Role of Endogenous Release of Norepinephrine in Muscle Spasms After Chronic Spinal Cord Injury

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INTRODUCTION

Spinal motoneurons contain low-voltage-activated persistent inward currents (PICs) that are comprised of both sodium (NaPIC) and calcium (CaPIC) components (reviewed in Heckman et al. 2005). When activated, PICs can amplify synaptic inputs, and importantly, they are essential for normal repetitive firing (Heckman et al. 2003; Lee and Heckman 1999; Li and Bennett 2003; Li et al. 2004a), making motoneuron PICs fundamental in the production and facilitation of movement. Both Na and Ca PICs are enhanced by the presence of serotonin (5HT) (Hounsgaard and Kiehn 1989; Perrier and Hounsgaard 2003) and norepinephrine (NE) (Conway et al. 1988). However, large motoneuron PICs and the self-sustained depolarizations they produce (plateau potentials) spontaneously recover in the weeks after an injury, and their gradual restoration closely parallels the development of debilitating muscle spasms (Harvey et al. 2006b; Li and Bennett 2003). The redevelopment of motoneuron PICs occurs despite the absence of descending monoaminergic drive possibly because motoneuron PICs develop supersensitivity to the 2–12% of 5HT (Newton and Hamill 1988) and/or 1–5% of NE (Magnusson 1973; Roudet et al. 1993, 1994) that are supplied by intraspinal and sympathetic neurons described in the preceding text.

Earlier suggestions that motoneuron PICs develop supersensitivity to residual sources of 5HT come from experiments where very low doses (20–100 mg/kg) of the agonist 5-hydroxytryptophan (5-HTP) increased spontaneous and reflex-evoked muscle activity in chronically spinalized rats in contrast to uninjured control animals (Barbeau and Bedard 1981; Tremblay and Bedard 1995). Similar facilitation also occurred in acutely transected animals that had chronic denervation of 5HT fibers, suggesting that enhanced responses to low-dose 5-HTP in chronic spinal animals were not simply due to general increases in neuronal excitability but rather to specific denervation supersensitivity to 5HT (Barbeau and Bedard 1981). Recently direct evidence of motoneuron PIC supersensitivity to 5HT after chronic spinal injury has been obtained in the rat (Harvey et al. 2006a,b). Facilitation of Na PICs and Ca PICs by 5HT, or from the 5HT2 receptor agonist DOI, at doses of 1 μM or less was 30 times greater in animals spinalized 2–4 mo previously compared with acutely lesioned animals (Harvey et al. 2006a; Li et al. 2006). Importantly, a combined blockade of 5HT2a, 5HT2c, and 5HT3 receptors completely eliminated the NaPIC and provides evidence that endogenous sources of 5HT and NE below the level of the lesion are necessary for the activation of Na PICs (Harvey et al. 2006b).

The main goal of the present study was to further investigate if endogenous sources of NE, like 5HT, facilitate motoneuron

PICs after chronic spinal cord injury. Specifically, we were interested in the NaPIC in generating long-lasting reflexes, or spasms, characteristic of the spastic syndrome (Bennett et al. 2001b; Li and Bennett 2003). Amphetamine, at low to moderate doses (<5 mg/kg), is known to specifically enhance the release of NE from presynaptic nerve terminals and inhibit its reuptake (de la Torre et al. 2004; Munkvad and Randrup 1966). Early evidence using amphetamines has already suggested a role for NE released from intraspinral sources in enhancing flexor reflex activity in chronically lesioned animals (Nozaki et al. 1980). In agreement with this, preserved NE intraspinal neurons can be found caudal to a lesion, albeit in reduced quantities, compared with pre lesion levels in the rat (Cassam et al. 1997).

In the present study, we examined if increasing endogenous release of NE from spinal sources below a lesion increases spinal excitability (spasms) after chronic injury to the sacral spinal cord of adult rats. To do this, we recorded both spasm behavior and cutaneous-evoked reflex responses in tail muscles of rats receiving a complete injury to the second sacral (S2) level of the spinal cord both before and after amphetamine administration. We then directly measured if amphetamine increases spinal excitability via direct actions on the motoneuron from intracellular recordings in the in vitro sacral spinal cord preparation. Reflex responses to dorsal root stimulation in this S2 in vitro preparation were also compared to dissociate neural versus potential vascular affects of amphetamine on reflex responses in the awake animal. Last we determined if long-lasting reflexes mediated by motoneuron PICs exhibit supersensitivity to NE by examining if facilitation of reflex activity in chronically injured animals occurs at appreciably lower doses of amphetamine compared with acutely injured animals.

**METHODS**

Motor responses to amphetamine administration in transected (S2) female Sprague-Dawley rats were measured under three experimental conditions: kinematic and surface EMG recording of spastic tail reflexes in the awake rat, ventral root recordings from an isolated (S2) spinal cord in vitro, and intracellular recordings from sacrocaudal motoneurons in vitro. Supersensitivity of spasm behavior and long-lasting reflex responses to amphetamines was compared between chronic (1.5–4 mo) and acutely (2–5 day) injured animals in the awake rat only. All procedures were approved by the University of Alberta animal welfare committee.

**Tail reflexes in the awake rat**

**MEASUREMENT OF SPASTICITY.** Reflexes in segmental tail muscles were recorded in chronic spinal and acute spinal rats, ranging in weight from 306 to 342 g (chronic) and 196 to 238 g (acute). All chronic rats used in the study exhibited clear spasticity in the tail muscles and had a spasticity score of ≥3 (Bennett et al. 1999). In preparation for surface EMG recordings, the tails were scrubbed with a soap solution and then wiped with a 70% ethanol solution to clear any debris and loose scales. After this, a 2-h rest period was given to allow any excitation resulting from the cleaning process to subside. To measure the degree of spasticity in the tail in response to mechanical stimuli, the rats were housed in a Plexiglas tube with the tail protruding and allowed to hang freely. The tail was then stimulated with a standardized stretch/rub maneuver (see Bennett et al. 2004 for details). Briefly, the base of the tail was held with one hand while the other gripped the tail with a damp piece of gauze held between the thumb and finger. The gauze was then quickly slid down the tail, maintaining a firm and consistent grip throughout. This stretch/rub maneuver was repeated three times in rapid succession after which the tail was allowed to move freely, hanging below the tube. The resultant response was video taped for 5 min, and the degree of spasticity was evaluated, off-line, through kinematic measurements as described in the following text.

**KINEMATIC MEASUREMENTS.** To quantify movement and spasms of the tail after a stretch/rub maneuver, flexion and extension angles of the tail were calculated as shown in Fig. 1. When viewing the animal’s left side, clockwise movements of the tail were defined as flexion (+ numbers), whereas counter-clockwise movements were defined as extension (or − flexion values). The maximum flexion and extension angles, relative to vertical, were measured at the location on the tail that exhibited the greatest degree of flexion or extension, respectively (for details, see Fig. 1, A and C). Most often the tail flexed into a pure

**FIG. 1.** Method for measuring maximum flexion and extension angles in spastic rat tail after standardized stretch/rub maneuver is applied. The tail is viewed from the left side of the animal. A: pure flexion spasm where the tail typically forms a coil. The maximum flexion angle is measured relative to vertical from a tangent line at the tip of the tail; the maximum extension angle here is zero as the tail is showing no extension. In a case of pure flexion coiling, the maximum flexion angle occurs at the tip of the tail (absolute tip angle). B: when flexion and extension both occur, the tail forms an inverted s-shape. To calculate the maximum extension angle, which occurs at the tip of the tail, the maximum flexion angle is first measured relative to vertical from a tangent drawn from the middle of the tail. The absolute extension angle of the tip of the tail is then measured relative to vertical from a tangent drawn from the tip of the tail. To obtain the maximum extension angle, the maximum flexion angle is then added to the absolute extension tip angle. C: when the tail is at rest, the maximum flexion angle occurs at the tip of the tail and therefore is identical to the absolute tip angle, and the maximum extension value is zero similar to the full flexion coil in A.
clockwise coil, and in these cases, the maximum flexion angle was the angle of the tip of the tail (Fig. 1A). At times, in very spastic animals, flexion and extension both occurred together, due to a co-contraction of flexor and extensor muscles. This caused the tail to form an inverted s-shape (Fig. 1B), with the proximal part of the tail flexing and the tip of the tail extending relative to this. In this case, the maximum flexion angle occurred near the middle of the tail, and was measured relative to vertical as in Fig. 1B. The maximum extension of the tail was measured relative to this maximum flexion angle (maximum flexion angle − tip angle; Fig. 1B) and in this case corresponded to the maximum extension angle. Less frequently, the tail extended into a counterclockwise coil, and accordingly, the maximum extension was measured at the point on the tail where maximum extension occurred, analogously to the above treatment of flexion coils (not shown in Fig. 1, but see Fig. 2A, small inset).

Maximum flexion and extension angles were measured every 5 s over the 5 min after the stretch/rub maneuver in chronic and acute spinal rats both before and after amphetamine. In addition to maximum flexion and extension angles, the number of discrete spasms (considered a jump of >50° in flexion or extension angles) was also tallied for the 5 min after the stretch/rub maneuver.

EMG–REFLEX TESTING PROTOCOL. After the stretch/rub maneuver, the rat was moved while still in the tube holder to rest on a temperature controlled water pad that kept the tail at 37°C for EMG recordings. To record surface EMG of segmental tail muscles, custom-built cuff electrodes consisting of Tygon tubing slit open and sewn with silver wire, were filled with conductive electrode gel and placed onto the tail of the rat. Electrode placement on the tail was standardized using the 12th coccygeal vertebra as a reference point. EMG recording electrodes were placed 1 and then 2.5 cm distal to this reference point with the recording ground 3 cm distal to this point. Two small stimulating cuff electrodes were placed on the distal tip of the tail, separated from each other by 1 cm. This portion of the tail is abundantly sensitive to touch and contains very little muscle, and thus this stimulation of the tip was used to provide a relatively pure cutaneous activation.

In each animal, the reflex threshold (RT) was determined, which was the minimum stimulation intensity required to evoke a small (usually 1.5–2 times larger than noise in signal) short-latency polysynaptic reflex, and varied from 0.05 to 0.30 mA in chronic animals (Isoflex stimulator, AMPI). In many cases, the RT could not be determined in acute animals, and thus the average chronic RT (0.07 mA) was used, in addition to a maximum 10-mA stimulation intensity controlled water pad that kept the tail at 37°C for EMG recordings.

DATA ANALYSIS: EMG RESPONSES. To measure reflex EMG activity in response to single or train stimulation to the tip of the tail, custom-written MatLab software (Release 14: The MathWorks, Natick, MA) was used for the analysis. After importing data, the program applied an additional high pass filter at 800 Hz (first-order Butterworth), and a low-pass filter at 1,750 Hz (first-order Butterworth), followed by rectification. Four specific reflex responses were measured. For single shock trials, the early latency, short-duration reflex (termed polysynaptic) was averaged 15–40 ms after the stimulus; the short-latency, longer-lasting reflex (termed tonic short) was measured 15–500 ms after the stimulus and the long latency, long-lasting tonic response (termed tonic long) was measured 500–5,500 ms after the stimulus. For stimulation trains, a 500- to 5,500-ms window was averaged (termed train) after the 500-ms stimulus train was applied. An interstimulus interval of 2 s was used for the polysynaptic and tonic short reflexes, 20 s for the tonic long reflexes, and 30 s for the train reflexes. Average EMG values were obtained for each reflex response by dividing the rectified EMG activity by the number of samples within the reflex interval for each trial (typically 5 trials were recorded) and then averaging the individual trial values together. Average background levels for each stimulus (single pulse or
train) were measured over the 200 ms before the stimulation and were matched across amphetamine doses to maintain comparable background levels of motoneuron excitability. In other measurements, background was not matched to examine effects of amphetamine on general motoneuron excitability. The average noise (i.e., average voltage with no EMG activity) was subtracted from all reflex values. In chronic animals, all postdrug reflex averages were normalized to the predrug condition (postdrug/predrug). In acute animals, the size of the reflex responses, especially the long-lasting reflexes, were an order of magnitude smaller than the reflexes recorded in chronic animals and at times, produced large normalization errors. Therefore the size of acute reflexes was thresholded to a minimum level (0.3 μV; ~10% of average noise) when required. As for chronic animals, postdrug reflexes in acute animals were normalized to the predrug condition (postdrug/predrug).

In vitro ventral root reflexes

A detailed description of these procedures can be found in Li et al. (2004a). Rats between 1.5 and 4 mo postinjury and exhibiting clear spasticity were used. Briefly, ventral and dorsal roots were mounted on chlorided silver wires and were suspended above the ACSF at root entry points for monopolar recording and stimulation, respectively. The root was wrapped around the wire in the air and then covered with a 1:1 mixture by weight of petroleum jelly/mineral oil to prevent the roots from drying out. Three additional wires were submerged in the ACSF for the stimulation-return, monopolar recording reference, and instrumentation ground.

Dorsal roots were stimulated with 0.1-ms current pulses for 0.5 s, 100-Hz trains usually at 0.05 mA, which was around 5 times sensory threshold ([ST]; Isoflex stimulator, AMPI). Sensory threshold was determined by transferring an unused dorsal root to the recording chamber and recording at one end while stimulating the other prior to each experiment (T = 0.007–0.01 mA; conduction velocity ranged from 16 to 24 m/s at 25°C). The anode was connected to the dorsal root, and the cathode to the stimulation-return wire in the ACSF. Ventral roots were recorded via a custom built differential preamplifier (QT5, Dean Charles, University of Alberta), with one lead connected to the root and the second to the reference wire in the ACSF [high-pass 300 Hz; low-pass 3 kHz; amplified by 2000 times; sampling rate 6.7 kHz (Axoscope, Axon Instruments)]. Dorsal root stimulation was repeated more than five times with an inter-stimulation interval set at 20 s to provide multiple ventral root reflexes for averaging.

ACSF and drug applications. Two kinds of artificial cerebrospinal fluid were used in these experiments: a modified artificial cerebrospinal fluid (mACSF) used during dissection and recovery to minimize neural and metabolic activity and a normal ACSF (nACSF) used during dissection and recovery to maintain a pH of 7.4.

Drug solutions. All solutions were made fresh daily and were not normally distributed according to the Kolmogorov-Smirnov test for normality, statistical differences were computed with a Student’s t-test at the 95% confidence level. Where the data were not normally distributed according to the Kolmogorov-Smirnov test for normality, statistical differences were computed using a Wilcoxon signed-ranks test at the 95% confidence level.

Statistics

Data are shown as the means ± SE throughout the text and figures. Where the data were normally distributed, as indicated by the Kolmogorov-Smirnov test for normality, statistical differences were computed with a Student’s t-test at the 95% confidence level. Where the data were not normally distributed according to the Kolmogorov-Smirnov test for normality, statistical differences were computed using a Wilcoxon signed-ranks test at the 95% confidence level.
RESULTS

Amphetamine induced changes in tail spasm behavior in the awake chronic spinal rat

Kinematic measurements in response to a rapid stretch/rub of the tail were compared before and after low (0.1–0.2 mg/kg; \( n = 11 \)) and high (0.6–1.0 mg/kg; \( n = 11 \)) doses of amphetamine. Prior to administration of amphetamine, the tail of a chronic spinal rat typically showed both flexion (Fig. 2A) and extension (Fig. 2B) movements in response to a manual stimulation, sometimes in isolation (e.g., pure flexion coil) and at other times simultaneously (e.g., inverted S-shape, see Fig. 2A, insets). The maximum predrug flexion and extension angles of the tail (see details in METHODS and Fig. 1) averaged over a 5-min period after stimulation are shown in Fig. 2, E and F (open bars; all animals averaged together). After a low dose of amphetamine, the maximum flexion of the tail increased dramatically (Fig. 2C) with an average increase of 41.7 \( \pm 12.2^\circ \) in flexion (\( n = 11 \); significant increase), nearly doubling the average flexion prior to amphetamine (Fig. 2E, gray bar). This increased flexion was necessarily associated with a decrease in extension with extension eliminated in some animals (Fig. 2D). On average the maximum extension angle significantly decreased by 17.0 \( \pm 6.9^\circ \) (\( n = 11 \); gray bar Fig. 2F). High doses of amphetamine produced similar significant increases in flexion and decreases in extension although these effects were slightly smaller (\( n = 11 \); solid bars in Figs. 2, E and F). Overall, with amphetamine application, there was a bias toward more tail flexion with more forceful flexion contractions overpowering the extensor muscles of the tail.

In addition to measuring maximum flexion and extension angles, the number of spasms, defined as sharp increases in flexion and extension angles (\( >50^\circ \) increase in 5 s as indicated by solid arrows in Fig. 2) were counted at low and high doses of amphetamine. Before drug administration, the average number of flexion spasms in the 5 min after a stretch/rub was 3.2 \( \pm 0.8 \) (\( n = 11 \)). The average number of spasms increased to 5.9 \( \pm 2.6 \) at low doses of amphetamine (\( n = 11 \); not significant) and, significantly, to 5.6 \( \pm 1.2 \) at high dose of amphetamine (\( n = 11 \)). Prior to amphetamine the average number of extension spasms was 2.4 \( \pm 0.6 \), which decreased significantly to 0.8 \( \pm 0.5 \) at low doses of amphetamine and to 1.6 \( \pm 1.0 \) at high doses of amphetamine. These data demonstrate that both low- and high-dose amphetamine administration in chronic spinalized rats leads to an overall increase in number of flexion spasms and a decrease in the number of extension spasms. This concurs with the kinematic data, which showed an increase in flexor and, as a consequence, a reduction in extensor angles after amphetamines in the chronic spinal rat.

Amphetamine induced changes in tail reflexes in the awake chronic rat

Single-shock electrical stimulation to the tip of the tail produced substantial polysynaptic (Fig. 3B) and long-lasting (Fig. 3A) reflexes in chronic spinal rats as measured by surface EMG over the segmental tail muscles. These reflexes, evoked by the stimulation of cutaneous afferents from the tip of the tail, increased in amplitude after administration of amphetamine. As shown for a representative
animal, both low (Fig. 3, C and D) and high (Fig. 3, E and F) doses of amphetamine increased long-lasting and polysynaptic reflexes. The effects of amphetamine were not limited to stimulus-locked reflex responses, as the amount of tonic background activity occurring prior to the stimulation also increased (compare background EMG before stimulation artifact in Fig. 3, B, D, and F).

Averaged group data (Fig. 4) demonstrate that amphetamine administration consistently increased reflex responses. The predrug polysynaptic reflex (Fig. 4A, left) was relatively large, on average, compared with longer-duration reflexes (compare absolute reflex responses, open bars on left of graphs in Fig. 4; grey and solid bars on right of graphs show reflex increases after amphetamine) and showed an increase in amplitude with both low ($n = 11$) and high ($n = 11$) doses of amphetamine when normalized to predrug values (Fig. 4A, right, grey and solid bars; significant in low dose only). The tonic-short reflex began at a lower average amplitude than the polysynaptic reflex (Fig. 4B; left) but still showed significant increases ($\approx 1.5$ times larger) at both low and high doses of amphetamine (Fig. 4B, right). The tonic long reflex (Fig. 4C) and the train reflex (Fig. 4D) increased by three- to fourfold at both low and high doses of amphetamine. Although the average amplitude of the tonic long and train reflexes was similar compared with the tonic short reflex before amphetamine administration (10–13 $\mu V$), these long-duration reflexes were substantially more sensitive to the drug as they demonstrated larger increases in average reflex amplitudes. The average level of accumulated background EMG activity recorded before single shock stimulation delivered in quick succession (10×RT every 2 s), also increased threefold after low and high doses of amphetamine (Fig. 4E).

Facilitation of ventral root reflexes in the chronic spinal rat

Increases in EMG responses by amphetamine in the awake chronic spinal rat may have been produced, in part, from changes in blood flow to the muscle in response to elevated blood pressure, tachycardia and/or selective vasodilation; known peripheral effects of amphetamine (de la Torre et al. 2004). To eliminate the contribution of these peripheral factors and dissociate neural from vascular effects, ventral root reflex responses to dorsal root stimulation in the sacral spinal in vitro preparation were also compared. Under in vitro recording conditions, large polysynaptic and short- and long-duration reflexes were evoked from dorsal root stimulation and showed dramatic increases with successively larger doses of amphetamine (Fig. 5; $n = 12$ root pairs tested). These ventral root reflexes were very similar to reflexes evoked in the awake animal (compare Fig. 5 with 3). In both cases, amphetamine induced particularly large increases in long-duration reflexes in low (Fig. 5C) and high (Fig. 5E) doses of amphetamine.

Averaged grouped data quantifies these reflex changes and demonstrates significant increases in tonic short (Fig. 4A, right), tonic long (Fig. 4C), train (Fig. 4D), and background (Fig. 4E) responses. Significant increases were seen in all groups at both low and high doses of amphetamine (Fig. 4A–E). The significant increase in background activity increased threefold after low and high doses of amphetamine (Fig. 4E).
B), tonic long (Fig. 6C), and train reflexes (Fig. 6D) with both low (0.1 μM; n = 12) and high (1–10 μM; n = 12) doses of amphetamine. High doses were much more effective in increasing reflex responses compared with the low dose especially in tonic short and long reflexes. The only exception to these substantial reflex amplitude increases is the polysynaptic reflex (Fig. 6A), which showed a small but significant decrease (24.3%) in amplitude at a low dose of amphetamine, and a small but significant increase (29.1%) in amplitude at a high dose of amphetamine.

**Effects of amphetamine on the motoneuron CaPIC in chronic spinal rats**

To examine if increases in reflex responses recorded in the awake animal and in vitro preparations were mediated, in part, from amphetamine’s action on motoneuron PICs, intracellular recordings were performed in the sacral spinal cord of chronic spinal rats (n = 9 cells from 9 rats). Voltage-clamp recordings of the motoneuron, made after the application of TTX, allow measurement of the CaPIC in isolation, which has been shown

![Graphs showing reflex amplitude changes with amphetamine](image-url)
to mediate the long-lasting reflexes (spasms) recorded in chronic spinal rats (Li and Bennett 2003). The application of a slow depolarizing voltage ramp in TTX (Fig. 7A, top trace) initially produced a measured current (Fig. 7A, bottom trace) that increased linearly until the CaPIC was activated, as reflected by a sharp downward deflection from the estimated leak current (solid line). The threshold of the CaPIC (reflected by a sharp downward deflection from the estimated leak current, which is measured from the leak current to the point of zero slope) persisted for many seconds as reflected by the sustained current in the current trace. This deviation from the leak current that is measured from the leak current to the point of zero slope (CaPIC) is indicated by the length of the solid arrow and the sustained CaPIC on the downward voltage ramp is indicated by the dashed arrow. A: response of the motoneuron in TTX, to isolate CaPIC, before amphetamine administration. Solid line on voltage ramp at −50 mV indicates spike threshold. \(v_{on}\) is the onset voltage of the CaPIC. \(I_{on}\) is the corresponding onset current. \(V_{off}\) is the offset voltage of the CaPIC. The size of the initial CaPIC, which is measured from the leak current, is indicated by the length of the solid arrow and the sustained CaPIC on the downward voltage ramp is indicated by the dashed arrow. B: response of the same motoneuron in A after administration of amphetamine. Amphetamine causes significant increases in both the initial CaPIC and sustained CaPIC. C: different motoneuron in TTX, which shows a smaller initial and sustained CaPIC than shown in A. D: response of same motoneuron in C after amphetamine application. The small CaPIC also shows substantial enhancement after application of amphetamine.

**FIG. 7.** Example recordings of the calcium component of the persistent inward current (CaPIC) from 2 motoneurons of chronic S. spinal rats measured during a slow triangular voltage ramp. Voltage command in top traces, resulting current in bottom traces with leak current shown as the thin triangular line on the current trace. A: response of a motoneuron in TTX, to isolate CaPIC, before amphetamine administration. Solid line on voltage ramp at −50 mV indicates spike threshold. \(v_{on}\) is the onset voltage of the CaPIC, \(I_{on}\) is the corresponding onset current. \(V_{off}\) is the offset voltage of the CaPIC. The size of the initial CaPIC, which is measured from the leak current, is indicated by the length of the solid arrow and the sustained CaPIC on the downward voltage ramp is indicated by the dashed arrow. A: response of the motoneuron in TTX, to isolate CaPIC, before amphetamine administration. Solid line on voltage ramp at −50 mV indicates spike threshold. \(v_{on}\) is the onset voltage of the CaPIC. \(I_{on}\) is the corresponding onset current. \(V_{off}\) is the offset voltage of the CaPIC. The size of the initial CaPIC, which is measured from the leak current, is indicated by the length of the solid arrow and the sustained CaPIC on the downward voltage ramp is indicated by the dashed arrow. B: response of the same motoneuron in A after administration of amphetamine. Amphetamine causes significant increases in both the initial CaPIC and sustained CaPIC. C: different motoneuron in TTX, which shows a smaller initial and sustained CaPIC than shown in A. D: response of same motoneuron in C after amphetamine application. The small CaPIC also shows substantial enhancement after application of amphetamine.

**TABLE 1. Effects of amphetamine on motoneuron properties and the motoneuron CaPIC**

<table>
<thead>
<tr>
<th>Average (n = 9)</th>
<th>(V_{on}, \text{mV})</th>
<th>(G_{on}, \text{\mu S})</th>
<th>(V_{off}, \text{mV})</th>
<th>Initial PIC, nA</th>
<th>Sustained PIC, nA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>−79.3 ± 2.8</td>
<td>0.19 ± 0.03</td>
<td>−60.2 ± 2.7</td>
<td>2.44 ± 0.40</td>
<td>2.14 ± 0.33</td>
</tr>
<tr>
<td>Amphet (1−10 (\mu)M)</td>
<td>−74.8 ± 4.1</td>
<td>0.25 ± 0.03</td>
<td>−58.8 ± 3.6</td>
<td>3.35 ± 0.48</td>
<td><strong>3.34 ± 0.41</strong></td>
</tr>
</tbody>
</table>

Intracellular recordings made from motoneurons in the sacrocaudal spinal cord of chronically injured, spastic rats (n = 9). \(V_{on}\), resting membrane voltage (mV); \(G_{on}\), resting membrane conductance (\(\mu\)S); \(V_{off}\), onset voltage or threshold of the CaPIC (mV); initial PIC, initial peak current for the CaPIC (nA); sustained PIC, sustained peak current of the CaPIC (nA). All values are shown as means ± SE, values in bold are significant (t-test; \(P < 0.05\)).

**Induction of very large CaPIC after amphetamine**

In all cells, the time for amphetamine to reach peak effect was 15–20 min; therefore all measurements (e.g., initial and sustained PIC, etc.) were made during the time when amphetamine had reached its peak effectiveness. Figure 8 documents the progression from the time of amphetamine application to when peak effectiveness was observed. An unexpected finding was uncovered in six of the nine motoneurons tested after the application of TTX and in the presence of amphetamine. Before the drug, the CaPIC was controlled throughout the voltage clamp and could be terminated at the hyperpolarizing end of the voltage ramp with the current coming back to control levels near the leak current (Fig. 8A). Eight minutes after amphetamine application, a moderate increase in CaPIC amplitude was visible (as noted for the sustained CaPIC, see arrow in Fig. 8B), and the CaPIC could again be terminated by the downward phase of the voltage ramp. A further 15 min after amphetamine application, when the drug’s full effect was achieved, a very large current was revealed (Fig. 8C). In this...
As the CaPIC remained fully activated after the original voltage ramp even when a large negative bias current, after which a CaPIC could again be evoked (data not shown). In cells where there was a loss of control of the PIC, the amphetamine-induced PIC was taken as the PIC measured just prior to this loss of control.

Further, these large, noninactivating CaPICs were not due to the presence of apamin which was sometimes present in the bath because they occurred equally in motoneurons with \( (n = 3) \) and without \( (n = 3) \) apamin \( (n = 6 \text{ total with these types of CaPICs}) \).

**Supersensitivity of tail spasms and reflexes to amphetamine in the awake chronic spinal rat**

Classic denervation supersensitivity is known to develop in chronic spinal cord injury. To determine if supersensitivity to endogenous NE present below the injury develops, responses to low and high doses of amphetamine were compared in chronic and acute animals beginning with kinematics. Maximum flexion and extension angles of the tail after the standardized stretch/rub maneuver were measured in the acutely spinalized rat in the same manner as for the chronically spinalized rat (Fig. 9). Representative data show that in acute animals, prior to amphetamine administration, only a small degree of extensor movements were present (Fig. 9A), and the spasms (if present) elicited by rubbing the tail primarily produced flexion (Fig. 9A). As acute animals predominantly show flexion spasms, with little to no interference of extension behavior, the maximum flexion angles are consequently larger compared with those in chronic spinalized animals; the balance of activity is flexor biased in acute animals. After a high dose of amphetamine, an increase in flexion movements was seen (Fig. 9C) as well as a modest increase in phasic extensor movements (Fig. 9D). Lower doses of amphetamine had no significant effects.

Averaged group data from the acute animals showed that the amount of maximum flexion before amphetamine was 37.2 ± 3.4° \( (n = 10) \), and a significant increase in maximum flexion occurred with high-dose amphetamine to 69.4 ± 10.8° \( (n = 10) \), whereas low-dose amphetamine showed no significant change in flexion (53.8 ± 13.2°; Fig. 9E; \( n = 10 \)). Extension, conversely, was present in only very small amounts, if at all, prior to amphetamine administration (maximum extension: 0.2 ± 0.1°) and did not show any significant change with subsequent doses of amphetamine (Fig. 9F; 0.0 ± 0.0 at low dose; 0.8 ± 2.0° at high dose). This indicates that when spasms are present in the acute spinal rat, they are composed mainly of flexor activity and only increase after high, but not low, doses of amphetamine. Unlike chronic animals, acute animals showed negligible maximum extension movements and extension spasms even after amphetamine administration. The fact that acute animals showed significant increases in maximum flexion only at high doses of amphetamine, whereas the chronic spinalized animal showed significant increases in maximum flexion even at doses 10 times lower (2.0 mg/kg in acute vs. 0.2 mg/kg in chronic), is indicative of a development of supersensitivity to NE that develops in chronic animals.

Cutaneous reflex responses in acute, compared with chronic, spinal rats showed similar trends in the development of supersensitivity as those shown in the kinematic data. After the application of a train of stimuli to the tip of the tail, acute rats
(having received a complete S₂ transection < 5 days prior to testing), showed minimal responses from EMG recorded at the segmental tail muscles (Fig. 10A) compared with chronically injured rats (Fig. 10D). On the administration of amphetamine at low doses (0.2 mg/kg), acutely injured rats showed no increase in the reflexes (Fig. 10B), whereas reflexes in chronically injured animals were facilitated at this dose (Fig. 10E). Only a high dose (2 mg/kg) of amphetamine in acute animals produced an augmentation of both short-latency and long-lasting reflexes (Fig. 10C).

Cutaneous reflexes recorded from the segmental tail muscles in the acute spinal rat were much smaller than those recorded from the chronic spinal rat before drug application (Fig. 11, compare absolute reflex responses, open bars on left of graphs; acute absolute reflexes significantly smaller). When the data collected from acute animals are grouped, significant increases

![Image](image-url)

**FIG. 9.** Similar format as Fig. 2 with example kinematic data from an acute spinal rat for predrug maximum flexion (A), predrug maximum extension (B), high dose (0.6 mg/kg) maximum flexion (C), and high dose maximum extension (D). Note that the amount of maximum flexion and the number of spasms counted increased with a high dose of amphetamine. E: group data: average maximum flexion predrug is 37.2 ± 3.4° and increases significantly by 163.2% at high doses of amphetamine. A trend for increased maximum flexion is evident, but not significant, at low doses (0.2 mg/kg) of amphetamine. F: same format as E but maximum extension. Maximum extension values are negligible prior to amphetamine, and do not show any significant increases with subsequent doses (note the scale). * = P < 0.05.

**FIG. 10.** Development of amphetamine supersensitivity in chronic S₂ spinal rats as seen by changes in cutaneous reflexes recorded in the segmental tail muscles evoked by train stimulation (1.5 x RT) applied to the tip of the tail. A: surface EMG response in acute spinal rat before amphetamine administration. B: same rat as in A but recorded after administration of a low dose of amphetamine (0.2 mg/kg). C: same rat as in A and B but recorded after a high dose of amphetamine (2.0 mg/kg). D: response of a chronic spinal rat to train stimulation prior to amphetamine administration. Reflex response is already much larger than in the acute spinal rat. E: same rat as in D but after a low dose of amphetamine (0.2 mg/kg). F: same rat as in D and E but after administration of a high dose of amphetamine (1.0 mg/kg). Despite doses 10 times that of the chronic spinal rat, the reflexes recorded in the acute spinal rat never reach similar increases in amplitude, pointing toward the development of a supersensitivity to endogenous NE present below the transection.
spinal rats are augmented by amphetamine (Nozaki et al. 1980). As discussed in the following text, the mechanism of action of amphetamine at the doses used in our experiments (<2 mg/kg in vivo; <10 mg in vitro) are primarily to increase the release of NE from presynaptic terminals. Thus it is very likely that enhancement of reflexes and spasm behavior after amphetamine administration in completely lesioned animals is mediated by increasing the endogenous release of NE from presynaptic terminals located below the level of the spinal injury. In particular, this study shows that amphetamine increases long-lasting reflexes through facilitated activation of the CaPIC in motoneurons; thus showing an overall increase in intrinsic motoneuron excitability leading to the production of larger and longer spasms in response to brief sensory stimulation. Finally, we have also shown that, similar to 5-HT, long-lasting reflexes can be potentiated by very low doses of amphetamine in chronic, but not acutely, injured animals; suggesting that motoneurons below the level of the injury become supersensitive to endogenous levels of NE. Development of supersensitivity to low levels of NE below the lesion may be responsible for the recovery of motoneuron PICs, known to mediate spasm generation in these animals after spinal cord injury.

Mechanism of amphetamine-induced increases in long-latency reflexes
Amphetamine has the combined ability to release NE and dopamine from presynaptic terminals and block its reuptake as
well as to inhibit the metabolism of NE by monoamine oxidase (MAO) (Aboul-Enein 1971; de la Torre et al. 2004; Florin et al. 1994; Randrup and Munkvad 1966).

Although the specific mechanism of action of amphetamine is dependent on the dose utilized (Elliott and Beveridge 2005; Seiden et al. 1993; Sulzer and Rayport 1990; Sulzer et al. 2005), the net effect of amphetamine at the doses used in this study is to increase the presynaptic release of NE via the reversal of the norepinephrine transporter (NET). Although amphetamine can act at both the dopamine (DAT) and serotonin transporters (SERT), producing a similar presynaptic release of these monoamines, amphetamine is 5- to 9-fold less potent at DAT and 200- to 500-fold less potent at SERT (Han and Gu 2006). Therefore amphetamine likely exerts its main effects through increasing the release of endogenous NE (Carlsson et al. 1965; Randrup and Munkvad 1966).

Based on the high potency of amphetamine for the NE transporter, increases in long-lasting reflexes from amphetamine is likely due to increases in the release of endogenous NE from presynaptic terminals below the spinal lesion. It has already been established that the CaPIC underlies the sustained portion of the PIC as well as long duration (>1 s) reflex responses (Bennett et al. 2001b; Li and Bennett 2003). For instance, when a hyperpolarizing current bias is applied to a motoneuron to eliminate the voltage-sensitive PIC, sustained depolarizations of the motoneuron in response to a single shock dorsal root stimulation are dramatically shortened from many seconds before the PIC block to <1 s during the PIC block (Bennett et al. 2001b). Facilitation of the CaPIC is known to be contingent on the monoamines 5HT and NE as acute spinal transection eradicates self-sustained depolarizations and firing normally associated with the PIC (Hounsgaard et al. 1988), whereas subsequent application of 5HT and NE agonists restores these properties (Bennett et al. 2001b; Conway et al. 1988; Hounsgaard et al. 1988). In contrast, the effect of dopamine agonists on the PIC is minimal (Bennett, personal communication). In this study, amphetamine dramatically increases the dendritic CaPIC, likely via its action of increasing release of endogenous NE. The increase of CaPIC, in turn, likely enhances the amplitude and duration of the long-lasting reflexes recorded both in vitro and in vivo.

In addition to facilitating the CaPIC, the increase in monoamines from amphetamine may also influence other postsynaptic currents such as the activation of a nondendritic calcium-activated cationic current. However, the majority of the PICs recorded in tail motoneurons are Na and Ca mediated (TTX- and nimodipine-sensitive currents, respectively) with the CaPIC mediating the long-lasting reflexes (Bennett et al. 2001b; Li and Bennett 2003). Alternatively, amphetamine may also activate postsynaptic receptors directly, such as the G-protein-coupled rat trace amine receptor, rTAR1 (Bunzow et al. 2001). However, cyproheptadine, which can also activate rTAR1, has been shown to have a depressive effect on motoneuron excitability and spasms (Barbeau et al. 1982; Tremblay and Bedard 1995), which is in direct contrast to the increased motoneuron excitability and spasms seen with amphetamine. Should the primary mechanism of action of cyproheptadine and amphetamine be through rTAR1-activated pathways, it would be expected that these drugs would produce similar effects on motoneuron excitability and spasms, whereas in fact they produce opposing effects. Thus given the opposing effects of amphetamine and cyproheptadine on spasticity, increases in CaPICs and long-lasting reflexes from amphetamine are unlikely to be a result of activation of rTAR1.

Amphetamine may also enhance long-lasting reflexes by directly binding to presynaptic α2 adrenergic receptors on internurons or sensory afferent terminals (Ritz and Kuhar 1989). After spinal transection in rats, it is known that the α2 adrenergic receptor is present caudal to the lesion in the distal dorsal horn and in fact is found to be present in increased density in the chronic spinal rat compared with uninjured controls (Roudet et al. 1994). However, experiments involving the α2 receptor agonist clonidine, known for its potent antispastic action (Anderson et al. 1982), have shown that clonidine has an inhibitory effect on polysynaptic pathways (Chau et al. 1998) and long-lasting reflexes (Li et al. 2004b), likely mediated by a blockade of polysynaptic excitatory postsynaptic potentials. Thus as the dominant effect of α2 adrenergic receptor activation is inhibitory in respect to reflexes, it is unlikely that there was a marked stimulation of this receptor by amphetamine given that the overall effect of amphetamine was to increase reflex responses.

Amphetamine biases spasm behavior to flexion

Following a standardized stretch/rub maneuver in chronic spinal rats, low and high doses of amphetamine increased long-lasting flexor reflexes spasms exhibited by the tail, and consequently, decreased extension reflexes and spasms. This is consistent with prior research by Nozaki et al. (1980) where an increase in the hindlimb flexor reflex of chronic spinal rats occurred after amphetamine. Our data are also in line with specific differences between 5HT and NE reported by several other groups. L-DOPA, a drug which leads to the synthesis and subsequent release of NE, has been reported to increase flexor reflexes elicited by toe pinching in acute spinal rats (Austin et al. 1976). Conversely, after selective 5HT denervation with 5,6-dihydroxytryptamine, subsequent application of 5-HTP (a serotonin precursor) enhances extensor reflexes elicited by tail pinching in rats (Nygren et al. 1974). Conway et al. (1988) also observed that L-DOPA application to decerebrate acute spinal cats increased flexor activity, whereas 5-HTP application increased extensor activity. Likewise, 5-HTP injection to acutely spinalized decerebrate cats reveals a bistable behavior in extensor motoneurons due to PIC activation (Hounsgaard et al. 1988), whereas flexor, and to a lesser degree extensor, motoneurons show these bistable properties after L-DOPA administration (Conway et al. 1988). These studies indicate that 5HT mainly affects extensor activity, whereas NE can affect both flexor and extensor activity, but its main effects are observed in flexors. The fact that NE preferentially increases bistable or PIC activation in flexor motoneurons may account for the amphetamine-induced increased in maximum flexion, rather than extension, movements after a stretch/rub stimulus in chronic spinal rats in this study.

Amphetamine causes confounding effects on short-duration reflexes

The in vitro ventral root reflexes and the reflexes recorded from the awake animal both show consistent increases in long-lasting reflexes at low and high doses of amphetamine but
The longer-duration reflexes (neuron inputs during short-duration reflexes, compared with 1988). These interneurons are inhibited by NE, which is likely mediand excitatory effects on motoneurons (Jankowska 1992). They are a heterogeneous population able to exert both inhibitory potentials lasting 1 s) (Bennett et al. 2001b; Conway et al. 1988).

Given that motoneurons are activated by afferent and interneuron inputs during short-duration reflexes, compared with the longer-duration reflexes (>1 s), the postdrug short-duration reflexes may be more variable as a result of the mixed excitatory and inhibitory effects that amphetamine has on interneurons and afferents (Jankowska 1992). It has already been shown that premotor interneurons evoked by group II afferents, as are involved in the cutaneous reflexes recorded in this study, are a heterogeneous population able to exert both inhibitory and excitatory effects on motoneurons (Jankowska 1992). These interneurons are inhibited by NE, which is likely mediated by the α2 receptor (Maxwell et al. 2000). The increased levels of NE released by amphetamine may cause inhibition of excitatory interneurons and result in reduced polysynaptic reflexes, such as seen during low doses in ventral root reflexes. Likewise, increases in polysynaptic reflexes at high doses in the in vitro preparation may result from a balance of increased excitation of afferents, interneurons, and motoneurons in response to increases in NE. The different degrees of facilitation of the polysynaptic reflex at low and high dose in the awake animal may reflect differences in this balance.

Sources of NE caudal to spinal transection

The amphetamine-induced increases in ventral root reflexes and cutaneous reflexes in the awake rat and motoneuron CaPICs observed in this study could not have occurred without an endogenous source of NE present caudal to the transection. The presence of intraspinal noradrenergic neurons below a complete transection has been shown, but in reduced quantities, when compared with pre lesion levels (Cassam et al. 1997). Another source of NE below the transection may originate from peripheral sympathetic fibers as histofluorescence studies in chronic spinal rats show that peripheral sympathetic fibers enter the spinal cord alongside blood vessels can dissociate from the blood vessels and be seen in the immediate vicinity of neurons (McNicholas et al. 1980). As discussed in the following text, the CaPIC, which mediates long-lasting reflexes, may become supersensitive to these residual sources to aid in the development of spasticity in chronic spinal injury.

Supersensitivity of motoneuron PICs to monoamines

It has already been suggested that after chronic spinal cord transection in rats, a supersensitivity of motoneurons to intraspinal sources of 5HT develops (Barbeau and Bedard 1981; Harvey et al. 2006a,b; Tremblay and Bedard 1995). More recently, a 30-fold supersensitivity of motoneuron PICs to 5HT was demonstrated in the chronic spinal rat (Harvey et al. 2006a). In this case, application of the 5HT2 receptor agonist DOI, at doses of 1 µM or less, facilitated both the NaPIC and the CaPIC, and this facilitation occurred at doses 30 times smaller than those required to produce a similar, but smaller, facilitation in acutely spinalized animals.

Although the development of denervation supersensitivity to 5HT after spinal transection has been thoroughly investigated (Tremblay and Bedard 1995), the same cannot be said for NE. Our results show that acute spinalized rats require a higher dose of amphetamine than chronic spinalized rats to achieve a significant increase in maximum flexion and reflex amplitudes. The reflex responses of the acute rats, with or without amphetamine, were still much smaller than those of chronic spinal rats. These data suggest that denervation supersensitivity to NE is occurring below the level of transection in chronic spinalized rats. Similar effects have been reported previously, where it was shown that amphetamine produced a greater facilitation of the flexor reflex in the hindlimb of the chronic spinal rat than the acute spinal rat (Nozaki et al. 1977), and this facilitation was abolished when catecholaminergic terminals were previously destroyed with 6-hydroxydopamine (6-OHDA) (Nozaki et al. 1980).

The leading theories regarding the mechanism of the development of this noradrenergic denervation supersensitivity are largely concerned with noradrenergic receptors on the postsynaptic membrane rather than from supersensitivity developing at the presynaptic NE transporter resulting in increased presynaptic release of NE at low doses of amphetamine. To support this, spastic reflexes in chronic spinal rats are themselves supersensitive to NE (Li et al. 2004b), indicating postsynaptic receptors likely become supersensitive. Also the hindlimb flexor reflex of the chronic spinal rat is known to become enhanced after the application of the NEα1 receptor agonist methoxamine, which again suggests a development of supersensitivity on the part of the noradrenergic postsynaptic receptors below the transection (Nozaki et al. 1980). An upregulation of the NEα1 receptor has been found in the spinal cord of chronically transected rats using autoradiographic techniques (Roudet et al. 1993, 1994). Receptor upregulation after transection is likely to be one mechanism in which denervation supersensitivity develops after spinal cord injury. Another possible mechanism may be that individual receptors located below the transection develop a higher affinity for ligands through a process of modification of the receptor itself as is known to occur with the 5HT2C receptor (Gurevich et al. 2002). Because both NaPIC and CaPIC are under the control of G-protein-coupled pathways, development of supersensitivity may also be due to changes or upregulation within these pathways (Alaburda et al. 2002; Cantrell and Catterall 2001). There is currently little research showing the mechanism by which denervation supersensitivity develops in the spinal cord after transection, but there is no reason the above mentioned mechanisms need be mutually exclusive or cannot function together with an as yet undiscovered mechanism.
Clinical implications

Amphetamine, which increases the presynaptic release of NE, increases long-lasting reflexes and the motoneuron CaPIC underlying them. This strongly implicates the role of NE in the development of spasticity after spinal cord injury. Our data also suggest a development of supersensitivity to NE in the chronic stages of spinal cord injury, whereas previous research has established that 5HT supersensitivity also develops (Harvey et al. 2006a). Given that a combined blockade of 5HT2a, 5HT2c, and NE2 receptors is required to eliminate NaPICs underlying spasticity (Harvey et al. 2006a) (see INTRODUCTION), it would seem that an ideal clinical approach to spasticity management would include drugs that target both the 5HT and NE systems, especially for modulation of the CaPIC, which mediates long-lasting reflexes. Specific control of 5HT or NE release from fibers below the lesion could be targeted pharmacologically as without these neuromodulators spastic symptoms are unlikely to develop. Alternately, the intraspinal administration of 5HT and NE immediately after injury may prevent the development of supersensitivity to these neurotransmitters in the motoneurons. At this point, it is essential that both 5HT and NE are taken into equal consideration when developing clinical spasticity management treatments.

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