Modulatory Actions of Serotonin, Norepinephrine, Dopamine, and Acetylcholine in Spinal Cord Deep Dorsal Horn Neurons

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Garraway, Sandra M. and Shawn Hochman. Modulatory actions of serotonin, norepinephrine, dopamine, and acetylcholine in spinal cord deep dorsal horn neurons. *J Neurophysiol* 86: 2183–2194, 2001. The deep dorsal horn represents a major site for the integration of spinal sensory information. The bulbospinal monoamine transmit-ters, released from sero-tonergic, noradrenergic, and dopaminergic systems, exert modulatory con-trol over spinal sensory systems as does acetylcholine, an intrinsic spinal cord biogenic amine trans-mitter. Whole cell recordings of deep dorsal horn neurons in the rat spinal cord slice preparation were used to compare the cellular actions of serotonin, norepinephrine, dopamine, and acetylcholine on dorsal root stimulation-evoked afferent input and membrane cellular properties. In the majority of neurons, evoked excitatory postsynaptic po-tentials were depressed by the bulbospinal trans-mitters serotonin, norepi-nephrine, and dopamine. Although, the three descending transmitters could evoke common actions, in some neurons, individual transmitters evoked opposing actions. In comparison, acetylcholine generally facilitated the evoked re-sponses, particularly the late, presumably *N*-methyl-D-aspartate receptor-mediated component. None of the transmitters modified neuronal passive membrane properties. In contrast, in response to depolarizing cur-rent steps, the biogenic amines significantly in-creased the number of spikes in 14/19 neurons that originally fired phasically (P, 0.01). Together, these results demonstrate that even though the deep dorsal horn contains many functionally distinct subpopulations of neurons, the bulbospinal monoamine transmitters can act at both synaptic and cellular sites to alter neuronal sensory integrative properties in a rather predictable manner, and clearly distinct from the actions of acetylcholine.

INTRODUCTION

Neurons within the spinal cord represent a primary site for the integration of somatosensory input. Spinal sensory integra-tion is a dynamic process regulated by factors that include multisensory convergence and pathway selection (Baldissera et al. 1981; Jan-kowska 1992; Lundberg 1979), activity-dependent plasticity (see Millan 1999), and neuromodulation (see Randic 1996). Neuro-modulatory responses within the spinal cord in-clude actions me-diated by monoaminergic systems that origi-nate in the brain stem. These bulbospinal monoaminergic nu-clei can be divided into three subtypes by their transmitter phenotype, serotonin (5-HT), norepinephrine (NA), or dopa-mine (DA). Neurons within these nuclei are characterized by

their widespread projections throughout the spinal cord (e.g., Clark and Proudfit 1991, 1993; Holstege et al. 1996; Marlier et al. 1991a).

The monoaminergic modulation of two prominent spinal cord functional systems has been examined in some detail. These are the control of motor output and nociception. Generally, the monoamines have been reported to facilitate motor activity and inhibit sensory systems (Basbaum and Fields 1984; Bell and Matsumiya 1981; Jacobs and Fornal 1993; Wallis 1994; Willis and Coggeshall 1991), consistent with a general hypothesis on 5-HT function in the CNS forwarded by Jacobs and Fornal (1993). Because serotoner-gic, noradrenergic, and dopaminergic systems have a simi-larly diffuse distribution in the spinal cord (Clark and Proudfit 1991, 1993; Holstege et al. 1996; Marlier et al. 1991a; Rajaofetra et al. 1989, 1992) and their monoamine transmit-ters frequently exert similar actions (Belcher et al. 1978; Bell and Matsumiya 1981; Headley et al. 1978; Weight and Salmoiraghi 1966), it is possible that these transmitter systems act at similar spinal sites and by similar mechanisms. For example, descending monoaminergic transmitters powerfully inhibit nociceptive informa-tion in neurons by activation of serotonergic 5-HT$_{1A}$, 5-HT$_{1B}$, a$_2$-adrenergic, and D$_2$-dopaminergic receptors (Kiritsy-Roy 1994; Per-tovaara 1993; Zemlan 1994) all of which are negatively coupled to adenylate cyclase (reviewed in Barnes and Sharp 1999; Bylund et al. 1994; Vallone et al. 2000). However, the existence of many bul-bospinal monoaminer-gic systems with heterogeneous transmitter phenotypes (in-cluding co-transmitters) that act on a variety of spi-nal metabotropic receptor subtypes (e.g., Huang and Peroutka 1987; Marlier et al. 1991b; Stone et al. 1998; van Dijken et al. 1996), sug-gest that neuromodulation in the spinal cord is a highly differenti-ated process. Indeed, more recent findings indicate that different noradrenergic or serotonergic nuclei can exert opposing modulatory actions on spinal cord noci-ceptive function (Calejesan et al. 1998; Martin et al. 1999). Further, the actions of 5-HT and NA on the afferent-evoked recruitment of functionally identified spinal neu-rons can differ considerably (Bras et al. 1989; Jankowska et al. 1997, 2000). For example, the recruitment of ascending tract neu-rons following primary afferent stimulation is commonly

facilitated by 5-HT yet depressed by NA (Jankowska et al. 1997).

The ester amine acetylcholine (ACh) also modulates spinal sensory processing in the dorsal horn (Myslinski and Randic 1977; Urban et al. 1989). As it appears that there are no descending cholinergic systems in the rat (refer to Willis and Coggeshall 1991, but see Bowker et al. 1983), these actions probably arise from a population of intrinsic cholinergic inter-neurons found in the dorsal horn (Barber et al. 1984; Todd 1991).

Several studies have compared the actions of these biogenic amine transmitters on the modulation of sensory input onto spinal neurons (Belcher et al. 1978; Bras et al. 1989; Headley et al. 1978; Jankowska et al. 1997; Skoog and Noga 1995; Todd and Millar 1983; Weight and Salmoiraghi 1966; Will-cockson et al. 1984). However, in these studies, only modifi-cations in extracellular spiking or field potentials were re-corded and transmitters were applied by iontophoresis (but see Bras et al. 1989). While it is apparent from these studies that the transmitters have both common and distinct actions on the modulation of spinal sensory input, the effects of monoamines on intrinsic cellular properties and synaptic potentials in indi-vidual neurons were not studied. Clearly, additional insight into monoamine transmitter function may be achieved by a more direct examination of their actions with intracellular recordings (e.g., Khasabov et al. 1998, 1999; Lopez-Garcia 1998; Lopez-Garcia and King 1996).

Therefore in this study, we compared the effects of bath-applied 5-HT, NA, DA, and ACh on cellular properties and primary afferent-evoked synaptic responses in individual deep dorsal horn (DDH) neurons using whole cell patch-clamp recordings (see Garraway et al. 1997; Hochman et al. 1997). Neurons in the DDH represent a functionally heterogeneous population, which include several classes of ascending tract cells and spinal interneurons (Willis and Coggeshall 1991) and may thereby account for the diversity in intrinsic and synaptic properties observed from neurons in this region (e.g., Jiang et al. 1995; Morisset and Nagy 1998, 1999; also refer to Hoch-man et al. 1997). Parts of these results have been presented in abstract form (Garraway and Hochman 1999).

METHODS

Preparation of spinal cord slices

All experimental procedures complied with the Canadian Council of Animal Care guidelines. Neonatal rats (Sprague-Dawley postnatal days 10–14) were first anesthetized with 10% urethan (2 mg/kg ip body wt) and decapitated, and spinal segments L_2–S_1 were removed. The isolated spinal cord was embedded in Agar, 2.5% wt/vol (Type E, Sigma), and sliced on a vibrating blade microtome in 500–600 mm transverse sections (Leica VT1000S or Pelco 101) in cooled (,4°C) oxygenated high sucrose-containing artificial cerebrospinal fluid (ACSF) containing (in mM) 250 sucrose, 2.5 KCl, 1 $CaCl_2$, 3 $MgCl_2$, 25 glucose, 1.25 NaH_2PO_4, and 26 $NaHCO_3$ at a pH of 7.4. Short dorsal rootlets remained attached to the spinal segments to allow for electrical stimulation of primary afferents.

Electrophysiology

Slices were incubated at 32°C for $1 h in normal ACSF containing (in mM) 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 glucose, 1.25 NaH_2PO_4, and 26 $NaHCO_3$ at a pH of 7.4 and oxygenated with 95%

O_2-5% CO_2. For experimentation, spinal cord slices were affixed to a recording chamber using platinum U frames with a parallel array of nylon fibers glued across (Edwards et al. 1989). Patch electrodes were prepared from 1.5-mm OD capillary tubes (Precision Instruments or Warner) pulled in a two-stage process (Narishige PP83) producing resistance values ranging from 4 to 7 MV with recording solution containing (in mM) 140 K-gluconate, 0.2 EGTA, 10 HEPES, 4 Mg-ATP, and 1 GTP; pH 7.3. The recording chamber was continu-ously superfused with oxygenated normal ACSF at a rate of ;2 ml/min. The whole cell "blind" patch-clamp recording technique (Blanton et al. 1989) was undertaken at room temperature (;20°C) using the Axopatch 1D amplifier (Axon Instruments) filtered at 5 kHz (4-pole low-pass Bessel). Voltage- and current-clamp data were ac-quired on computer with the pCLAMP acquisition software (v 6.0; Axon Instruments).

Determination of cell membrane properties

Immediately following rupture of the cell membrane (in voltage clamp at 290 mV), the current-clamp recording configuration was used to determine resting membrane potential. Series resistance was subtracted in current-clamp mode (bridge balance), and junction po-tentials were measured and subtracted off-line. For the duration of the experiment, leak conductance and bridge balance were monitored; if their values were largely unaltered, the experiments were continued. Mean electrode series resistance was 33 6 4 (SD) MV (n 5 37). At an adjusted membrane potential of 270 mV, a series of hyperpolar-izing and depolarizing current steps were undertaken to obtain esti-mates of membrane time constant, cell resistance, rheobase, voltage threshold, action potential height, and action potential duration at half-maximal amplitude (half-width). For details on the estimation of these mem-brane properties, refer to Hochman et al. (1997).

Primary afferent stimulation

Primary afferents were stimulated electrically with a constant cur-rent stimulator (Eide 1972), using bipolar tungsten electrodes. In the present comparative study, we used high stimulation intensities to recruit the highest threshold unmyelinated afferents and, hence, the majority of afferent fiber types, irrespective of age (typically $500 mA, 500 ms) (see Thompson et al. 1990). In the present sample, 29% of the neurons received synaptic responses at intensities ,500mA, 100ms; 49% received synaptic input at 500mA, 100ms, while the remaining 22% of neurons only received input at intensities $500 mA, 500 ms. Generally, the evoked synaptic responses were first char-acterized as excitatory by determining their reversal potential prior to collec-tion of baseline events. Neurons with short-latency inhibitory synaptic re-sponses were not included in this study. Excita-tory postsynaptic potentials (EPSPs) were evoked at low frequencies (once every 20–60 s) by stimulating dorsal rootlets for a baseline period of 10–15 min while maintaining the neuron at a holding potential of 290 mV. In all cases, membrane potential was carefully monitored, and any alterations in membrane potential were noted, then countered with intracellular current injection to maintain a hold-ing potential of 290 mV.

Application of agonists

5-hydroxytryptamine HCl (5-HT), norepinephrine bitartrate (NA), dopa-mine HCl (DA), and acetylcholine chloride (ACh) were obtained from RBI/Sigma. The solutions were prepared on the day of the experiment from 10 mM frozen stock solutions and bath applied at a final concentration of 10 mM. Ascorbic acid (100 mM), an antioxidant, was added to solutions con-taining 5-HT, NA, and DA to prevent their oxidation (Krnjevic et al. 1978). A 10 mM concentration was chosen based on previous studies involving bath application of NA, 5-HT, and ACh (e.g., Baba et al. 2000; Lopez-Garcia and King 1996;

Miyazaki et al. 1998; Urban et al. 1989). While this concentration may (e.g., Lopez-Garcia and King 1996) or may not have a maximal physiological response (e.g., Baba et al. 2000), it is likely to be below the concentration range where nonspecific binding and actions have been observed (Chesnoy-Marchais and Barthe 1996; van Wijngaarden et al. 1990). All agonists were dissolved in normal ACSF and bath applied from independent perfusion lines. Each agonist was applied for a period of 7–15 min during which time, EPSPs were continually recorded at the baseline parameters described in the preceding section.

To compare the actions of more than one agonist on the primary afferent-evoked synaptic responses and cellular properties of the neu-rons, we allowed a washout/recovery period of 10–20 min before subsequent drug application. Due to the restrictions in recording duration with patch electrodes, we were often unable to observe the effects of all four agonists on a given neuron. However, in all cases, the actions of at least two drugs were compared. The following three combinations of drugs were compared in most cases: 5-HT and ACh, 5-HT, NA, and DA, and 5-HT, NA, DA, and ACh. These transmitters were applied in random order, and evoked EPSPs were always re-corded at baseline parameters both during drug application and wash-out. In separate experiments, we compared the magnitude of modu-latory actions evoked by independent application of 5-HT and NA to their co-application (5-HT/NA).

Analysis

Recordings were analyzed using pCLAMP (v 6.0, Axon Instru-ments). Both the maximum amplitude of the synaptic response and the changes in synaptic charge transfer calculated as the integral of the synaptic response (area under the curve) of individual traces were measured. Primary afferent-evoked synaptic responses in dorsal horn neurons are generally glutamater-gic consisting of both early and late components; presumably (6)-a-amino-3-hydroxy-5-methylisoxasole-4-propionic acid (AMPA)/kainate and N-methyl-D-aspartate (NMDA) receptor-mediated respectively (e.g., Gerber and Randic 1989). To determine whether, as a first approximation, the drugs differentially modulated these components of the evoked responses, we calculated area under the curve (AUC) at two time intervals that approxi-mately separate these events: early (,200 ms) and late ($200 # 750 ms). These periods were chosen to approximately separate AMPA/kainate from NMDA receptor-mediated actions as described in an earlier study (Garra-way and Hochman 2001a) (Fig. 3A). Synaptic events generally returned to baseline before 750 ms. Analysis of EPSPs within this time interval ex-cludes actions from the long-latency pep-tidergic component (Urban and Randic 1984).

The applied transmitters were considered to have a modulatory action if they altered EPSP amplitude $10%. Because multiple drugs were added in most experiments, and in several experiments evoked responses did not return to naïve EPSP baseline values, the change in synaptic amplitude was measured as a difference in the mean peak amplitude or AUC during drug application compared with the mean values just prior to drug applica-tion (control or washout/recovery). Similarly, the estimated membrane properties and firing properties of the neurons were compared before, dur-ing, and following washout of the drugs tested. Following analysis, graphs were constructed using Sigma Plot (SPSS) and imported into CorelDRAW (Corel) for final editing. Both AUC and peak amplitude were measured, and as re-ported later, similar results were obtained for the actions of 5-HT, NA, and DA. Thus unless stated, all values are reported as means 6 SD of peak changes (maximum amplitude) of the synaptic response. Unless otherwise stated, the effects of the monoamines are statistically com-pared with con-trol values using the Students's t-test. Multiple pairwise comparisons on differences in values between the monoamine trans-mitters are not reported.

RESULTS

Sample population

A total of 41 deep dorsal horn neurons (laminae III–VI) were recorded. The approximated location of 36 of these neurons, de-termined using visual landmarks (King et al. 1988), is pre-sented in Fig. 1A. The membrane properties of the neurons are summa-rized in Table 1. Neurons can also be grouped by their firing prop-erties in response to current injection. Phasically firing neurons could not be induced to fire repetitively regard-less of the magnitude of current injection. Repetitive firing neurons always fired repetitively in responses to larger mag-nitude current injections. In the present sample, 15 neurons fired spikes repeti-tively, while the remaining 26 neurons fired
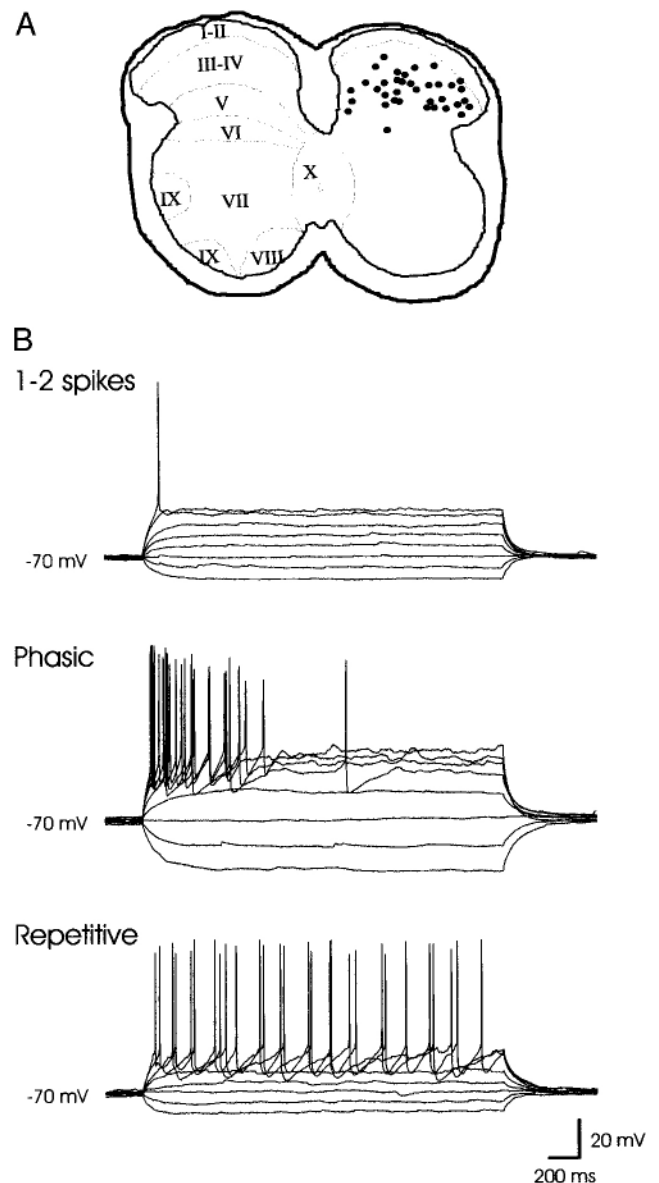

FIG. 1. Location and firing properties of recorded neurons. A: approximate topog-raphical distribution of 36 of the 41 neurons used in the present analysis. Left side of cord presents outline of Rexed's laminae for the lumbar enlarge-ment. B: firing patterns of deep dorsal horn (DDH) neurons in response to current injection. In response to current steps, neurons of the DDH fired 1–2 spikes, phasically or repeti-tively. Neurons were held at 270 mV and hyper-polarizing and depolarizing current steps were delivered in 5-pA increments.

TABLE 1. *Membrane properties of recorded neurons in the presence or absence of a biogenic amine*

	E_{MR},mV	R_{in},mV	t_m,ms	Rheobase, pA	V_{TH},mV	AP Height, mV	AP½-Width, ms
Control (33–41)	259 ± 18	507 ± 250	44 ± 19	57 ± 42	26 ± 7	94 ± 12	3.7 ± 2.0
5-HT (16–28)	258 ± 18	509 ± 187	42 ± 16	57 ± 43	26 ± 8	78 ± 19	4.3 ± 2.0
NA (20–26)	259 ± 18	606 ± 288	46 ± 26	60 ± 47	30 ± 12	82 ± 12	4.1 ± 1.3
DA (16–20)	259 ± 19	583 ± 335	46 ± 21	72 ± 56	33 ± 11	82 ± 12	4.8 ± 2.4
ACh (14–18)	257 ± 19	541 ± 246	47 ± 26	69 ± 51	30 ± 6	78 ± 16	4.2 ± 1.4

Values reported are means ± SD. The range of sample size used to calculate mean values is bracketed in left column. E_{MR}, resting membrane potential; V_{TH}, voltage threshold; R_{in}, cell input resistance; t_m, membrane time constant; AP height, action potential height; AP½ width, action potential duration at half maximal amplitude. These properties were estimated at an adjusted membrane potential of -70 mV. 5-HT, serotonin; NA, norepinephrine; DA, dopamine; ACh, acetylcholine.

only phasically, 10 of which fired no more than two spikes (1–2 spikes) (refer to Lopez-Garcia and King 1994). Examples of neuronal firing patterns are illustrated in Fig. 1*B*. The effects of the monoamines on neuronal membrane properties will be considered later.

Effects of the monoamine transmitters on primary afferent-evoked EPSPs

The effects of bath-applied 5-HT, NA, DA, and ACh on the dorsal root stimulation-evoked EPSPs are summarized in Tables 2 and 3 and illustrated in Fig. 2. In the majority of neurons, 5-HT and NA depressed EPSP amplitude ($P < 0.05$), and a similar trend was observed for DA. In comparison ACh facilitated EPSP amplitude ($P < 0.05$; Table 3, Fig. 2*A*). When EPSP AUC values were compared, 5-HT, NA, and DA decreased while ACh increased AUC values ($P < 0.05$). These observations demonstrate that despite the heterogeneous population of neurons found in the DDH, the biogenic amine neurotransmitters altered afferent-evoked EPSPs rather predictably with the bulbospinal monoamines having similarly depressant actions. The relative incidence of the transmitters at modulating peak EPSP amplitude were 5-HT > ACh > NA > DA (Table 2). Following washout of NA and DA, partial recovery of EPSP amplitudes generally occurred (Fig. 2*B*). However, in some neurons, during the washout that followed 5-HT-evoked synaptic depression, a rebound potentiation of EPSP amplitude occurred, usually exceeding control values. In addition, EPSP amplitudes generally remained facilitated following washout of ACh.

We also compared the differential effects of the agonists on the early versus the late occurring components of the evoked EPSPs by measuring the AUC for early (<200 ms) and late ($\geq 200 \leq 750$ ms) components of the EPSP (Table 4), periods that approximately separate AMPA/kainate from NMDA receptor-mediated actions (e.g., Fig. 3*A*) (see also Garraway and

Hochman 2001a). There were no differences in depression between early and late AUC values for 5-HT, NA, and DA (Table 4), supporting a uniform depression of both AMPA/ kainate and NMDA receptor-mediated responses. However, AUC measures show that ACh preferentially facilitated the late component of the EPSP supporting a preferential facilitation of the NMDA receptor-evoked response (Table 4). Figure 3*B* illustrates a representative example of the effects of 5-HT and ACh on evoked EPSPs in a given cell. Figure 3*C* compares the AUC increases for early and late components of the EPSP following application of ACh in individual neurons. Note that in many neurons, the later component of the EPSP is dramatically facilitated compared with the early component of the EPSP.

The modulatory actions of ACh and 5-HT were compared in 12 neurons (Fig. 4). In six of the nine neurons where EPSPs were depressed by 5-HT, ACh facilitated the EPSPs. Thus 5-HT and ACh have different ($P < 0.01$; Wilcoxon signed-rank test) and predominantly opposite actions on spinal neurons in the deep dorsal horn.

The generally depressant actions of the three descending transmitters 5-HT, NA, and DA were compared in 15 neurons (Fig. 5). With few exceptions, none of the transmitters had competing modulatory actions on the evoked EPSPs. In 6 of the 15 neurons, all three transmitters produced synaptic depression while in 1 neuron, all three drugs produced synaptic facilitation. In 8 of 15 neurons, common modulatory responses were not produced by all three transmitters, but only in 3 of these neurons were opposite actions >10% observed. An example of the common neuromodulatory actions of the brain stem monoamines in a single neuron is presented in Fig. 5*B*. Thus unlike ACh, which generally supported synaptic facilitation, the three descending monoamines 5-HT, NA, and DA commonly exerted similar functions on spinal cord sensory input to a given cell.

TABLE 2. *Incidence of neurons having EPSP amplitude depression or facilitation evoked by 5-HT, NA, DA, and ACh*

Agonist	EPSP Amplitude ↓	EPSP Amplitude ↑	No Change
5-HT (28)	14	79	7
NA (26)	15	62	23
DA (20)	15	55	30
ACh (20)	70	15	15

Values are percentage of sample size indicated in brackets. Shaded boxes indicate predominant action for each transmitter. EPSP, excitatory postsynaptic potential.

TABLE 3. *Effects of 5-HT, NA, DA, and ACh on the evoked synaptic responses*

Agonist	Peak Amplitude	Total AUC
5-HT (28)	-30 ± 49*	-36 ± 48*
NA (26)	-13 ± 42*	-20 ± 66*
DA (20)	-10 ± 33	-17 ± 37*

Values are mean percentages ± SD of change in EPSP peak amplitude and total AUC. Minus indicates depression. Note that with the exception of DA, the transmitters had significant actions on both peak amplitudes and areas under the curve (AUC). * Overall effect of transmitter on changes in EPSP amplitude and AUC is
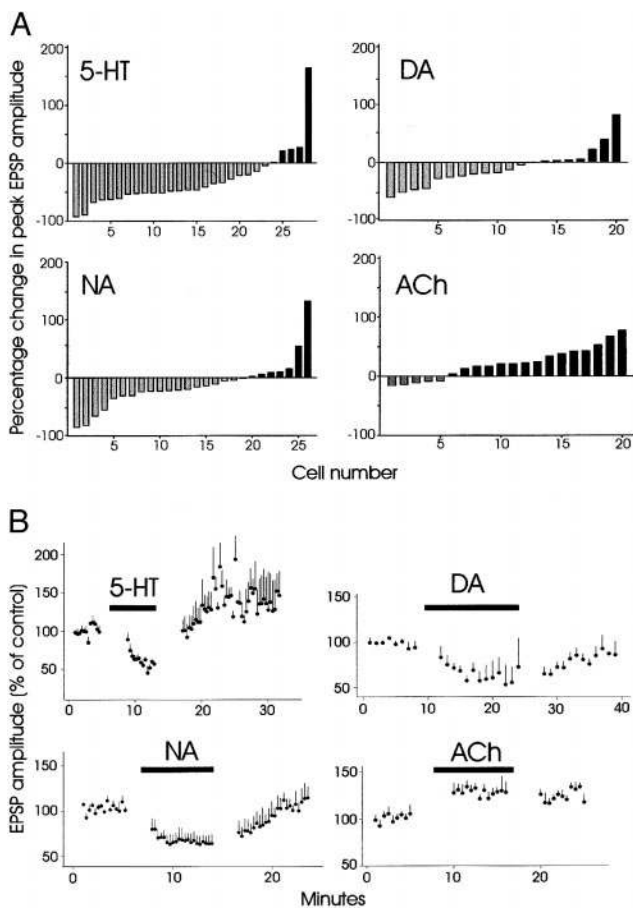
FIG. 2. Effects of the monoamine transmitters on excitatory postsynaptic potential (EPSP) amplitude. *A*: histograms presenting the modulatory actions of the monoamines on EPSP amplitude in individual cells presented in order from maximal depression to maximal facilitation. Note that the predominant actions of serotonin (5-HT), norepinephrine (NA), and dopamine (DA) were depression while acetylcholine (ACh) generally caused synaptic facilitation. *B*: normalized data on the time course of synaptic depression (5-HT, NA, DA) or facilitation (ACh) from 6 representative cells in each. Values are presented as means 6 SE, and the duration of transmitter application is indicated with a horizontal bar.

In 10 neurons, 5-HT and NA were applied individually as well as co-applied. Co-application of 5-HT and NA evoked synaptic depression of a greater magnitude than the sum of 5-HT and NA applied alone in 3 of the 10 neurons tested (Fig. 6*A*, asterisks). The effects of co-applied 5-HT and NA were not significantly different from the effects of 5-HT alone but significantly greater than effects of NA alone (P, 0.05; Tukey test), suggesting a prominent contribution from

TABLE 4. *Comparison of changes in total AUC and AUC for early and late components of the evoked responses*

	Total AUC	AUC (,200)	AUC ($200)
5-HT (22)	256 6 20	253 6 18	257 6 25
NA (16)	249 6 30	242 6 25	251 6 41
DA (11)	233 6 25	230 6 17	234 6 34
ACh (14)	75 6 48	42 6 26	121 6 97*

The comparison involves only the EPSPs that were *depressed* by 5-HT, NA, and DA and *facilitated* by ACh. Values expressed as mean percentage 6 SD. Minus indicates depression. * Effect of ACh is significantly greater on late versus early
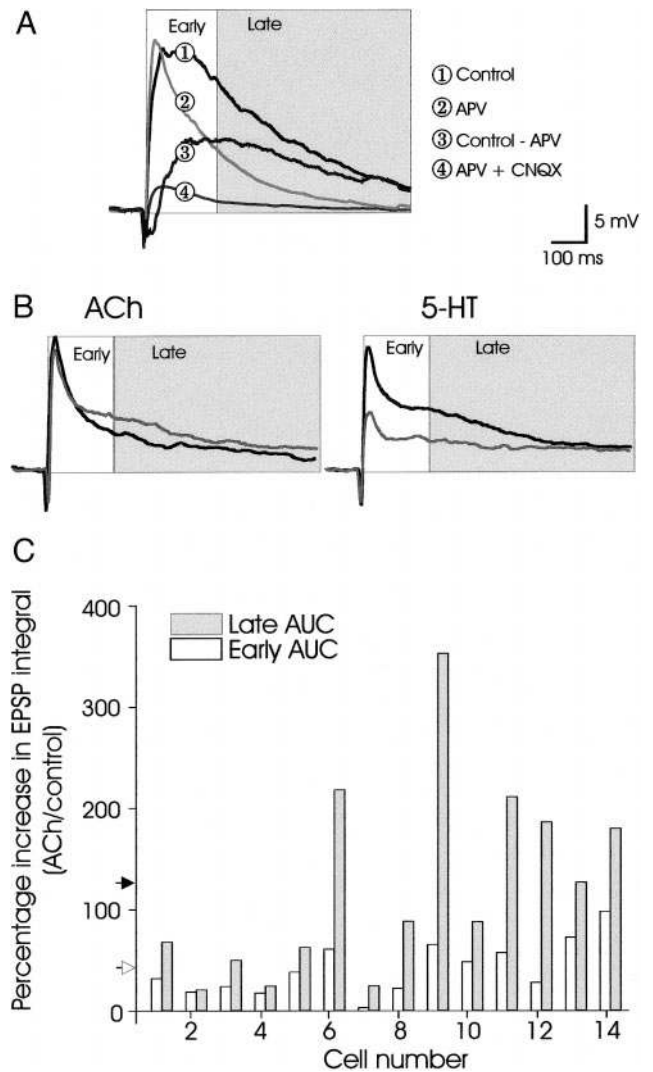

FIG. 3. ACh preferentially facilitates the late component of the evoked EPSP. *A*: actions of ionotropic glutamate receptor antagonists on evoked EPSPs. Block of *N*-methyl-D-aspartate (NMDA) receptors with 2-amino-5-phosphonovaleric acid (APV; 50 mM; 1) reveals the remaining AMPA/kainate component that dominates the early phase of the EPSP (white box; 2). Algebraic subtraction of APV from control trace (3) reveals the NMDA receptor-mediated component of the EPSP whose actions dominate the longer duration aspect of the EPSP (shaded box). White and shaded boxes enclose 0-to 200-ms and 200- to 750-ms periods used to approximately separate AMPA/ kainate from NMDA receptor evoked actions in *B* and *C*. In the presence of APV and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 mM), most of the evoked EPSP is blocked (④). *B*: average EPSPs from a sample neuron comparing modulatory actions of ACh and 5-HT. While ACh preferentially facilitates the later phase of the EPSP (52%), 5-HT depresses both early and late phases similarly (35 and 34%, respectively). Black traces represent control mean EPSP value prior to application of transmitter. *C*: histogram showing the effect of ACh on the shorter (0–200 ms)- vs. longer-latency (200–750 ms) components of the evoked EPSP (measured as area under the curve, AUC) in the 14 cells facilitated by ACh. Arrows on ordinate present mean percentage increase in AUC for the short (open arrow; 42%1)- and long (closed arrow; 121%1)-latency components.

5-HT on synaptic depression when transmitters are com-bined.

Effects of the agonists on cellular properties

As a population, none of the transmitters had significant effects on cell passive membrane properties, rheobase or volt-
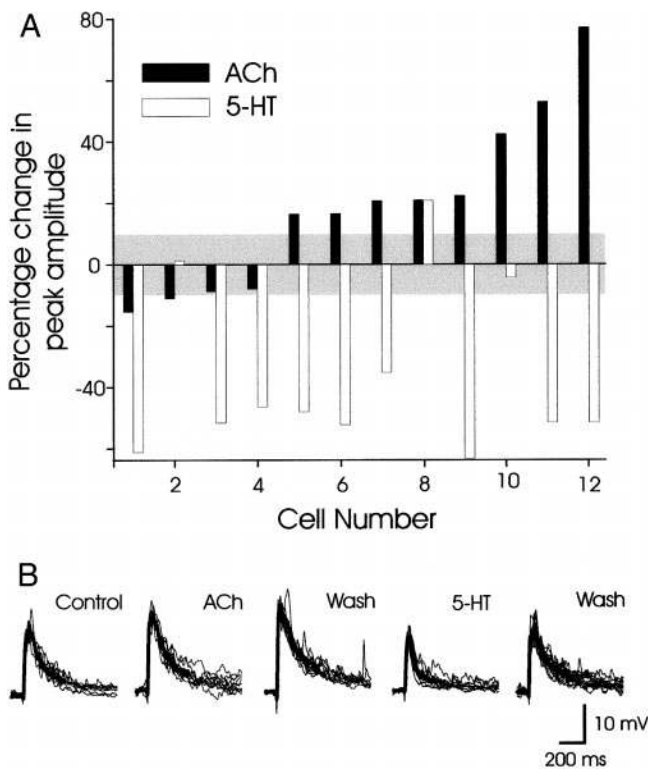
FIG. 4. Comparison of the effects of ACh and 5-HT on evoked synaptic responses in individual neurons. *A*: ACh evokes synaptic facilitation, while 5-HT causes synaptic depression in the majority of cells co-tested. In this and the following figures, the shaded area around baseline represents the region of changes in EPSP amplitude #10%. *B*: example of the evoked EPSP in a given neuron where 5-HT produces synaptic depression (52%) following ACh-induced facilitation (53%) of the evoked responses. Individual traces are presented superimposed in gray, while the average response is presented overlaid as a thick-black trace.

age threshold (Table 1). The effects of the agonists on EPSP amplitude were plotted against cell input resistance (R_{in})to identify corresponding actions that would support a postsynaptic site of action (Fig. 7*A*). However, with the exception of ACh, where a relatively weak relationship existed (r5 0.18), no relations were found.

The actions of the biogenic amines on firing properties were examined in 32 neurons by counting spike numbers during 1.2-s depolarizing current pulses at approximately twice rheobase current intensities. Overall, the monoamines significantly increased firing in these neurons (P , 0.05). However, when neurons were divided into those that initially fired either phasically (*n* 5 19) or repetitively (*n* 5 13) in response to current injection, only the population of neurons that fired phasically had an increased spike number (P , 0.01). In addition, when the actions of the individual transmitters were separated and compared in this population significant increases were produced only by 5-HT (P , 0.01). In this population, 14 of the 19 cells had increased number of spikes during current injection in the presence of the transmitters (5/6 from neurons

initially firing 1–2 spike population; Fig. 7*B*,■

). This increase
in spike number was largely attributable to the observation that the cells that originally fired phasically were converted into neurons that fired repetitively during monoamine transmitter application. An example is presented in Fig. 7*C*. In contrast, in cells initially

evident (Fig. 7*B*,u). No relationship existed between the type of neuronal firing observed in response to current injection (e.g., phasic vs. repetitive) and the effects of the agonists on EPSP amplitude.

DISCUSSION

Summary

In this study, we investigated the effect of each biogenic amine transmitter on primary afferent-evoked synaptic re-sponses and membrane properties in deep dorsal horn neurons. First, we observed that 5-HT, NA, and DA generally had common actions on evoked EPSPs in individual neurons, with the dominant action being depression. In contrast, ACh gener-ally increased EPSP amplitude, even in the same neurons where synaptic depression was evoked by the bulbospinal monoamines. The order of the incidence and magnitude of evoked modulatory actions on EPSP amplitude was 5-HT . ACh . NA . DA. Second, while 5-HT, NA, and DA tended to uniformly depress short- and long-latency components of the evoked EPSPs, the ACh-induced facilitatory response was significantly greater for the later, presumably NMDA receptor-mediated component of the EPSP. Third, we observed that co-application of 5-HT and NA could produce a much greater synaptic depression than either transmitter applied indepen-dently. Finally, while passive membrane and threshold prop-erties of neurons were unaffected by the monoamines, mem-brane firing properties in the subpopulation of neurons initially expressing a phasic firing pattern were converted to repetitive.
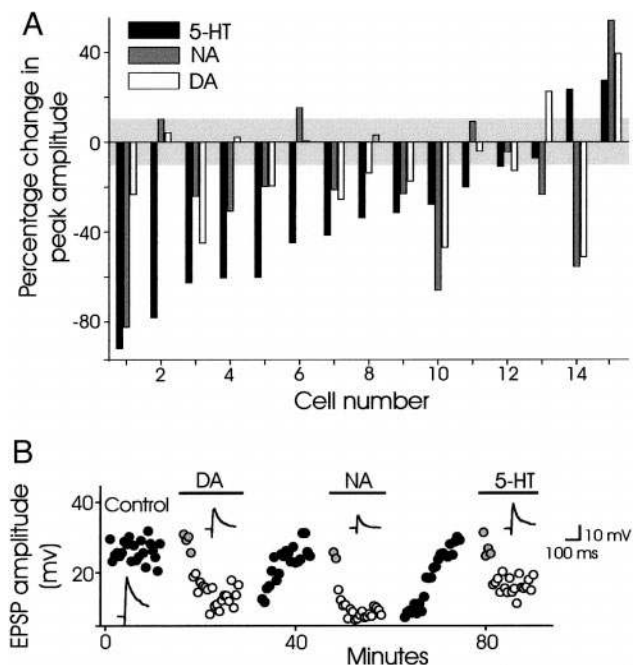

FIG. 5. 5-HT, NA, and DA generally exert common actions. *A*: histogram showing the population of cells tested with 5-HT, NA, and DA. Excluding changes #10% (1), these transmitters had similar actions in most cells tested. 5-HT generally produced depression of the greatest magnitude. *B*: example of transmitter-induced alterations in EPSP amplitude over time in a neuron. F, EPSPs evoked during control and wash-out periods;E, EPSP values obtained during agonist application. The averaged EPSP waveforms obtained during control, DA, NA, and 5-HT are also presented. The magnitudes of depression evoked in this neuron were 47% (in DA), 28% (in 5-HT), and 66% (in NA).
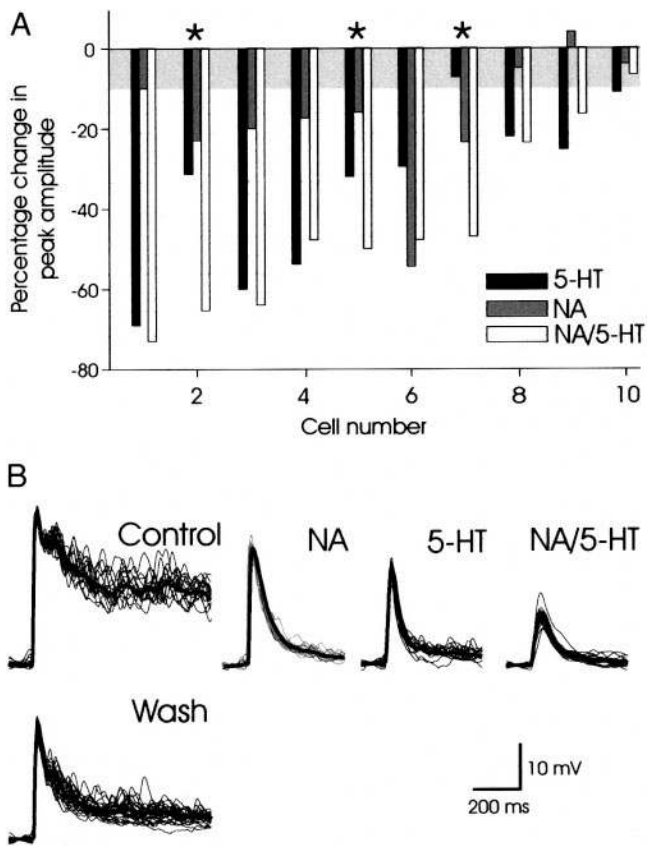
FIG. 6. Co-application of NA and 5-HT can produce greater depression than either 5-HT or NA alone. *A*: histogram showing population of cells tested with NA/5-HT. In 3 of 10 cells (asterisk), NA/5-HT-induced a synaptic depression greater than the sum of each agonist applied alone. *B*: evoked EPSPs obtained from *cell 2* in histogram *above*. Note that the depression of the EPSP is greater during co-application of 5-HT and NA (65%) than either drug applied alone (31 and 23%, respectively). Individual traces are presented superimposed in gray, while the average response is presented overlaid as a thick-black trace.

## Common actions of the monoamines on evoked EPSPs in individual neurons

Consistent with previous in vitro studies, we observed that 5-HT generally produced synaptic depression (e.g., Khasabov et al. 1999; Lopez-Garcia 1998; Lopez-Garcia and King 1996) although facilitation was observed in a few cells. Like 5-HT, bath application of NA and DA also produced synaptic depres-sion in most cells, consistent with depressant actions observed in previous studies that monitored alterations in firing fre-quency (Fleetwood-Walker et al. 1988; Headley et al. 1978; Skoog and Noga 1995; Willcockson et al. 1984). An important observation in this study is that 5-HT, NA, and DA commonly produced the same modulatory action on EPSPs when applied independently to the same neuron. Thus it appears that despite the diverse functional heterogeneity of the spinal cord dorsal horn (Baldissera et al. 1981; Jankowska 1992; Willis and Coggeshall 1991), the bulbospinal monoamine transmitters can produce a widespread depression of sensory synaptic input onto deep dorsal horn neurons. It is, however, necessary to mention, that in addition to the common actions generally evoked by the three transmitters arising from brain stem nuclei on individual neurons, these transmitters were also capable of inducing distinct modulatory actions on some neurons.

The bulbospinal monoamine transmitter systems project widely throughout the spinal cord (Holstege et al. 1996; Mar-lier et al. 1991a; Rajaofetra et al. 1992), and there are many serotonergic, noradrenergic, and dopaminergic receptors in the dorsal horn (e.g., Huang and Peroutka 1987; Marlier et al. 1991b; van Dijken et al. 1996). Hence, the effects of the bath-applied bulbospinal monoamine transmitters observed here probably reflect the actions of these transmitters on their respective families of receptors. However, it is also possible that part of the observed depression of the longer-latency portion of the EPSP is due to the transmitter having a direct voltage-dependent block of the NMDA receptor ionophore (Chesnoy-Marchais and Barthe 1996). That 5-HT, NA, and DA had common actions in most cells suggests that receptors for all three transmitters are co-localized on many neurons and/or primary affer-ent terminals. In another study, we have demon-strated that the synaptic depression produced by 5-HT in sim-ilarly located neurons is only partly mediated by 5-HT_{1A} receptors while 5-HT_7 receptors are predominantly responsible for producing synaptic facilitation (Gar-raway and Hochman 2001b).

In contrast to the depressant actions of the monoamines observed here, Jankowska and colleagues (1997, 2000) dem-onstrated that nonnociceptive afferent input from different afferents to different groups of spinal interneurons or ascend-ing tract cells is modulated by NA and 5-HT in a highly differentiated manner. Depending on the neuron and afferent fiber type, they observed that NA and 5-HT could have com-mon facilitatory, inhibitory, or opposing modula-tory actions on synaptic input strength as measured using peristimu-lus time histogram measures of extracellular spike latency and fre-quency. An explanation for the observed differences between their results and ours is our stimulation at high intensities to also recruit high-threshold C and Ad fibers, which comprise the largest fraction of primary afferent fibers (Snider and McMa-hon 1998; Willis and Coggeshall 1991). Therefore the strong depressant actions of the monoamines in our study may result from a dominating activation of high-threshold afferents that mask more subtle differential modu-latory actions on the low-threshold afferents studied by Jankowska and colleagues. An-other explanation for our observed differences may relate to our finding that EPSP amplitude can decrease con-comitant with increases in firing postsynaptically. Together, these stud-ies support a complex and functionally differentiated modula-tion of sensory-evoked firing properties in spinal cord neurons.

Despite the similar actions of the three transmitters arising from the brain stem, the magnitudes of synaptic depression differed in order of 5-HT . NA . DA. In addition, the depression evoked by 5-HT was more widespread as a greater proportion of cells underwent synaptic depression by 5-HT. The differences in depression may reflect the relative effec-tiveness of different bulbospinal systems in mediating modu-latory actions. Alternatively, the relative magnitude of effects may be due to differences in potency, as the transmitters may not be working at the same relative point on their dose-response curves (e.g., Elliot and Wallace 1992).

The actions of 5-HT and NA on antinociception have been exten-sively studied (Basbaum and Fields 1984; Fitzgerald 1986; Jones 1991; Millan 1995). Previous studies have dem-onstrated that nox-ious input leads to the release of both NA and 5-HT in the spinal cord (Satoh and Omote 1996; Tyce and
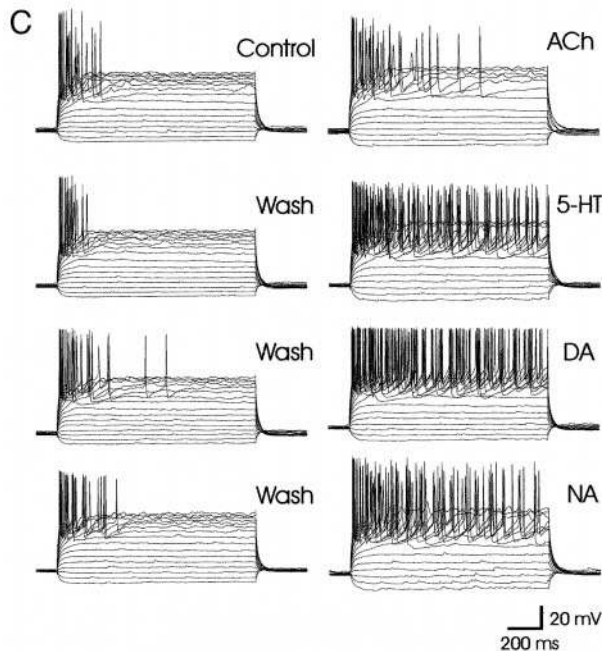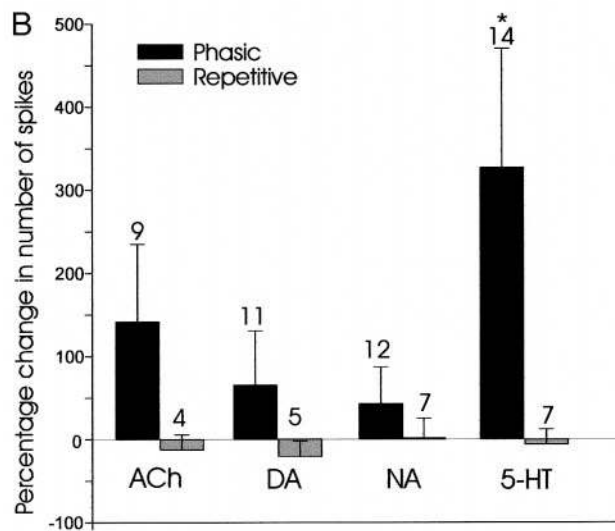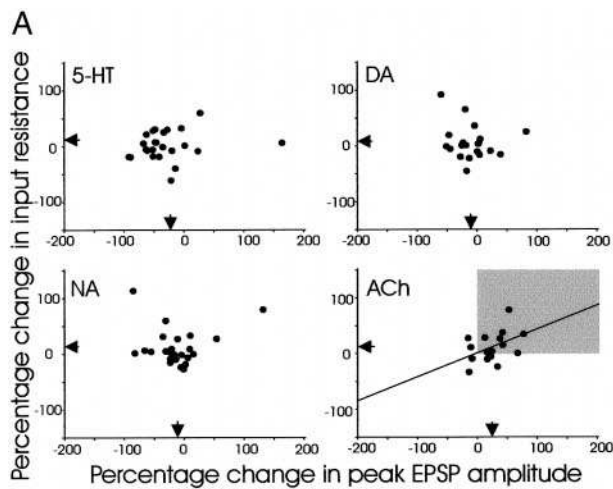
Yaksh 1981; Yaksh and Tyce 1981), and these transmitters can also produce antinociception following release after stimula-tion of specific supraspinal sites (e.g., Cui et al. 1999; Sorkin et al. 1993). In this study, we observed that co-application of 5-HT and NA could produce synaptic depression of a greater magnitude than 5-HT or NA applied alone. It is possible that both transmitters are co-released physiologically under condi-tions where a maximal sensory depression is sought. While nociceptive input does not appear to evoke release of dopamine (Satoh and Omote 1996), both stimulation of the A11 dopa-minergic cell group and exogenous application of DA can elicit antinociception (Fleetwood-Walker et al. 1988). Overall, the role of DA in mediating antinociception remains poorly stud-ied, and it is possible that DA may play a different role in the modulation of primary afferent input than 5-HT and NA.

Facilitatory actions of ACh

In contrast to the bulbospinal transmitters, ACh generally facili-tated primary afferent-evoked responses. Although some studies have reported inhibitory or antinociceptive effects of cholinergic agonists (e.g., Bleazard and Morris 1993), facilita-tory or excita-tory actions, consistent with our observations, have also been re-ported. For instance, Urban et al. (1989) reported an increase in excitability of spinal cord dorsal horn neurons by ACh, while Baba et al. (1998) reported a musca-rinic-induced facilitation of GABA release in substantia gelati-nosa neurons of the rat. We also observed that ACh caused significantly greater facilitation of the late, largely NMDA receptor-mediated, component of the evoked EPSP. This is consistent with previous studies demonstrating the facilitatory effects of cholinergic agonists on NMDA receptor-mediated events in various CNS regions including hippocampus (Marino et al. 1998) and striatum (Calabresi et al. 1998) via M_1-like receptor activation. M_1 receptor activation leads to an increase in protein kinase C (PKC), and PKC enhances NMDA receptor activity (e.g., Chen and Huang 1992; Xiong et al. 1998).

Modulation of neuronal firing properties

The values of membrane and threshold properties obtained here are comparable to those obtained from an earlier study on the properties of similarly located neurons (Hochman et al. 1997). The monoamine transmitters did not significantly alter

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FIG. 7. Effects of the monoamine transmitters on neuronal membrane prop-erties. *A*: relationship between changes in R_{in} and changes in EPSP peak amplitude. Regres-sion line for ACh is shown (r5 0.18; NS).4, mean values. In the presence of ACh, many data points fall within the 1, indicative of a correspondence between increases in peak amplitude and increases in input resistance. *B*: several neurons initially having phasic repetitive firing properties in response to current injection preferen-tially undergo increases in spike numbers. Spike numbers were counted during a 1.2-s depolarizing current pulses at approximately twice rheobase current intensities. Histograms present percentage change in firing number produced by the monoamine transmitters for neurons originally displaying phasic (■) or repetitive firing (1) in response to current injection. Although all 4 transmitters tended to increase the firing of phasic neurons, only 5-HT produced a significant increase in the number of spikes (P, 0.01). Note that neurons originally firing repetitively in response to current injec-tion are unaffected by application of agonist. *C*: reversible modulation of the monoamine transmitters on membrane firing properties. Current steps were delivered at 20-pA increments and voltage responses are presented superimposed. Note that the bulbospinal monoamines converted firing patterns from phasic to repetitive.

the passive membrane or threshold properties of DDH neurons. However, in neurons that originally fired phasically, the mono-amines could reversibly transform firing behavior from phasic to repetitive. These neurons did not display accelerated dis-charges in response to injection of depolarizing current steps or continued membrane depolarization and firing after current step termination. Thus the increased duration of firing is un-likely to be due to the activation of a plateau potential (see Morisset and Nagy 1998). Interestingly, Lopez-Garcia and

King (1994) showed that firing patterns in response to current injec-tion are functionally correlated to the source of primary afferent input. For example, wide dynamic range (WDR) neu-rons receive convergent input from both low- and high-thresh-old afferents, and these cells generally fire repetitively. If we applied this classification to the neuronal firing properties observed following the application of monoamines, the mono-amines could convert the functional properties of neurons from those that previously had restricted sen-sory convergence to a WDR profile. Interestingly, the majority of neurons in the dorsal horn of awake sheep, obviously having normal bul-bospinal activity, are WDR (Herrero and Headley 1995). It is also possible that the monoamines do not alter convergent proper-ties to DDH neurons but rather, the classification scheme developed by Lopez-Garcia and King (1994) does not apply to the behavior of neurons in the presence of monoamine trans-mitters.

It may seem inconsistent that the changes in neuronal firing prop-erties were unrelated to the observed changes in EPSP amplitude. However, since different receptor subtypes may be found at pre- and postsynaptic sites, it is not surprising that the monoamine transmit-ters exert different actions on the synaptic and firing properties of spinal neurons. For instance, while one class of receptor may de-press sensory input (e.g., Khasabov et al. 1999), another class may increase the excitability of these neurons (e.g., Wallis et al. 1991). Opposing pre- and postsyn-aptic actions may be a widely employed strategy to alter the network properties of spinal neurons. For ex-ample, a depressed sensory input with increased neuronal respon-siveness could support a transfer of control from peripheral to de-scending command systems.

Possible sites of action

Although the bulbospinal monoamines are capable of exert-ing direct postsynaptic actions, several observations suggest that the depression they produced on EPSPs is mediated via presynaptic mechanisms. First, none of these transmitters had effects on the passive membrane properties that would support a reduction in EPSP amplitudes by postsynaptic mechanisms (i.e., a decreased t_m or R_{in}). In addition, the identical percent depression on early, presumable AMPA/kainate and late, pre-sumably NMDA receptor-mediated, components can be ex-plained by a reduction in gluta-mate transmitter release. Con-sistent with a presynaptic site of ac-tion, all three transmitters, 5-HT, NA, and DA, have been shown to depress synaptic responses presynaptically as a result of their ability to increase potassium conductance (e.g., North and Yoshimura 1984, see also Barnes and Sharp 1999) or by inhibiting both N- and L-type calcium channels (e.g., Wikströ̈m et al. 1999). Other studies suggested that 5-HT and NA may mediate presynaptic inhibition of glutamate release from primary afferents in the

guinea pig (Travagli and Williams 1996), while presynaptic D_2 do-pamine receptor mediates depression of spinal reflexes (Ga-jendiran et al. 1996). In addition, many monoaminergic recep-tors are pre-sent on primary afferent terminals (e.g., Daval et al. 1987; Hamon et al. 1989; Kidd et al. 1993; Ridet et al. 1994; Stone et al. 1998), and application of 5-HT has been shown to generate a primary afferent depolarization (PAD) that corre-sponds to presynaptic inhibition of primary afferents (Khas-abov et al. 1998, 1999; Lopez-Garcia and King 1996). Inter-estingly, Khasabov et al. (1998) demonstrated that capsaicin treatment, which selectively destroys unmyelinated pri-mary afferent fibers, significantly reduced 5-HT-induced PAD in the neonatal rat. Thus 5-HT may mediate spinal inhibitory actions by reducing transmitter release from nociceptors. The ability of the monoamine transmitters to reduce nociceptive transmitter release at the first CNS synaptic input site supports a critical role in antino-ciceptive function.

In contrast to the bulbospinal monoaminergic transmitters, ACh probably mediates its facilitatory actions predominantly post-synaptically. ACh had a preferential facilitatory action on the late, presumably NMDA receptor-mediated, component of the EPSP, consistent with observed modulatory actions of M_1 muscarinic re-ceptor activation on NMDA receptor activity (Calabresi et al. 1998; Marino et al. 1998). If facilitation in synaptic strength involved only presynaptic mechanisms that increase glutamate release, one would expect to see a uniform facilitation of both AMPA/kainate and NMDA receptor-medi-ated responses. Interestingly, the presumed postsynaptic action of ACh in this study is consistent with the dominant postsyn-aptic localization of cholinergic receptors in the spinal cord dorsal horn (Gillberg and Askmark 1991), particularly the muscarinic receptors which are expressed about two to three times higher than the nicotinic receptors (Gillberg et al. 1988).

Experimental issues

There are several potential limitations to our experimental ap-proach. *1*) Bath-applied transmitters will activate all their respective receptors simultaneously, whereas physiologically, it is possible that there is some degree of preferential activation of receptor subtypes by separate descending serotonergic sys-tems (Wei et al. 1999). Thus physiological conditions may exist where synaptic facilitation dominates over the depression observed in this study. Nonetheless, for 5-HT at least, the dorsal horn contains many 5-HT varicosities that end blindly and do not form conventional synapses (Poulat et al. 1992; Ridet et al. 1994) and in this regard have been suggested to modulate spinal activity via volume transmission (Zoli and Ag-nati 1996). Thus if 5-HT and the other descending mono-amine transmitters act via volume transmission, their actions would non-specifically activate all receptor subtypes. Interest-ingly, in this re-gard, the net effect in most neurons would be a significant synaptic depression. *2*) Our use of high-intensity electrical stimulation to recruit the majority of afferents (Thompson et al. 1990) prevents a comparison of modulatory actions between specific afferent fiber populations. For exam-ple, Jankowska and co-workers (Jankowska et al. 1995, 1997, 2000; Noga et al. 1992; Riddell et al. 1993) dem-onstrated that 5-HT and raphe-spinal stimulation can exert a differ-ential control over primary afferents of different modalities. *3*) The DDH spinal region is functionally heterogeneous, yet we

treated all dorsal horn neurons as a single population. Jankowska and co-workers (2000) have demonstrated that the actions of 5-HT may depend on the functional identity of the spinal neuron studied. *4*) The animals were recorded from immature rats (P10–14), and hence the actions may be differ-ent in adult rats. However, this is the preferred age range to study sensory integrative mechanisms in vitro due to its near-intact circuitry, and near mature developmental status (Fitzger-ald 1985; Fitzgerald and Koltzenburg 1986; Lopez-Garcia and King 1994; Thompson et al. 1990; Woolf and King 1987).

Furthermore, the modulatory actions observed here are un-likely to differ from those seen in the adult. If we take 5-HT as an example, 5-HT immunoreactivity is found in the gray matter at all spinal cord levels at birth, and staining density increases, peaking at P7 in cer-vical and P14 in lumbar cord (Bregman 1987). In addition, Wallis et al. (1993) showed that the strong descending inhibition of the monosynaptic reflex in the P1 rat is mediated by serotonin. Thus at least for 5-HT, descending modulatory systems are present at birth (but see Fitzgerald and Koltzenburg 1986).

The aforementioned limitations notwithstanding, the present ob-servations demonstrate that the descending monoamine transmitters 5-HT, NA, and DA have broadly similar depres-sant actions in the spinal cord and on the same neurons, and these actions are opposite to the facilitatory actions of ACh, which is normally released from intrinsic spinal neurons. That these effects were similar in the ma-jority of neurons sampled suggests that descending monoaminergic systems are capable of modulating spinal sensory integration in a diffuse and gen-eral matter.

Significance

In conclusion, we provide the first comparative analysis of the actions of the biogenic amine transmitters on synaptic and cellular properties of DDH spinal neurons. 5-HT, NA, and DA are involved in the descending control of spinal sensory inte-gration, and the present observations suggest that the separate brain stem monoaminergic systems can affect spinal sensory integration in a remarkably similar manner. With respect to the control of sensory input, these studies increase our understand-ing of how these trans-mitters act, perhaps to maximize antino-ciceptive actions.

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