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SPATIAL AND TEMPORAL PATTERNS OF SEROTONIN RELEASE IN THE RAT'S LUMBAR SPINAL CORD FOLLOWING ELECTRICAL STIMULATION OF THE NUCLEUS RAPHE MAGNUS

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Abstract

The monoamine neurotransmitter serotonin is released from spinal terminals of nucleus raphe magnus (NRM) neurons and important in sensory and motor control, but its pattern of release has remained unclear. Serotonin was measured by the high-resolution method of fast cyclic voltammetry (2 Hz) with carbon-fiber microelectrodes in lumbar segments (L3–L6) of halothane-anesthetized rats during electrical stimulation of the NRM. Because sites of serotonin release are often histologically remote from membrane transporters and receptors, rapid emergence into aggregate extracellular space was expected. Increased monoamine oxidation currents were found in 94% of trials of 50-Hz, 20-s NRM stimulation across all laminae. The estimated peak serotonin concentration averaged 37.8 nM (maximum 287 nM), and was greater in dorsal and ventral laminae (I–III and VIII–IX) than in intermediate laminae (IV–VI). When measured near NRM-evoked changes, basal monoamine levels (relative to dorsal white matter) were highest in intermediate laminae, while changes in norepinephrine level produced by locus ceruleus (LC) stimulation were lowest in laminae II/III and VII. The NRM-evoked monoamine peak was linearly proportional to stimulus frequency (10–100 Hz). The peak often occurred before the stimulus ended (mean 15.6 s at 50 Hz, range 4–35 s) regardless of frequency, suggesting that release per impulse was constant during the rise but fell later. The latency from stimulus onset to electrochemical signal detection (mean 4.2 s, range 1–23 s) was inversely correlated with peak amplitude and directly correlated with time-to-peak. Quantitative modeling suggested that shorter latencies mostly reflected the time below detection threshold (5–10 nM), so that extrasynaptic serotonin was significantly elevated well within 1 s. Longer latencies (>5 s), which were confined to intermediate laminae, appeared mainly to be due to diffusion from distant sources. In conclusion, except possibly in intermediate laminae, serotonergic volume transmission is a significant mode of spinal control by the NRM.

Keywords

spinal cord; raphe; serotonin; monoamines; fast cyclic voltammetry; volume transmission

The monoamine neurotransmitter serotonin is an important modulator of sensory and motor pathways in the spinal cord. It is released from axon terminals whose cell bodies reside mostly in the hindbrain raphe nuclei (Törk, 1990; Jones and Light, 1992; Kwiat and Basbaum, 1992), especially the nucleus raphe magnus (NRM). One of its major effects in the dorsal horn

is to depress ascending nociceptive transmission (Furst, 1999; Millan, 2002). In the intermediate laminae and ventral horn it enhances locomotor rhythms and modulates various reflex pathways (Schmidt and Jordan, 2000; Hochman et al., 2001). Although numerous functional and anatomical studies of the spinal serotonergic system and its brainstem origins have appeared, measurements of spinal serotonin release on temporal and spatial scales relevant to its extracellular dynamics have not been previously reported. A significant proportion of both serotonin receptors and membrane serotonin transporters are located remotely from release sites in the brain and spinal cord (Descarries et al., 1990; Zhou et al., 1998; Ridet and Privat, 2000; Doly et al., 2004). Thus extrasynaptic changes on scales of seconds and tenths of a millimeter are highly pertinent to serotonin's acute neurophysiologic actions, and are also a major consideration in designing therapies for transmitter replacement therapy in spinal cord injury.

Measurement of chemical concentrations in tissue at high resolution requires a detection method that is non-extractive and minimally depleting. The practical choices are presently limited to electrochemical (i.e. voltammetric) methods, in which the substance of interest is caused to oxidize at a microelectrode and its concentration is inferred from the resulting current flow. Prior work with voltammetric measurement of serotonin in the spinal cord has used differential normal pulse voltammetry (Rivot et al., 1995; Kato et al., 1996). This involves applying a sequence of gradually increasing pulses to scan a range of oxidation potentials, giving measurements at a minimum interval of about 5 min. Another form of voltammetry, fast cyclic voltammetry (FCV), allows much faster repetition rates (up to 10 Hz) while maintaining adequate sensitivity (Stamford, 1989). It employs a brief (<30 ms), triphasic saw-tooth waveform scanning through a wide range of oxidation and reduction potentials at several hundred volts per second. We previously reported the use of FCV to map dynamic release of norepinephrine in the rat's lumbar spinal cord following electrical stimulation of the midbrain locus ceruleus (LC) (Hentall et al., 2003). Subsequently, we reported measurements of basal monoamine levels in various laminae, assayed relative to the dorsal cord surface, and acute changes in these levels caused by thoracic spinal block by cooling or transection (Noga et al., 2004). The present paper describes the use of FCV to map the spinal release of serotonin by electrical stimulation of the hindbrain in or near the NRM. Sometimes the effect of LC stimulation or the basal level referenced to the dorsal white matter was also determined at the same location or nearby, as in our aforementioned reports, in order to directly compare the different spinal monoaminergic control systems.

EXPERIMENTAL PROCEDURES

Animal preparation

Adult male rats (200–300 g, Fisher-CDF strain) were obtained from Charles River Laboratories, Inc. (Wilmington, MA, USA). Anesthesia was induced in a small, transparent chamber containing 3% halothane vaporized in a mixture of 60% nitrous oxide and 40% oxygen, after which the trachea was intubated. For the remainder of the experiment, the anesthetic was delivered through the tracheal tube at a level of 1–1.3% halothane. A cannula was inserted in the left common carotid for blood pressure measurement and the right external jugular vein was cannulated for administration of fluids. A bicarbonate solution (100 mM NaHCO₃ with 5% glucose) was infused i.v. at 0.5 ml/h. Blood pressure, blood oxygenation and exhaled carbon dioxide were monitored continuously. Experiments were carried out in accordance with the U.S. National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996). The number of animals used, and their pain and distress, were minimized.

A laminectomy was performed to expose the lumbar spinal cord. The head was positioned in a stereotaxic holder, and clamps were placed on the adjacent vertebrae on either side of the

exposed spinal cord to stabilize it mechanically. The exposed lumbar segments were covered by a pool of phosphate-buffered saline (PBS), the walls of which were formed with a thin layer of Reprosil (Dentsply Caulk, Milford, DE, USA) covering the exposed muscles of the back. The area between muscle and skin (which was tied to form an outer pool) was filled with agar to keep exposed tissue moist. The temperature of the pool was maintained at 37 °C by a thermistor-controlled heating lamp. Core body temperature was maintained at 37 °C by a second heating lamp controlled by a rectal thermistor. Openings were drilled in the skull to allow stereotaxic positioning of stimulating electrodes in the rostral medial medulla and the dorsal mesencephalon.

FCV

Microelectrodes for voltammetry consisted of 33- μ m diameter carbon fibers (Textron Systems, Lowell, MA, USA) inserted into 2-mm diameter borosilicate glass micropipettes. The small gap between the glass tip and the carbon fiber was sealed with an epoxy resin (Epon 815) that was then hardened for 48 h. The carbon fiber was connected to its output wire by electrically conductive silver epoxy, which was allowed to cure for 12 h at room temperature followed by 2 h at 60 °C. The microelectrodes were beveled at a 30° angle from horizontal, a process that left the surface of the carbon fiber flush with the tip and gave a final inside average diameter of 40–50 μ m. The surface was cleaned after beveling in ethyl alcohol followed by rinsing in de-ionized water. To increase sensitivity to monoamines, the microelectrodes were electrically pretreated (Stamford et al., 1992) in PBS (10 ml, pH 7.4) just prior to use by applying a 71 Hz triangular waveform in three consecutive 10-s phases, in which the voltage range was first –0.6 to +3.0 V, second –0.6 to +2.0 V and finally –0.6 to +1.0 V.

A three-lead voltage clamp amplifier (Millar Voltammeter, P.D. Systems Ltd., UK) was connected to the carbon-fiber working electrode, to a carbon-based reference electrode internally filled with KCl (Dri-Ref: WPI, Sarasota, FL, USA) and to a silver–silver chloride auxiliary electrode. The auxiliary electrode delivered the current needed to maintain a fixed voltage between the reference and working electrodes. The reference and auxiliary electrodes were positioned several centimeters from each other in the pool of saline covering the spinal cord. A three-phase (negative–positive–negative) triangular voltage scan, sweeping at ~450 V/s between –1 V and +1.4 V for roughly 15 ms, was applied to the carbon-fiber microelectrode every 0.5 s. The amplifier's output signal was proportional to the current flowing through the microelectrode. At the start of an experimental trial, the output of a single scan was stored. Subsequent output scans were subtracted from this stored one in real time, to show the change in redox current (Faradaic current) during a trial. This signal is referred to below as a subtracted voltammogram.

Calibration and *in vivo* confirmation of voltammetric signals

Working microelectrodes were calibrated in PBS previously purged of air by bubbling with nitrogen. Norepinephrine or serotonin, dissolved in the same medium, was added in small volumes, and stirred in rapidly to achieve uniform final concentrations of 0.5–2.5 μ M. Both norepinephrine and serotonin caused an upward deflection in the subtracted voltammogram during the first ascending phase of the applied waveform, representing the current flow consequent to their oxidation at the carbon-fiber microelectrode. For serotonin, this oxidation current was maximal when the applied input waveform was near 920 mV and fell off within ± 200 mV (Fig. 1A). Norepinephrine gave a similar current deflection peaking near 940 mV. The amplitude of these peaks was quantified by subtracting a reference value occurring just before the deflection or, in the case of sloped subtracted voltammogram, by subtracting the mean of the current values just beyond (before and after) the deflection as described previously (Hentall et al., 2003). During the subsequent descending section of the input waveform, serotonin produced two reduction-current deflections, one peaking near –100 mV and the other

near -800 mV, presumably reflecting reduction of the electrode oxidation reaction products that had remained nearby. Norepinephrine, in contrast, was associated with only one reduction peak, which fell near -400 mV. Each microelectrode was calibrated before and after its use in an experiment. The mean post-experimental sensitivity, estimated by linear regression of the peak oxidation currents (Fig. 1D), was 7.3 nM/nA (± 1.03 , $n=6$) to serotonin and 60.9 nM/nA (± 16.3 , $n=6$) to norepinephrine. Since recording noise was about 0.5 nA *in vivo*, detection thresholds were about 1 nA, which translates to 5 – 10 nM in the case of serotonin. Further technical details concerning substance identification and interference by ascorbate or hydrogen ions can be found in two previous papers (Hentall et al., 2003; Noga et al., 2004).

Two sorts of tests, superfusion and microinjection, were performed to ascertain whether the FCV output scan produced by serotonin during *ex vivo* calibration would be replicated *in vivo*. Serotonin was microinjected (100 μ M, 2 – 4 μ l, 1 μ l/min, $n=3$ rats) through a beveled micropipette of 10 – 25 μ m tip diameter, positioned 400 – 800 μ m rostral to the carbon-fiber microelectrode and at the same depth (600 – 800 μ m) in the L3 dorsal horn. For the superfusion, a carbon-fiber microelectrode was positioned in the L3 dorsal horn (600 – 800 μ m depth) and the surrounding PBS was withdrawn and replaced rapidly (<5 s) with a solution of serotonin (10 μ M to 2 mM) dissolved in PBS at the same pH and temperature. Following microinjection of serotonin, delays in the oxidation and double reduction peaks (which were greater with higher concentration) and diminished relative amplitude of the paired reduction peaks (Fig. 1B) were observed. At the lowest extracellular concentrations of serotonin, double reduction peaks were not distinguishable from the baseline levels (Fig. 1B, bottom trace). Superfusion also produced a delay in the oxidation peak of serotonin ($n=4$ rats; 12 measurements). On average, a measured concentration of 84.3 ± 97.0 nM showed a delay of about 0.4 ms (183.7 ± 115.8 mV on the input voltage waveform) over *ex vivo* calibrations. Paired reduction peaks were also seen *in vivo*, but these were weaker relative to the size of oxidation peaks than seen *ex vivo*. Both the delay in oxidation peak and the weaker reduction currents can be accounted for by the different redox and diffusional environment *in vivo* (Hentall et al., 2003).

Electrical stimulation and mapping protocol

Electrical stimulation was applied through monopolar tungsten electrodes (A-M Systems, Inc., Carlsborg, WA, USA). These had gradually tapering tips and an exposed final length of around 50 μ m. The hindbrain stimulating electrode was placed within 0.5 mm lateral of the midline, between 2 mm dorsal and 1 mm ventral to the interaural line and between 2.1 – 2.7 mm caudal to the interaural line. These coordinates included the NRM and parts of adjacent regions (Törk, 1990). The midbrain electrode was directed caudally at an angle of 25° in the sagittal plane, aiming stereotactically at the LC: 1.1 mm caudal, 3 mm dorsal, and between 1.2 and 1.7 mm medial in the reference frame. Before spinal cord mapping, midbrain and hindbrain electrodes were moved within their stereotaxic ranges to find sites producing the highest monoamine signal in the dorsal horn (600 – 800 μ m from the dorsal surface). At the end of each experiment, locations of stimulating electrodes were marked electrolytically (1 mA, 8 – 10 s).

In a typical experimental trial, monoamine levels were sampled by FCV at 2 Hz for 200 s. After the first 10 s of sampling, the NRM or LC stimulus was applied for 20 s. This stimulus normally consisted of rectangular cathodal pulses of 50 Hz frequency, 0.5 ms width and 150 μ A, except when the effect of the stimulus parameters themselves was being studied. An interval of at least 4 min was allowed between trials. Lumbar segments L3–L6 were examined along microelectrode penetrations located 350 and 750 μ m lateral to the midline and 0 – 2.2 mm below the white matter. Measurements were made at vertical spacings of either 100 μ m or 200 μ m. In order to analyze the influence of brainstem stimulation on the shapes of subtracted voltammograms, 10 – 20 -S averages were taken of the peak responses to the 20 -s stimulation period.

Histology and statistical analysis

Both the spinal cord and brainstem were immersion-fixed postmortem in 10% buffered formalin. Frozen sections were cut transversely at 100 μm thickness, and stained with Toluidine Blue or Cresyl Violet. Brainstem stimulation sites were reconstructed on standardized sections from the observed locations of electrolytic lesions. The depths of spinal recording sites were estimated from micromanipulator readings. Their lateral positions were estimated from micromanipulator readings and verified by the slight tissue damage that indicated a microelectrode track. Laminar locations were specified by mapping onto the histologically observed cytoarchitecture, after allowing for tissue shrinkage.

Spatial differences in measured monoamine levels and other dependent variables were analyzed statistically from samples in which uncompleted microelectrode trajectories had been removed to avoid spatial bias. Multi-way analysis of variance (ANOVA) or co-variance (ANCOVA) was performed with post hoc Bonferroni comparison at a two-tail significance level of $P < 0.05$. Repeated-measures ANOVA was used when comparing measurements taken consecutively at different depths along microelectrode penetrations. The comparison of spatial profiles for different kinds of measures (e.g. responses to LC and NRM stimulation) was done by first standardizing the sample distributions (subtracting the sample mean and dividing by the sample standard deviation). For categorical data, the Pearson correlation matrix with Bonferroni-adjusted probabilities and the chi-square test were used. All statistical testing was done with commercial software (Systat, version 10.2, Point Richmond, CA, USA).

RESULTS

Voltammetric output signals caused by NRM stimulation

Stimulation in the NRM with the standard 20-second train (50 Hz, 150 μA , 0.5 ms pulses) caused an increase in monoamine oxidation current in 94% of trials across all laminae (Fig. 2). Only rarely was a decrease over baseline levels observed in the subtracted voltammograms (Fig. 2, L3 map: at 400 μm deep). The average change in serotonin across all laminae measured during systematic mapping was 37.8 nM (± 3.25 S.E.M., $n=61$; maximum 287 nM), according to post-experimental calibration of the microelectrodes. This estimate of concentration change assumes that the oxidation peaks were entirely due to serotonin (see Discussion for the basis for this assumption). When subtracted voltammograms were averaged over the 20-s period of stimulation, the oxidation current peaked at a point corresponding to 1150 mV ($n=77$) on the first ascending ramp of the input waveform. Thus oxidation peaks were delayed compared with where they fell during calibration *ex vivo* (Fig. 1C). This delay was similar to the delay seen during *in vivo* superfusion or microinjection of serotonin (Fig. 1B), as described in the Experimental Procedures section.

The reduction currents produced by NRM stimulation, as a proportion of the oxidation currents, were considerably smaller than observed *ex vivo*. This difference from the *ex vivo* voltammogram was also noted during *in vivo* microinjection or superfusion of serotonin. As a consequence of this effect, when the NRM-evoked oxidation peak was small (about 2 nA or less, equivalent to about 15 nM serotonin) it was not possible to discern reduction peaks within the electrical recording noise. However, when noise was relatively low, two reduction peaks were often found in the subtracted voltammogram averaged during NRM stimulation. In contrast, reduction peaks in the mean subtracted voltammograms recorded during LC stimulation ($n=50$) were always unpaired, as reported previously (Hentall et al., 2003). There was a significant difference in the laminar distribution of NRM-evoked double reduction peaks, very few being found in laminae IV and V (Fig. 3). This may have been due either to lower amounts of serotonin being liberated or to a very high proportion of norepinephrine in the total

released monoamine (the microelectrodes were about eight times less sensitive to norepinephrine than serotonin).

Optimization of stimulation site and parameters

Effects of the standard 20-s stimulus train (50 Hz, 150 μ A, 0.5 ms) were tested at different locations in the hindbrain while the carbon-fiber microelectrode position remained stationary in the dorsal horn (600–800 μ m deep in different preparations). In the vertical axis, the more effective hindbrain stimulation sites were found to lie 0–1 mm above the pyramidal tract. Sites located more dorsally produced weak or transient responses (Fig. 4). No systematic differences were noted in effectiveness along the rostrocaudal axis within the stereotaxic ranges examined, nor within 1 mm of the midline ipsilateral or contralateral to spinal measurement site.

Stimulus frequencies were tested in the range 10–100 Hz with pulses of 150 μ A amplitude and 0.5 ms width. A strong direct correlation between the peak oxidation current and stimulus frequency in this range was found (ANCOVA, $P < 0.00005$). This correlation was due to changes between 20 and 100 Hz, since there was a slight drop in mean response between 10 and 20 Hz (Fig. 5). When different stimulus amplitudes (≤ 200 μ A) were examined (50 Hz and 0.5 ms), amplitudes below 50 μ A were usually found to be ineffective. Increasing the pulse width from 0.1 ms to 0.5 ms (50 Hz, 150 μ A pulses) caused a higher peak oxidation current, but a further increase from 0.5–1 ms produced little additional rise in peak oxidation current.

Time course of changes in monoamine level evoked by NRM stimulation

A marked delay (latency) of several seconds always appeared between the start of the NRM stimulation train and the emergence of a clear change in monoamine oxidation current (Fig. 2). This latency was expressed numerically as the point when the monoamine oxidation signal exceeded twice the highest signal measured in the last 10 voltammograms before stimulation. The mean latency was 4.2 s (± 0.36 , $n = 61$, range 1–23 s) for trials with the standard stimulus train. It was significantly reduced by raising the stimulus frequency (linear regression, $P < 0.05$) when the microelectrode was at a fixed site in the dorsal horn. The oxidation current usually showed little change during the second half of the 20-s stimulus train, and indeed often declined before the stimulus ceased. Thus, the peak response occurred on average at 15.6 s (± 0.93 S.E.M., $n = 61$, range 4–35 s) from the onset of the stimulation. The latency was directly correlated with this time to peak, and was inversely correlated with peak concentration (both $P < 0.005$, Pearson correlation matrix with Bonferroni adjusted probabilities). The relation between latency and peak concentration is also visible in their respective spatial maps (Fig. 6: graphs A and B).

The monoamine level was sampled for 170 s following the NRM stimulation. During this period, several patterns were observed. In 46% of cases, a second, more prolonged wave of increased monoamine signal appeared on average 72.3 s (± 7.4 S.E.M.) after stimulus onset (e.g. Fig. 2, L3 map, at 800, 1600 μ m deep; L6 map at 200, 300 and 400 μ m deep). This second wave was more frequent in lamina II/III and VII than elsewhere (Fig. 7; chi-square test $P < 0.05$, $n = 29$). Second waves were also produced by LC stimulation, as noted and discussed with regard to causation in a previous report (Hentall et al., 2003). In the remaining trials, either the prior resting level was approximately restored (e.g. Fig. 2, L3 map, at 1200 μ m deep) or a new, stable level was established that was considerably lower than before stimulation (e.g. Fig. 2, L3 map, at 1800 μ m). A higher steady level after a positive response to NRM stimulation was seen when the microelectrode was in white matter (e.g. Fig. 2, L3 and L6 map, at 200 μ m).

Laminar differences in brainstem-evoked and resting levels of monoamines

Significant differences occurred among the peak concentrations in different laminae (repeated measures ANOVA $P = 0.044$). As can be observed in the graph of Fig. 6A, mean peak levels were highest in laminae I and VIII/IX, moderately high in II/III and VII and lowest in laminae

IV–VI. The peaks at dorsal (laminae I) and ventral (lamina VIII/IX) locations were reflected in a significant 2nd order polynomial contrast ($P=0.02$). The latency of the oxidation response following NRM stimulation also showed significant laminar variation (repeated measures ANOVA $P=0.037$), and was largest in the intermediate laminae (Fig. 6B). Effects on peak concentration or latency of mediolateral and rostrocaudal locations, analyzed by three-way ANOVA along with the laminar location, could not be differentiated from random differences among rats or electrodes.

Basal monoamine oxidation currents were measured at the same location as the response to NRM stimulation in four animals. Basal current values were calculated as differences from the dorsal column white matter. That is, the subtracted voltammograms were referenced to a point in the dorsal column (Noga et al., 2004). Basal currents were generally several times higher than the change in oxidation currents evoked by the NRM (Fig. 8A). Furthermore, whereas NRM-evoked currents were lowest in the intermediate laminae (IV–VI), basal currents were higher here than elsewhere (repeated measures ANOVA on standardized data: $P=0.02$). These differences were also found in larger samples based on unpaired measurements, for example in comparing the present NRM profiles (Fig. 6A) with previously published basal profiles (Noga et al., 2004). Oxidation currents were not converted to concentrations for this comparison, since the contributions of different monoamine species were less certain in basal than in brainstem-evoked voltammograms.

LC and NRM stimulation was tested consecutively in three spinal cord tracks. The estimated change in serotonin concentration elicited by NRM stimulation was generally much smaller than the estimated change in norepinephrine concentration elicited by LC stimulation (Fig. 8B). The normalized spinal profiles for NRM and LC stimulation resembled each other in most laminae, the main difference being that NRM-evoked signals were lowest in the intermediate laminae while LC-evoked signals were lowest in lamina II/II and VII (repeated measures ANOVA on standardized data: $P=0.04$). These laminar differences replicate those in larger, unpaired samples taken from the present measurements on NRM-evoked responses (Fig. 6A) and previously reported measurements on the LC-evoked responses (Hentall et al., 2003).

DISCUSSION

Chemical basis for NRM-evoked changes in oxidation current

Most of the NRM-evoked change in oxidation current seen in these experiments is readily explained as an increase in extrasynaptic serotonin following its release from the spinal terminals of activated raphe neurons. Electrical stimulation excites nearby NRM neurons both directly and indirectly via activation of presynaptic fibers; both have been observed by concurrent (coincident) recording through stimulating electrodes (Hentall and White, 1997). Descending serotonergic axons are abundant in the rat's NRM (Jones and Light, 1992; Kwiat and Basbaum, 1992). There is no published evidence for spinally projecting NRM neurons containing other monoamines in significant amounts. The wide-spread occurrence of increases in oxidation current in the lumbar spinal cord when stimulation was applied at one site in the NRM is consistent with the extensive collateral branching of typical raphe–spinal axons. Their terminals enter various laminae in many spinal segments (Huisman et al., 1981; Fields et al., 1995). A non-neural route to the spinal cord from the brainstem is unlikely to have mediated the rapid initial rise of the oxidation signals or the rapid fall occurring soon after the stimulus train was stopped (Fig. 2).

Release of serotonin in the dorsal horn following electrical stimulation of the NRM had previously been shown by differential normal pulse voltammetry through carbon-fiber microelectrodes (Rivot et al., 1982) and by off-line chromatographic analysis of microdialysates (Bowker and Abhold, 1990; Sorkin et al., 1993). Electrical stimulation of the

periaqueductal gray (PAG) region, a major input region to the NRM, had produced similar results with the same methods: differential normal pulse voltammetry (Kato et al., 1996) and microdialysis (Cui et al., 1999). Although superior to FCV in qualitative discrimination of molecular species, these two methods are unsuitable for mapping because of their low temporal and spatial resolution. Moreover, they both require prolonged brainstem stimulation in order to yield any signal. The studies of PAG stimulation cited above, for example, used frequencies of at least 100 Hz and amplitudes of at least 200 μ A pulses applied for at least 3 min (Kato et al., 1996; Cui et al., 1999).

Since the NRM can activate noradrenergic descending pathways via the nucleus cuneiformis (Clark and Proudfit, 1991), and the timing of norepinephrine and serotonin oxidation peaks in FCV is very similar, the measured NRM-evoked oxidation currents may have included a norepinephrine component. Both substances have been detected in microdialysates following prolonged, high-frequency stimulation of the NRM or PAG (Bowker and Abhold, 1990; Cui et al., 1999), and also in spinal cord superfusates in the presence of reuptake inhibitors during NRM stimulation (Hammond et al., 1985). However, serotonin can be differentiated from norepinephrine by possession of paired reduction peaks (Fig. 1A). Such pairing was often observed during NRM stimulation (Fig. 1C) but never during LC stimulation, and was significantly more common in dorsal and ventral laminae than in intermediate laminae (Fig. 3). This laminar distribution accords with anatomical studies that have demonstrated the greatest density of serotonergic raphe-spinal terminals in dorsal laminae in the rat (Jones and Light, 1992), whereas noradrenergic fibers predominate in intermediate laminae (Rajaofetra et al., 1992).

Extrasynaptic release of serotonin and stimulation frequency

Monoamine release sites in the CNS are generally not located opposite postsynaptic receptors or membrane transporters (Descarries et al., 1990; Zhou et al., 1998; Doly et al., 2004). Solely on this anatomical basis, volume transmission has been proposed to be important in monoaminergic systems (Ridet and Privat, 2000; Vizi et al., 2004). Volume transmission was inferred previously to occur in spinal neurons after LC-evoked norepinephrine release (Hentall et al., 2003). The present findings suggest that the raphe-spinal system also uses volume transmission, because NRM stimulation produced a large extrasynaptic overflow of the neurotransmitter. It might be argued that such overflow results only from relatively prolonged and rapidly repeated stimulation, such as the 20-second train of stimulation at a frequency of 50 Hz that was applied most commonly. However, lower stimulation frequencies (10 and 20 Hz) were also found to produce overflow starting within a few seconds of stimulation onset (Fig. 5). Indeed, extrasynaptic serotonin level probably climbed much sooner, but was below the measurement threshold, as discussed below. The prolonged stimulus (20 s) was selected deliberately to increase the chance of achieving a steady-state concentration, which would allow independent variation in rise-time and response amplitude to be distinguished. The frequency of 50 Hz was selected to optimize the signal-to-noise ratio. Both choices were largely vindicated by the results. Another, related question arising in connection with the stimulus frequencies used is whether they were above what is normal in NRM neurons. NRM neurons can be classified according to their response to noxious stimuli, as inhibited (off-cells), excited (on cells) or unaffected (neutral cells). Off-cells have been found with resting rates of 2–20 Hz and neutral cells 0.2–2 Hz, while prolonged painful stimuli can raise the sustained rates of on-cells to 10 Hz (Hentall et al., 1993, 2000). In the awake, freely moving rat, most NRM neurons have irregular spontaneous activity of 2–16 Hz (Oliveras et al., 1989). In sum, stimulation within the upper range of firing frequencies normally found in NRM neurons probably produces an almost immediate extrasynaptic overflow of serotonin in spinal gray matter.

Modeling the time-course of release

The latency in the measured monoamine signals evoked by NRM stimulation could have resulted from several factors: axonal conduction time, compartmentalized diffusion spaces, time to detection threshold, and uneven distribution of sources. The first two of these almost certainly made a minor contribution. Even if it is assumed that only the slowest, unmyelinated fibers conducting at <1 mm/ms were involved, a delay of at most 100 ms is expected from axonal conduction along the raphe–spinal pathway. The most obvious compartmentalization is between synaptic clefts and general extracellular space, but diffusion in clefts reaches a steady-state within a millisecond (Eccles and Jaeger, 1958). Unfortunately, no techniques are available for making real-time measurements sub-synaptically, although optical dye–based measurements are in principle feasible. Carbon-fiber microelectrodes, due to their size, inevitably sample from aggregate extracellular space only.

The time required for the extracellular concentrations to attain detection threshold was probably a major cause of the observed latencies, especially the shorter ones. Thresholds can be estimated from the calibration data and peak-peak noise *in vivo* as 5–10 nM. Dorsal laminae (I–III) showed a mean peak serotonin release of about 50 nM. The roughly linear rise was about 90% complete after 10 s. Hence the time needed to reach detection threshold readily explains the mean latency in these laminae (about 2.5 s, Fig. 6B). However, the 10 longest latencies (6–23 s), which all arose in intermediate laminae (IV–VII) where the mean peak concentration was about 30 nM (Fig. 6A), cannot be explained by this technical limitation alone. Because these longer latencies were associated with longer times-to-peak, diffusional delay was likely a major fraction of their total length.

A simple model was created to assist in understanding the time-course of extracellular serotonin. As described in detail for the legend of Fig. 9, the microelectrode was regarded as being situated within a uniform field of axon terminals firing sufficiently rapidly so that their boutons approximate continuous sources of serotonin. The constant of diffusion, the tortuosity and the volume fraction were given typical values (Sykova et al., 1994; Rice and Nicholson, 1995). The plot of concentration against time shows an increasing growth rate for about the first 20 s as more distant sources come into play, followed thereafter by a constant growth rate (Fig. 9B). The model was modified to make all sources distal, as suggested above to be true of intermediate laminae, by imposing a void (“hole”) of 0.2 mm radius around the measurement site (Fig. 9B). An additional lag of several seconds then emerged before the period of constant rise, which had the same rate as in the uniform case since this was determined mainly by the distant sources. A stronger distant source imposed on a uniform distribution caused no additional lag, but the final rate of rise was greater than in the other two cases (“ring,” Fig. 9B).

No distribution of constantly emitting sources, regardless of the complexity of their arrangement, can produce a decline in concentration in this model. The addition of continuous sinks, whether formed by blood vessels or active membrane transporters, does not alter this. An additional mechanism is required to explain the decline in concentration commonly observed during the stimulus train. An increase in conduction block in the raphe–spinal projection or a gradual diminution of transmitter release due to depletion of vesicles at higher stimulus frequencies is among the possibilities, but such mechanisms are difficult to reconcile with the observed effect of frequency. For example, release approximately doubled and time-to-peak changed little when the frequency was raised from 50 Hz to 100 Hz (Fig. 5) in the 20-s stimulus train. Linear release of serotonin with no failure has previously been noted at frequencies as high as 100 Hz from terminals in the substantia nigra reticulata, dorsal raphe and suprachiasmatic nucleus (O’Connor and Kruk, 1991; Bunin and Wightman, 1998). Evidence is similarly lacking for conduction block. NRM neurons can be antidromically stimulated at 100 Hz for many seconds (Hentall et al., 1984b). Functionally, the threshold for

inhibiting the heat-evoked tail-flick reflex by 20 s of NRM stimulation drops consistently between 10, 20 and 50 Hz, and is constant between 50 and 200 Hz (Hentall et al., 1984a). The best explanation, therefore, is that there is a slight reduction in transmitter release per impulse later in the 20 s train at all frequencies from 10 to 100 Hz, due to an unclear mechanism. Similar peaking in dopamine release from nigrostriatal and mesolimbic pathways has been proposed to result from altered ion fluxes at the releasing terminals during higher frequency firing (Stamford et al., 1988; Williams et al., 1995).

Implications for role of monoamines in modulation of spinal functions

Several neurophysiological studies have compared descending serotonergic and adrenergic effects. There are many similarities but some subtle differences too. For example, both serotonin and norepinephrine facilitate transmission from Ia and Ib muscle afferents to various interneurons in the cat's lumbar dorsal and intermediate laminae (Jankowska et al., 2000). Cutaneous and muscle group II afferent responses can be facilitated or inhibited in a similar or opposite way by serotonin and norepinephrine, depending on the location, and nature of the input and output (Noga et al., 1992; Jankowska et al., 1997, 2000). In lamina VII of the cat, both substances facilitate the activation by reticulospinal fibers of commissural neurons involved in interlimb coordination, whereas their activation by group II fibers is blocked by norepinephrine and facilitated by serotonin (Hammar et al., 2004). The differences likely depend on the receptor subtypes involved and their presynaptic or postsynaptic locations (Bras et al., 1990; Maxwell et al., 2003; Dougherty et al., 2005), which remain poorly delineated. Among serotonin subtypes, 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-HT₃ and 5-HT₇ all seem to be important for spinal motor function (Schmidt and Jordan, 2000; Hochman et al., 2001), and among catecholamine receptor subtypes α_1 , α_{2A} and α_{2C} may all be critical (Bras et al., 1990). The detection threshold of our measurements (5–10 nM) was above the K_m of most 5-HT receptor subtypes. The one with the exception is the 5-HT_{2A} receptor, whose EC₅₀ is 13 micromoles (Hochman et al., 2001), and is therefore the only subtype unlikely to be involved in volume transmission.

If any broad principles can be formulated for the complex and varied roles of monoamines in the spinal cord, they may emerge from the intermediate scales of time and space over which these substances operate (on the order of a few seconds and few hundred microns). These times and distances are not well suited for conveying rapid, specific commands, but can serve to set up segmental circuits to respond with specific motor patterns to descending glutamatergic motor commands under a particular state of peripheral input (Jankowska et al., 1993). On the sensory side, these scales imply that monoamines modulate ascending transmission of information over longer times and larger somatotopic regions than encompassed in the typical dynamics of afferent input. Besides their effects on sensory events and motor commands, monoamines can also operate at the opposite spatiotemporal extreme, initiating trophic changes. For example, serotonin alters cyclic AMP levels via 5-HT₁ and 5-HT₇ receptors (Raymond et al., 2001), and major trophic effects of cyclic AMP can be seen in its influence on recovery from spinal cord injury (Spencer and Filbin, 2004). Slow, widespread trophic changes and fast, localized sensory and motor responses could be linked through the feedback they provide to monoaminergic control systems, and a future effort to better characterize such feedback may therefore be productive. Yet regardless of the influences on them, the sum of the evidence suggests that descending monoaminergic systems employ volume transmission to act at intermediate scales and govern spinal cord functioning at these two scalar extremes.

Abbreviations

ANCOVA, analysis of co-variance; ANOVA, analysis of variance; FCV, fast cyclic voltammetry; LC, locus ceruleus; NRM, nucleus raphe magnus; PAG, periaqueductal gray; PBS, phosphate-buffered saline.

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Fig. 1.

(A) Input waveform (above) and subtracted voltammograms (below) averaged from 20 to 30 scans during ex vivo calibration in serotonin or control medium (PBS, pH 7.4). Concentrations are given in μM . (B) *In vivo* subtracted voltammograms seen when serotonin (100 μM) was microinjected near a carbon-fiber microelectrode located at the same depth in the dorsal horn. Traces 1–3 indicate progressively increasing concentrations of 5-HT as the bolus of 5-HT approaches and envelops the CFME. Note the delay in oxidation peaks and reduction peaks *in vivo* (A) compared with those obtain *in vitro* (B), as marked by the broken lines through them. At the lowest concentrations, the double reduction peaks observed *in vivo* (arrowheads) were indistinguishable from baseline noise levels. Output traces in A and B are all at the same

gain. (C) Comparison of the subtracted voltammograms from an *in vitro* calibration trial (top) and, in the same microelectrode, from an *in vivo* (bottom) response in the dorsal horn (600 μm deep) to raphe magnus stimulation. Note the delayed oxidation and reduction peaks in the *in vivo* recording. (D) Calibration points determined ex vivo in serotonin and corresponding linear regression lines for all carbon-fiber microelectrodes used in the experiments.

Fig. 2.

Example of responses to raphe magnus stimulation (150 μA , 100 Hz, 0.5 ms cathodal pulses) seen along one electrode track in the L3 and L6 spinal segments. Sampling was done every 200 μm for the L3 example and every 100 μm for the L6. Thick horizontal bars indicate the duration of the stimulus train. Vertical line indicates the concentration of 5-HT determined from post-experimental calibration of the microelectrode *ex vivo* on the assumption that all of the oxidation current at the peak was due to this substance. Locations of recording sites were determined from histological reconstructions. In all cases but one (second trace in L3 segment), NRM stimulation resulted in an increase in oxidation current over subtracted baseline values. At 2000 μm in L3, the stimulus train was erroneously given for longer than 20 s.

Fig. 3.
Frequency of occurrence of double reduction peaks within average voltammograms produced during NRM stimulation.

Fig. 4. Effect of stimulating ($150 \mu\text{A}$, 100 Hz , 0.5 ms) four different hindbrain locations (A, B, C and D) on the peak monoamine oxidation current measured by a microelectrode in the dorsal horn at $600 \mu\text{m}$. The arrows pointing up and down mark the beginning and end of stimulation. Concentrations (dotted horizontal lines) were calculated from post-experimental calibration in serotonin. Inset: Voltammograms illustrating ex vivo response (middle trace) to 400 nM 5-HT and *in vivo* response (lower trace) to electrical stimulation of the NRM (point C). The input waveform is illustrated in upper trace. Arrowheads indicate delayed reduction peaks.

Fig. 5.

Effect of stimulus frequency in the NRM on the time course of change in monoamine level measured in the dorsal horn. (A) Time courses observed at one point in one experiment. The up and down arrows mark the beginning and end of stimulation. (B) Mean peak serotonin concentration plotted against stimulus frequency, derived from measurements made in the dorsal horn of five rats. Error bars depict the standard error of the mean.

Fig. 6.

NRM-evoked concentration increases and response latencies in different laminae. Averaging was carried out over all segments and lateral positions. Error bars depict the standard error of the mean. To achieve a roughly equal number of points per region, lamina II was combined with lamina III and lamina VIII was combined with lamina IX. (A) Concentration. Lamina I was significantly different from both lamina IV and lamina V (Bonferroni-adjusted pairwise comparison, $P < 0.05$), and the overall distribution was significantly non-uniform (see text). (B) Response latency. The combination of laminae II/II (combined) was significantly different from lamina V and lamina VII (Bonferroni-adjusted pairwise comparison, $P < 0.05$). Significant pairwise differences are marked by the asterisks.

Fig. 7. Frequency of occurrence of second waves of increased monoamine concentrations following raphe magnus stimulation. The distribution was significantly non-uniform (chi-squared test, $P < 0.05$).

Fig. 8.

Basal and evoked monoamine levels in different laminae. Bars depict the standard error of the mean. (A) Comparison of NRM-evoked monoamine oxidation currents (averaged) with average basal current levels mapped at the same location. Both transmitters and metabolites probably contribute to the basal current level measured at each location (Noga et al., 2004). (B) Comparison of average NRM-evoked serotonin release with LC-evoked norepinephrine release measured at the same location in a given preparation. Concentrations for norepinephrine and serotonin were estimated from post-experimental calibration.

Fig. 9.

Simple models of NRM-evoked changes in serotonin in the spinal cord as measured by a carbon-fiber microelectrode. (A) An idealized view of rat's spinal cord (L3 segment) in transverse section, with a recording electrode in lamina V. The intensity of extrasynaptic serotonin release in each lamina (or grouping of II/III and VIII/IX) is shown by the density of release sites. This density is proportional to the mean peak NRM-evoked monoamine oxidation signal, as presented in Fig. 6A, but may be an overestimate of serotonin release in intermediate laminae due to the role of distant sources there. (Inset) A higher magnification view of the box illustrated at left, depicting the level of serotonin diffusing from extrasynaptic release sites shortly after an action potential. The numerous axonal terminals causing the release are not

illustrated. Two blood-filled capillaries are also drawn, in order to highlight the primacy of diffusion and circulation in clearance. (B) Quantitative models of time-course. In the simplest (“uniform”) version of the model, release sites have a grossly uniform density within a large volume of uniform diffusion constant ($D=10^{-5}$ cm²/s), tortuosity ($\lambda=1.5$) and volume fraction ($\alpha=0.2$). The measurement site is 1 μ m from the closest of the point sources, which are scattered randomly. NRM stimulation at any frequency in the range 10–100 Hz is assumed to release serotonin essentially continuously from a given site with respect to the temporal resolution of the voltammetric measurements. The concentration at a given location is obtained by linear superposition of contributions from the randomly distributed sources: $C(r,t)=(Q\lambda^2/4\pi D\alpha r)\text{erfc}(r\lambda/2D^{1/2}t^{1/2})$ (Rice and Nicholson, 1995), where r is radial distance in cm, t is time in seconds, the source (Q) and concentration (C) are expressed in arbitrary units and erfc is the complementary error function. The curve labeled “hole” models the situation in which sources are absent within a certain radius of the measurement site ($r<200$ μ m), but are uniform elsewhere and have the same intensity as the uniform model. The curve labeled “ring” was obtained by placing a source of eight times normal intensity at radial distances 200–250 μ m from the measurement site in an otherwise uniform source distribution. Conclusions derived from these models are presented in the Discussion section.