIC with riluzole (or a low dose of TTX) eliminates the ability of motoneurons to fire repetitively during slowly increasing current ramps (Harvey et al. 2006b). Thus we evaluated whether the indirect elimination of the Na PIC seen in the monoamine receptor blockade had a similar detrimental effect on firing. Before drug applications, motoneurons in acute (Fig. 4A) and chronic (Fig. 4D) spinal rats could readily fire repetitively during slow triangular current injec-
tions (see details in Harvey et al. 2006b), whereas in the monoamine receptor blockade, repetitive firing ability during a slow current ramp was lost in all motoneurons of acute spinal rats (Fig. 4B; n = 6/6) and all but one motoneuron of chronic spinal rats (Fig. 4E, n = 6/7). Current ramps that were two to three times faster than in control conditions still did not initiate firing (Fig. 4B). This firing ability was lost only at the time when the Na PIC was completely eliminated (>45 min after antagonist application; Fig. 1), consistent with the link between the Na PIC and firing ability described above. As mentioned above, the antidromic spike evoked from rest was not affected by the monoamine receptor blockade (see insets in Fig. 4, B and E; solid and dotted lines, representing before and after antagonist application, respectively). Thus the loss of repetitive firing was not from a loss of the sodium spike, but instead from a loss of the Na PIC that helped initiate spikes during a slow ramp.

It was previously described that without a fast-activating persistent current like the Na PIC spikes are not initiated during a slowly increasing current ramp because inactivation of the transient sodium current immediately follows its activation and readily keeps pace with activation during slow or sustained depolarizations (see introduction; Lee and Heckman 2001). The Na PIC normally serves to accelerate the depolarization of the membrane sufficiently to minimize Na-channel inactivation and maintain sufficient Na-channel availability to produce a spike. Indeed, any rapid depolarization (exceeding a critical dV/dt) that replaces this function of the Na PIC can initiate spikes when the Na PIC is not present (see Harvey et al. 2006b). Consistent with this understanding, current steps (Fig. 4, C and F) or very fast ramps (10 times normal speed in Fig. 5Dii) were still able to initiate spikes when the Na PIC was eliminated by the monoamine receptor blockade. On average, in this blockade, the minimum rate of rise of potential needed to evoke spiking with fast current ramps was dV/dr = 16.1 ± 9.5 mV/s (n = 11 tested; acute and chronic combined), which was significantly greater than the rate of rise of potential induced by the standard slow current ramp before Na PIC activation in control conditions (2.6 ± 0.9 mV/s, n = 39; U test, acute and chronic not different and combined) and similar to the rate of rise of potential induced by the Na PIC during the subthreshold acceleration in control conditions 10–100 ms before the first spike (without monoamine blockade; dV/dr = 12.7 ± 15.6 mV/s; n = 39; U test). In control conditions (without blockade) the rate of rise of potential accelerated rapidly just before the spike, and at 1–10 ms before the spike it was 293 ± 153.3 mV/s (n = 39), although this might be partly the transient sodium channel activating as well as the Na PIC.

In the absence of a Na PIC (in monoamine receptor blockade), after initiation of one spike on a step or fast ramp, subsequent spikes arose when there was a sufficiently fast upswing in potential at the end of each afterhyperpolarization (AHP; i.e., during fast firing), together with a ramp increase in current. A dramatic demonstration of this is the fast repetitive firing initiated by a single antidromically evoked spike (at * in Fig. 5Diii) during a slow current ramp, whereas the same ramp could not by itself evoke firing (Fig. 5D; n = 5). During a current step, the firing usually stopped after a few spikes (Fig. 4).
Monoamine receptor antagonists increase depolarization-induced sodium channel inactivation

As stated above, the antidromic spike evoked from rest was not affected by the monoamine receptor antagonists (Fig. 5, Ai and Bi). However, the first spike evoked during a slow current ramp was subtly affected by these antagonists (when firing was still present; Fig. 5Bi), likely resulting from increased sodium channel inactivation. As just mentioned, during a slow current ramp, the transient sodium current is normally partly inactivated by the slow depolarization before the first spike. Changes in the first spike’s amplitude and maximum rate of rise (dV/dtmax) are all sensitive indicators of the sodium channel availability after such partial inactivation; thus changes in these parameters have been used to infer changes in sodium channel inactivation (Schlué et al. 1974; Schmidt and Stampfli 1966). The slow onset of the action of the monoamine receptor blockade (all three antagonists) gave us an opportunity to study the changes in these sodium channel inactivation-related parameters that occurred in parallel to the decrease in the Na PIC. That is, when the Na PIC was only partly eliminated by the monoamine receptor blockade (Fig. 5B, firing still present), during a slow current ramp there was a significant decrease in the height of the first spike (spike overshoot decreased to 11.0 ± 4.8 mV, n = 7, compared with 18.6 ± 5.7 mV for control chronic spinal motoneurons, n = 12; see Fig. 5Bi), and a significant decrease in dV/dtmax (to 119.6 ± 44.6 V/s, n = 7, compared with 161.1 ± 34.1 V/s, n = 12 under control conditions; chronic spinal), consistent with increased sodium channel inactivation in the subthreshold region. Part of this increase in spike overshoot and dV/dtmax might simply be linked to an increase in spike threshold Vth, although this is unlikely to be a major factor because, on average, Vth was not significantly increased compared with control (−57.5 ± 6.4 mV, n = 7, compared with −56.9 ± 4.2 mV, n = 12 under control conditions).

Once the Na PIC was eliminated (at full monoamine receptor block; Fig. 5, C and D), firing was not evoked by the ramp alone, but could still be triggered by an antidromic stimulation during a current ramp (as described above). The antidromically evoked spikes measured near the normal spike threshold (Vth) had a lower amplitude (Fig. 5Civ) and dV/dtmax suggested again that increased sodium channel inactivation occurred during the ramp at this point. That is, across all motoneurons from chronic spinal rats the spike amplitude (overshoot) and dV/dtmax measured for antidromic spikes triggered near Vth were significantly lower in the full monoamine receptor blockade (11.8 ± 3.9 mV and 138.4 ± 22.8 V/s respectively; n = 7), compared with that in control conditions (19.8 ± 5.3 mV and 172.5 ± 32.1 V/s; n = 12). Taken together, the above results suggest that, with the monoamine receptor blockade, there is greater sodium channel inactivation during a slow current ramp. This occurs in parallel to the decrease in Na PIC, which is likely not a coincidence, because in general the degree of sodium channel inactivation helps determine the size of the Na PIC (see DISCUSSION; Taddese and Bean 2002).
Tonic activity at any single monoaminergic receptor is sufficient to maintain repetitive firing

With the exception of a few motoneurons, we did not see the loss of repetitive firing during slow current ramps unless all three antagonists (WB 4101, ketanserin, and RS 102221) were added to the bath. In total, 19 of 22 motoneurons (including 14/17 chronic and 5/5 acute spinal motoneurons) measured in combinations of any two out of three antagonists still exhibited repetitive firing in response to slow current ramps (<0.8 nA/s) and had some Na PIC remaining. This was the case when we omitted either ketanserin (n = 4/4 still firing, three chronic and one acute), RS 102221 (n = 6/8; two chronic did not fire) or WB 4101 (n = 9/10, one chronic did not fire) from the combination of the three antagonists. Thus it appears that, in most cases, tonic background activity at any one of the three monoamine receptors investigated here is sufficient to allow the motoneuron to trigger a spike during a slow current ramp and maintain repetitive firing. This suggests that all three receptors are involved in enabling repetitive firing and this is related to the corresponding changes in the Na PIC described above.

Motoneurons fire faster with monoamine receptor antagonists

During the experiments, a hallmark of the monoamine receptor antagonists taking effect was that firing occurred at higher rates (before full block of the Na PIC and firing). This was made especially clear by evaluating the minimum firing rate, calculated from the last interspike interval on the down ramp during current-clamp recordings (right of Fig. 6B; rate higher in antagonists, solid symbols). On average, under control conditions (in nimodipine) motoneurons of chronic spinal rats had a minimum firing rate of 4.0 ± 1.3 Hz (n = 12 tested; left of Fig. 6A). With one monoamine receptor antagonist applied to the bath the minimum firing rate in chronic spinal rat motoneurons was increased significantly to 7.1 ± 3.6 Hz (3.1 Hz increase; Fig. 6A; n = 10; seven with WB 4101 and three with RS 102221, data combined; steady-state response). This was associated with a significant reduction in the Na PIC amplitude (see above) and elimination of the negative-slope region (NSR) produced by the Na PIC (depth of NSR reduced from 0.56 ± 0.46 to 0.13 ± 0.18 nA), consistent with the importance of the NSR in enabling steady slow firing (<6 Hz; see Harvey et al. 2006b). Likewise, with any of the two monoamine antagonists in the bath (n = 14 chronic spinal tested) the minimum firing rate was significantly increased to 6.6 ± 2.4 Hz (2.6 Hz increase compared with control; U test) and the NSR was again eliminated (reduced to 0.05 ± 0.01 nA).

In motoneurons of acute spinal rats the minimum firing rate was 7.4 ± 2.5 Hz (n = 26), significantly larger than that in chronic spinal rats (4.0 ± 1.3 Hz, n = 12, U test), resulting from the smaller Na PIC in acute spinal rats (see Harvey et al. 2006b). In these acute spinal rats, with two antagonists in the bath, the minimum firing rate was significantly increased to 11.0 ± 6.3 Hz (4 Hz increase; n = 5; right of Fig. 6A; single antagonists not tested).

Not only was the minimum firing rate increased by 3–4 Hz in the presence of monoamine antagonists, as just described, but overall the firing rate was increased. That is, the initial firing rate (at recruitment) was increased by nearly 50% to 9.5 ± 3.1 Hz with two antagonists present (n = 14, chronic spinal) compared with 6.5 ± 1.6 Hz in control conditions (n = 12). Likewise for motoneurons of acute spinal rats, the initial rate increased significantly from 10.0 ± 5.3 Hz under control conditions (n = 27) to 15.1 ± 7.0 Hz with two antagonists present (n = 5). This increase persisted throughout firing, as shown in Fig. 6B. Interestingly, the monoamine antagonists usually did not significantly change the firing frequency–current (F–I) slope, so that the F–I relation was simply shifted vertically upward (parallel shift as in Fig. 6, B and C; plots in 6C aligned at recruitment current, which was always higher in the antagonists, Fig. 6B). That is, compared with the F–I slope in control conditions in chronic spinal rats (5.91 ± 1.46 Hz/nA, n = 12), the F–I slope was not significantly different with one (7.42 ± 2.69 Hz/nA, n = 6), two (7.36 ± 1.49 Hz/nA, n = 7), or all (5.97 ± 2.73 Hz/nA, n = 5; just before full block of firing) antagonists present. Acute spinal rats had fairly high F–I slopes initially under control conditions (9.48 ± 6.22 Hz/nA; see Bennett et al. 2001c), and application of two monoamine antagonists did not significantly increase this slope (9.17 ± 2.14, n = 5).
Be facilitated by 5-HT2 receptor agonists (and/or 5HT2 antagonists), subsequent application of high doses of monoamine antagonists that reduced the Na PIC (WB 4101 agonists known to facilitate the Na PIC. That is, in the presence of monoamine receptor antagonists (Melena et al. 2000; Riccioppo Neto 1979), we demonstrated that the effects of the monoamine receptor blockade does not directly block Na channels.

It is unlikely that the monoamine antagonists directly affected the sodium channels but, instead, likely acted indirectly on the sodium channels by the monoamine receptors because the antidromic sodium spike evoked from rest was not significantly affected by these antagonists (see above). However, to rule out any possible direct block of the Na PIC channels by the monoamine antagonists (Harvey et al. 2006b; Li and Bennett 2003). However, in some additional motoneurons from chronic spinal rats (n = 7), we also examined the action of the monoamine receptor blockade without nimodipine present (Fig. 9). In these cells, there was a PIC seen in the monoamine receptor blockade, likely a Ca PIC, because with nimodipine present this same monoamine receptor blockade eliminated the Na PIC (see above; total PIC is made up of only Na and Ca PICs). Also, the mean PIC amplitude and onset threshold (V_{START}) recorded in the monoamine receptor blockade were 1.39 ± 0.97 nA and −61.5 ± 9.4 mV (n = 7), not significantly different from the amplitude (1.82 ± 0.78 nA) and V_{START} (−57.1 ± 7.1 mV; n = 8; U test) previously reported for the Ca PIC in chronic spinal rats (Harvey et al. 2006b). Finally, the PIC activation threshold was considerably more depolarized than its deactivation (Fig. 9A), resulting in a significant voltage hysteresis of −7.4 ± 3.6 mV (n = 7), which is a distinctive characteristic of the Ca PIC, not generally seen with the Na PIC (Li and Bennett 2003).

In these cells recorded in the monoamine blockade without nimodipine (n = 7), firing was severely impaired, consistent with slow current ramps.

Without nimodipine, monoamine receptor blockade does not block Ca PIC, but still disrupts firing

All the cells described above were recorded in the presence of nimodipine to block the Ca PIC, a current whose size is similar to that of the Na PIC (Harvey et al. 2006b; Li and Bennett 2003). However, in some additional motoneurons from chronic spinal rats (n = 7), we also examined the action of the monoamine receptor blockade without nimodipine present (Fig. 9). In these cells, there was a PIC seen in the monoamine receptor blockade, likely a Ca PIC, because with nimodipine present this same monoamine receptor blockade eliminated the Na PIC (see above; total PIC is made up of only Na and Ca PICs). Also, the mean PIC amplitude and onset threshold (V_{START}) recorded in the monoamine receptor blockade were 1.39 ± 0.97 nA and −61.5 ± 9.4 mV (n = 7), not significantly different from the amplitude (1.82 ± 0.78 nA) and V_{START} (−57.1 ± 7.1 mV; n = 8; U test) previously reported for the Ca PIC in chronic spinal rats (Harvey et al. 2006b). Finally, the PIC activation threshold was considerably more depolarized than its deactivation (Fig. 9A), resulting in a significant voltage hysteresis of −7.4 ± 3.6 mV (n = 7), which is a distinctive characteristic of the Ca PIC, not generally seen with the Na PIC (Li and Bennett 2003).

In these cells recorded in the monoamine blockade without nimodipine (n = 7), firing was severely impaired, consistent with slow current ramps.
with a lack of Na PIC; that is, five of seven cells were not able to produce steady repetitive firing at all, and the remaining produced only fast firing. Of the five cells that could not produce steady repetitive firing, three did not even fire transiently and two could produce transient firing at the rapid onset of the Ca PIC (Fig. 9B). In the latter two cells, the Ca PIC onset acted like a current step (as in Fig. 4, C and F, or fast ramp as in Fig. 5Dii) and triggered the onset of rapid firing in the absence of a Na PIC (see expanded onset in Fig. 9C, with rapid Ca PIC–induced depolarization indicated). After the transient firing, there emerged a classic calcium plateau (Fig. 9B). In contrast, under control conditions, all motoneurons in chronic spinal rats produced sustained firing during the period where the Ca PIC was activated (8/8) and never showed the transient firing pattern seen in Fig. 9B (not shown; but see Li et al. 2004a).

Prolonged treatment with cadmium also eliminates the Na PIC

The elimination of the Na PIC by the monoamine receptor blockade can be interpreted in two ways that are not mutually exclusive: 1) it may arise from a block of receptors activated by endogenous monoamines and/or 2) it may arise from a block of constitutively active receptors in the absence of monoamines. To distinguish these two we attempted to block presynaptic release of monoamines. Presynaptic vesicular release of transmitter, whether or not induced by action potentials, is mediated by elevated calcium levels in the axon terminal (Angleson and Betz 2001; Katz and Miledi 1969). Nimodipine is not likely to block presynaptic actions because it does not block excitatory postsynaptic potentials (Li and Bennett 2003) and does not interfere with the N- and P/Q-type calcium channels that normally mediate vesicular release from synapses. However, cadmium blocks all calcium channels, leading to nearly complete elimination of calcium-mediated neurotransmitter release (Cooper and Manalis 1984; Forshaw 1977). Thus if the source of monoamines is from presynaptic terminals, then we should see a reduction of the Na PIC after 45 min in cadmium, similar to the delay in reversing the effects of exogenously applied 5-HT (Harvey et al. 2006a) or applying the combination of three monoamine receptor antagonists.

In motoneurons of chronic spinal rats without nimodipine, both persistent sodium and persistent calcium components contribute to the total PIC (Fig. 10A; see also Harvey et al. 2006b; Li and Bennett 2003). Cadmium (Cd²⁺) acts quickly (<5 min) to eliminate the persistent calcium component, but initially leaves a large Na PIC (measured at 5–20 min after application; Fig. 10B; TTX sensitive; see also Li and Bennett 2003). However, motoneurons recorded after >45 min (≤4 h) in cadmium did not exhibit a significant Na PIC (as in Fig. 10C; n = 7 chronic spinal; average Na PIC = 0.10 ± 0.40 nA). Also, steady repetitive firing was very poor (Fig. 10C; firing absent or transient in five of seven cells), even though antidromic spikes were still present (Fig. 10C, inset). Together these results are consistent with the idea that this long-term exposure to cadmium blocked the presynaptic release of monoamines that were facilitating the Na PIC.

Motoneurons recorded in long-term cadmium exposure were significantly more depolarized (mean −50.9 ± 10.1; n = 7) than in control chronic spinal rats (−76.0 ± 8.4 mV, n = 12), raising the concern that cadmium exposure had postsynaptic effects that contributed to the loss of the Na PIC. To rule out the possibility that the sodium currents (Na PIC and spikes) were directly damaged by cadmium, we applied 5-HT (10 μM) in these cadmium-treated cells and found that the Na PIC and repetitive firing during slow ramps was recovered (Fig. 10D). Thus long-term exposure to cadmium results in a loss of the Na PIC, which is not attributed to direct sodium channel block or toxicity because it can be restored by exogenous application of 5-HT to stimulate 5-HT receptors on the motoneurons.

DISCUSSION

Our results demonstrate that three major monoamine receptors are responsible for the spontaneously occurring Na PIC seen in spinal motoneurons recorded in the in vitro whole sacrocaudal spinal cord: 5-HT₂A, 5-HT₂C, and α₁-NE receptors. These receptors are endogenously activated, likely by endogenous monoamines in the spinal cord (or constitutive activity), because blocking them with their respective antagonists (ketanserin, RS 102221, and WB 4101) completely eliminates the Na PIC. Furthermore, blocking presynaptic release...
Even in chronic spinal rats, where there are scant endogenous monoamines available in the spinal cord, blockade of the monoamine receptors with the three antagonists eliminated the Na PIC and repetitive firing. This finding, together with the recent finding that the Na PIC is supersensitive to monoamines in chronic spinal rats (Harvey et al. 2006a), supports the hypothesis that the small amounts of residual monoamines left in chronic spinal rat spinal cords below the lesion act on supersensitive monoamine receptors to produce the large Na PIC seen in motoneurons of chronic spinal rats. Ultimately, it is this supersensitivity to residual monoamines that likely explains spastic activity (muscle spasms) in chronic spinal rats, and even humans, because these spasms have been shown to result from PICs on motoneurons (Bennett et al. 2004; Gorassini et al. 2004; Li et al. 2004a), as elaborated further below.

**Endogenous monoamines and/or constitutively active receptors are necessary for enabling the Na PIC**

Although the elimination of the Na PIC and firing with a monoamine receptor blockade clearly indicates that the Na PIC is normally facilitated by endogenously active monoamine receptors, the reason for this endogenous receptor activity is less clear. It could include two possibilities: 1) endogenous monoamines in the spinal cord activating receptors on motoneurons and/or 2) constitutively active monoamine receptors on motoneurons. The elimination of the Na PIC and firing by long-term exposure to cadmium (see Fig. 10; RESULTS) suggests an important role of endogenous monoamines (1) because cadmium eliminates most calcium-mediated transmitter release (Cooper and Manalis 1984; Forshaw 1977) and thus should reduce the level of monoamines. However, these cadmium experiments are not definitive because cadmium has postsynaptic effects (including depolarization and loss of AHP) and should reduce postsynaptic calcium-dependent processes. For this reason, our finding that 5-HT can rescue the Na PIC and firing after long-term cadmium is important because it suggests that postsynaptically cadmium does not block the sodium channels or the intracellular pathways that facilitate the sodium channels.

5-HT<sub>2a</sub>, 5HT<sub>2c</sub>, and α1-NE receptors have all been shown to have isoforms that are constitutively active (active without the presence of monoamines; Rossier et al. 1999; Vanover et al. 2006; Weiner et al. 2001). Also, the monoamine antagonists ketanserine and WB4101 that we used are known to block such constitutively active receptors (Rossier et al. 1999; Weiner et al. 2001), and thus their action on the Na PIC could be explained by this mechanism. Constitutively active monoamine receptors have been reported to be much more sensitive to 5-HT and NE than normal monoamine receptors (Egan et al. 1998; Rossier et al. 1999). Thus the supersensitivity of motoneurons to 5-HT in chronic spinal rats (Harvey et al. 2006a) might result from increased constitutively active receptors.

**5-HT<sub>3</sub> and α1-NE receptors both play a role in facilitating the Na PIC**

Consistent with our finding that 5-HT<sub>3</sub> antagonists inhibit the Na PIC, we previously showed that 5-HT<sub>3</sub> receptor activation strongly facilitates the Na PIC (Harvey et al. 2006a).
However, we had not expected NE receptors to play such a major role in facilitating the Na PIC as they do, with the α1-NE receptor antagonist WB 4101 being the most effective antagonist in reducing the endogenous Na PIC (Fig. 3). In hindsight, the importance of NE receptors makes sense, given that the α1-NE receptor antagonist methoxamine facilitates PICs in decerebrate animals (Hounsgaard et al. 1988; Lee and Heckman 1999) and the PIC-mediated long-lasting ventral root reflexes are strongly enhanced by NE and methoxamine (Li et al. 2004b).

The combined block of 5-HT2A, 5-HT2C, and α1-NE receptors is generally required to achieve a full elimination of the Na PIC and firing because residual unblocked activity at any one of the three receptors is sufficient for motoneurons to exhibit small Na PICs and generate steady repetitive firing. As a result of all three receptors being coupled to Gq proteins (Hille 2001; Lucaites et al. 1996), they likely converge to activate the same intracellular pathway. This convergence of three separate receptors from two different monoamine systems indicates a redundancy in mechanisms by which motoneurons maintain Na PICs and repetitive firing, consistent with the importance of reliable firing in motoneurons.

The Na PIC plays a critical role in firing

The loss of repetitive firing ability that occurs when the Na PIC is eliminated with a monoamine blockade is consistent with previous reports that the Na PIC is necessary for sustaining repetitive firing in neurons (Harvey et al. 2006b; Hu and Hvalby 1992; Jahnsen and Llinas 1984; Lee and Heckman 2001; Staffstrom et al. 1982; Taddeese and Bean 2002). Importantly, this action of the monoamine receptor blockade on the firing behavior is fairly selective and thus likely results from the loss of the Na PIC because the blockade does not affect the input resistance, resting membrane potential, or spikes evoked from rest. As outlined in the Introduction, the Na PIC is a relatively fast-activating current that plays a critical role in triggering spikes, producing the rapid acceleration just before each spike, thus enabling repetitive firing during steady or slowly increasing (ramp) current injections (see Fig. 1A and Harvey et al. 2006b; Lee and Heckman 2001). The slow voltage ramps used to quantify the Na PIC in the present paper do not quantify the speed of activation of the Na PIC; they just quantify the persistent nature of the Na PIC. However, the rapid onset of the Na PIC was previously demonstrated in these same motoneurons with voltage steps (Li and Bennett 2003). Whereas most of the Na PIC is very persistent, there is a small portion of the Na PIC that inactivates within 1 s after a voltage step (Li and Bennett 2003). This partial inactivation is not possible to see with the slow voltage ramps used in the present experiments. This may explain why repetitive firing is still present when there remains only a very small Na PIC evoked by voltage ramps (Fig. 5B); the inactivating portion of the Na PIC is probably still present, until a full monoamine receptor blockade takes hold.

When the Na PIC is selectively reduced by monoamine antagonists, there is a characteristic increase in the overall firing rate and increase in the minimum firing rate, confirming the importance of the Na PIC in slow firing and lowering firing rates in general (Li et al. 2004a), and consistent with the opposite action of monoamine receptor agonists (Harvey et al. 2006a). Interestingly, monoamine-related changes in the Na PIC are not associated with much change in the F–I slope (Fig. 6C), consistent with our previous conclusion that the Na PIC does not regulate the F–I slope (Harvey et al. 2006b). However, this is inconsistent with the results of Lee and Heckman (2001), where the F–I slope was correlated with changes in the Na PIC. However, perhaps this is simply a chance correlation and the F–I slope is regulated by a mechanism separate from that of the Na PIC.

Possible mechanisms by which monoamines modulate the Na PIC

Associated with the reduction of the Na PIC by the monoamine receptor blockade, we have presented evidence for increased inactivation of the transient sodium current underlying the spike during slow ramp depolarizations. That is, during these slow depolarizations, there appears to be greater sodium channel inactivation before recruitment than normal, and thus fewer channels are available for the spike. Ultimately, this inactivation is seen as smaller and slower-rising spikes evoked from depolarized levels (around −50 mV; Harvey et al. 2006b; Miles et al. 2005; Schlue et al. 1974; Schmidt and Stampfli 1966). In contrast, the spike evoked from rest (about −70 mV) by an antidromic stimulation or current step is not affected by the monoamine antagonists, suggesting that the activation of the transient sodium current is fairly normal. Therefore endogenous (see Results) and exogenous (Harvey et al. 2006a) activation of monoaminergic receptors by 5-HT and NE must both increase the noninactivating sodium current (Na PIC) and subsequently reduce the tendency for inactivation of the transient sodium current. Monoamine antagonists reverse these effects of monoamines on sodium channel inactivation.

Considering that the Na PIC is believed to be carried by the same channels as the transient sodium current underlying the spike (Crill 1996), ultimately the mechanism by which monoamine receptor activity modulates both currents may prove to be identical. The voltage-gated sodium channels mediating the transient sodium current normally inactivate rapidly after opening. However, the persistent sodium current appears to flow through a small percentage of these channels that do not inactivate (1–3%), potentially by switching to a modal “persistent” state (Alzheimer et al. 1993) or arising from allosteric gating kinetics (Taddeese and Bean 2002). Any modification of the voltage-gated sodium channel that increases the probability of the channel entering a persistent state (i.e., less likely to inactivate) ultimately increases the total Na-channel availability. This generates a larger Na PIC and spikes with less tendency to inactivate after depolarization (i.e., less tendency for spikes evoked from depolarized potentials to be diminished in height and rate-of-rise).

Implication of slow action of 5-HT2 receptors

The monoamine receptor facilitation of the Na PIC is remarkably slow to reverse, with about 1 h required to fully reverse the facilitation of the Na PIC after removal of a bath-applied 5-HT receptor agonist (Harvey et al. 2006a) and >1 h for the monoamine receptor blockade to eliminate the Na PIC. These results are consistent with the very long lasting facilitation of spinal reflexes seen hours after removing 5-HT2

J Neurophysiol • Vol. 96 • September 2006 • www.jn.org
receptor agonists (Machacek et al. 2001). The slow reversal of action of 5-HT receptors is likely not simply explained by diffusion delays in the relatively large adult in vitro sacrocaudal spinal cord because channel blockers like TTX can rapidly diffuse into the cord (Harvey et al. 2006b; Li et al. 2004a). Consistent with Machacek et al. (2001), the activation of the 5-HT2 (and α1-NE) receptors seems to involve an intracellular switch that is difficult to reverse (or turning on receptors that remain constitutively active), and thus leaves the Na PIC available for long periods after removal of 5-HT. In contrast, 5-HT1A effects on spinal reflexes reverse quickly (Machacek et al. 2001). Inasmuch as 5-HT1A receptors regulate the resting membrane potential (Takahashi and Berger 1990), we have also seen this fast reversal of action of 5-HT1A receptors because motoneurons depolarize quickly in 5-HT and this is reversed quickly (Harvey et al. 2006a). Further, 5-HT1A receptors appear to rapidly counter many of the effects of the 5-HT2 receptors, including reducing spinal reflex facilitation (or even producing inhibition of reflexes; Machacek et al. 2001) and reducing 5-HT2-mediated increases in the Ca PIC (see Perrier and Hounsgaard 2003) and rapidly reducing the Na PIC and related firing properties (Harvey and Bennett, unpublished findings). Thus whereas 5-HT2 receptor activation excites motoneurons for hours with some kind of intracellular switch, the 5-HT1A receptor may be able to quickly reverse this. This is probably of critical functional importance, without which it would be impossible for the brain stem to regulate PICs and normal motoneuron excitability on a minute-by-minute scale, and uncontrolled contractions, like spasms, would be common in normal animals. Whether 5-HT1A receptors become supersensitive to 5-HT with chronic injury is unknown, although this seems unlikely because it would tend to cancel the large PICs and spasms caused by supersensitive 5-HT2 receptors.

Other endogenous transmitters could also regulate the Na PIC

As discussed above, the endogenous monoamines likely play the major role in facilitating the Na PIC because the Gq-coupled monoamine receptor antagonists completely eliminate the Na PIC. However, there is still a remote possibility that other endogenous transmitters (nonmonoamine) also play a significant role in facilitating the Na PIC. That is, the induction of a Na PIC may involve a threshold phenomenon, whereby the underlying changes in the sodium channel inactivation properties (see above) or upstream intracellular signal (e.g., PKC) must exceed a certain threshold before a substantial Na PIC is made available to activate. Thus many endogenous transmitters, including glutamate, acetylcholine (ACh), γ-aminobutyric acid (GABA), 5-HT, and NE could regulate the sodium channel and related intracellular signals, and together exceed the threshold for making a Na PIC available. Blocking only the action of two of these transmitters (NE and 5-HT) may be adequate to drop below this hypothetical threshold and eliminate the possibility of a Na PIC occurring. The finding that the sum of the individual reductions in the Na PIC induced by either 5-HT2 or α1-NE receptor antagonists was >100% (nonlinear sum) suggests that at least there may be a threshold above which the Na PIC is readily altered by these two monoamines. Application of muscarinic and mGluR1 receptor agonists, both of which are Gq coupled, facilitate the Ca PIC in motoneurons (Svirskis and Hounsgaard 1998), although we do not know about their action on the Na PIC. Also, application of the GABAa receptor agonist baclofen facilitates the Na PIC in motoneurons (Li et al. 2004c).

Thus in principle, endogenous glutamate, ACh, and GABA may contribute to the spontaneously occurring PICs in spinal motoneurons. However, we do not favor this interpretation because the PICs measured in motoneurons are not significantly changed by a complete block of fast synaptic transmission (Harvey et al. 2006b), which should dramatically alter the availability of glutamate and GABA because it blocks all spontaneous or evoked synaptic events observed on the motoneurons. Further, we have not been able to see changes in PICs after intense/prolonged dorsal and ventral root stimulation (unpublished observations), which should increase glutamate and ACh availability. Thus we tentatively conclude that monoamine receptor activation plays the major role in regulating the Na PIC and firing, although antagonists to other transmitters need to be tested to verify this conclusion.

The present experiments were mostly performed in the presence of nimodipine (Ca PIC blocker), and CNQX, AP5, picrotoxin, and strychnine (fast synaptic transmission blockade). These drugs may have somehow affected nonmonoamine transmitters that normally facilitate the Na PIC, by for example affecting intraspinal neurons that facilitate the Na PIC. However, this did not appear to be an important factor because the clear elimination of repetitive firing seen with the application of the monoamine receptor antagonists was seen both with and without nimodipine present (Fig. 9). The Na PIC could not be measured directly without nimodipine, but the loss of repetitive firing is strongly suggestive of a loss of the Na PIC. Finally, neither nimodipine nor the drugs involved in the blockade of fast synaptic transmission (CNQX, etc.) directly reduce the Na PIC (Bennett et al. 2001a; Harvey et al. 2006b), and thus these drugs do not appear to block transmitter systems that facilitate the Na PIC.

Monoamines in the acute spinal state likely arise from cut descending terminals

Normally, with an intact brain stem and spinal cord, PICs are mostly maintained by innervation from descending monoaminergic axons originating in the brain stem (Alvarez et al. 1998; Heckman et al. 2004; Schroder and Skagerberg 1985). However, after acute spinal transection a small Na PIC usually remains, and this is eliminated by the antagonists to monoamine receptors, as we have shown, suggesting some residual source of monoamines. Likely this source arises from acutely cut descending monoaminergic axons. Although isolated from the cell soma, axon terminals are still able to release, take up, and resynthesize neurotransmitter for lengthy periods before degeneration (Andén 1977; Karobath 1972; Patrick and Barchas 1974). Monoamines may be released as a bolus at the time of injury or slowly leak from the terminals. The former is likely to be of minimal importance because the monoamines released would either be washed out of the dissection chamber or cleared by reuptake mechanisms of the axon terminals and surrounding glia (Blakely et al. 1994; Inazu et al. 2001). Slow leak of neurotransmitter from the injured monoaminergic terminals throughout the course of the experiment is more likely to be the source of the monoamine tone. Potentially, an
injury-induced depolarization and rise in intracellular calcium concentrations (Schanne et al. 1979) promote such spontaneous release of neurotransmitter from vesicles (Angleson and Betz 2001), followed by reuptake.

**Endogenous intraspinal monoamines likely act on supersensitive receptors to produce a large Na PIC**

In the spinal cord of chronic spinal rats, the transected descending monoaminergic terminals have long since degenerated (Haggendal and Dahlstrom 1973; Newton et al. 1986) and so can no longer serve as a source of 5-HT and NE. However, several investigators have reported a residual level of monoamines (2–15%; reviewed in Schmidt and Jordan 2000) remaining below the injury, as measured by immunohistochemistry (Cassam et al. 1997; Newton et al. 1986), fluorometric analysis (Clineschmidt et al. 1971), or high-pressure liquid chromatography—electrochemical detection (Hadjiconstantinuo et al. 1984). Although this source of monoamine is small, motoneurons and the Na PIC become extremely sensitive to monoamine receptor activation (Harvey et al. 2006a) and so these endogenous monoamines may be capable of strongly exciting motoneurons. The present finding that a blockade of the action of these monoamines with receptor antagonists indeed supports the hypothesis that endogenous monoamines acting on supersensitive receptor pathways produce the large Na PIC that occurs in chronic spinal rats (although constitutively active receptors may also play a role).

Although it is clear that there is a residual source of monoamines independent of brain stem innervation in the chronic spinal sacrocaudal cord, the origin of these monoamines is subject to debate. A likely source of monoamines below a chronic spinal transection is the intrinsic intraspinal monoaminergic neurons. The existence of intraspinal serotonergic neurons, albeit in very small numbers, has been reported in the low lumbar and sacrocaudal spinal cords of rats (Newton et al. 1986). These cells are associated with the autonomic system and have long, spindle-shaped cell bodies that can be readily distinguished only in longitudinal sections. These neurons are not easily observed in cross sections of spinal cord because of their shape and orientation, and thus have been missed in other investigations (Holets and Elde 1982; Kojima and Sano 1983; Steinbusch 1981). The numbers of these intraspinal serotonergic neurons may increase with loss of descending 5-HT terminals (Branchereau et al. 2002) and may sprout to innervate ventral motoneurons to compensate for the loss of descending serotonergic innervation. Intraspinal noradrenergic neurons are also present below a complete transection and increase tenfold in number within 2 wk after chronic spinal injury (Cassam et al. 1997). Indirect evidence for a neural supply of NE comes from the effectiveness of amphetamines in facilitating the flexor reflex in chronic spinal rats (Nozaki et al. 1977). The fact that amphetamines have actions below a chronic spinal transection suggests that there is a neural source of these monoamines below the injury because amphetamines affect presynaptic NE release and reuptake, but have no major postsynaptic effects (Carlsson et al. 1965; Hansch 1967; Randrup and Munkvad 1966). Thus motoneurons in chronic spinal rats seem to receive tonic monoaminergic innervation, likely from intraspinal serotonergic and noradrenergic neurons, perhaps associated with the autonomic system.

An additional possibility is that sympathetic efferents provide NE to the injured spinal cord. McNichols et al. (1980) has shown some sprouting of sympathetic fibers from blood vessels into the spinal cord below a spinal transection. Removal of the cord for our in vitro recording would denervate these sympathetic fibers from their ganglion bodies, and thus NE could leak only from these terminals.

**Summary and implications for management of spasticity**

In summary, endogenous activation of 5-HT₂A, 5-HT₂C, and α₁-NE receptors (likely by endogenous 5-HT and NE) is necessary for spinal motoneurons to exhibit a Na PIC, and therefore is required for initiating and sustaining repetitive firing. After chronic spinal injury, endogenous intraspinal monoamines likely act on supersensitive monoamine receptors to induce the very large Na PIC observed (although we cannot rule out the involvement of constitutively active receptors). Intrinsic intraspinal serotonergic and noradrenergic neurons are a likely source of these endogenous monoamines (Cassam et al. 1997; Newton et al. 1986) and these provide a supply of monoamines unregulated by descending control. As such, after chronic spinal injury, motoneurons exhibit a large Na PIC most of the time and could even have further enhanced PICs in response to modulation of these intraspinal monoaminergic neurons, perhaps resulting from their association with the autonomic system (Newton et al. 1986). Large, uncontrolled Na PICs and Ca PICs have been shown to underlie uncontrolled contractions (spasms) associated with spasticity in chronic spinal rats (Li et al. 2004a) and in humans with spinal cord injury (Gorassini et al. 2004). The present results suggest that intraspinal monoamines may be responsible for these large PICs, ultimately suggesting that spasticity may be a condition resulting from a developed supersensitivity to residual monoamines that persist after injury. Thus inhibiting PICs using monoamine receptor antagonists may be an effective treatment for spasticity; indeed the nonselective 5-HT antagonist cyproheptadine has been used clinically to treat spasticity, although not without side effects on appetite and mood (Norman et al. 1998; Wainberg et al. 1990). More selective monoamine receptor antagonists may prove more effective in treating spasticity. However, preventing the development of the supersensitivity of motoneurons to monoamines—and possibly related constitutively active monoamine receptors—may offer a new approach to management of spasticity.

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