Serotonergic Inhibition of Action Potential Evoked Calcium Transients in NOS-Containing Mesopontine Cholinergic Neurons

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Leonard, Christopher S., Sanjai R. Rao, and Takafumi Inoue. Serotonergic inhibition of action potential evoked calcium transients in NOS-containing mesopontine cholinergic neurons. J Neurophysiol 84: 1558–1572, 2000. Nitric oxide synthase (NOS)-containing mesopontine cholinergic (MPCn) neurons of the laterodorsal tegmental nucleus (LDT) are hypothesized to drive the behavioral states of waking and REM sleep through a tonic increase in firing rate which begins before and is maintained throughout these states. In principle, increased firing could elevate intracellular calcium levels and regulate numerous cellular processes including excitability, gene expression, and the activity of neuronal NOS in a state-dependent manner. We investigated whether repetitive firing, evoked by current injection and N-methyl-D-aspartate (NMDA) receptor activation, produces somatic and proximal dendritic [Ca\(^{2+}\)] transients and whether these transients are modulated by serotonin, a transmitter thought to play a critical role in regulating the state-dependent firing of MPCn neurons. [Ca\(^{2+}\)] was monitored optically from neurons filled with Ca\(^{2+}\) indicators in guinea pig brain slices while measuring membrane potential with sharp microelectrodes or patch pipettes. Neither hyperpolarizing current steps nor subthreshold depolarizing steps altered [Ca\(^{2+}\)]. In contrast, suprathreshold currents caused large and rapid increases in [Ca\(^{2+}\)], that were related to firing rate. TTX (1 μM) strongly attenuated this relation. Addition of tetrodynammonium (TEA, 20 mM), which resulted in Ca\(^{2+}\) spiking on depolarization, restored the change in [Ca\(^{2+}\)], to pre-TTX levels. Suprathreshold doses of NMDA also produced increases in [Ca\(^{2+}\)], that were reduced by up to 60% by TTX. Application of 5-HT, which hyperpolarized LDT neurons without detectable changes in [Ca\(^{2+}\)], suppressed both current- and NMDA-evoked increases in [Ca\(^{2+}\)], by reducing the number of evoked spikes and by inhibiting spike-evoked Ca\(^{2+}\) transients by ~40% in the soma and proximal dendrites. This inhibition was accompanied by a subtle increase in the spike repolarization rate and a decrease in spike width, as expected for inhibition of high-threshold Ca\(^{2+}\) currents in these neurons. NADPH-diaphorase histochemistry confirmed that recorded cells were NOS-containing. These findings indicate the prime role of action potentials in elevating [Ca\(^{2+}\)] in NOS-containing MPCn neurons. Moreover, they demonstrate that serotonin can inhibit somatic and proximal dendritic [Ca\(^{2+}\)], increases both indirectly by reducing firing rate and directly by decreasing the spike-evoked transients. Functionally, these data suggest that spike-evoked Ca\(^{2+}\) signals in MPCn neurons should be largest during REM sleep when serotonin inputs are expected to be lowest even if equivalent firing rates are reached during waking. Such Ca\(^{2+}\) signals may function to trigger Ca\(^{2+}\)-dependent processes including cfos expression and nitric oxide production in a REM-specific manner.

INTRODUCTION

The change in concentration of cytoplasmic free calcium regulates a remarkable array of cellular processes, including ion channel activity, receptor-effector coupling, enzyme activity, secretion, neurite elongation, differentiation, and gene expression (for review see Ghosh and Greenberg 1995). In neurons, voltage-gated and ligand-gated ion channels can effect large, rapid, and spatially discrete increases in cytoplasmic Ca\(^{2+}\) levels which can be evoked by both subthreshold (Eilers et al. 1995; Markram and Sakmann 1994) and suprathreshold (Jaffe et al. 1992; Tank et al. 1988) membrane depolarization. Mammalian central neurons differ substantially in their morphology and electrophysiological properties (Llinas 1988), and therefore patterns of intracellular Ca\(^{2+}\) signaling are likely to vary across neuronal classes. Little is known about the regulation of [Ca\(^{2+}\)], by mesopontine cholinergic (MPCn) neurons of the laterodorsal tegmental (LDT) nucleus, although it is expected that the dynamics of this process is of particular importance since virtually all of these cells express high levels of the Ca\(^{2+}\)/calmodulin-dependent enzyme neuronal nitric oxide synthase (NOS) (Bredt et al. 1991; Hope et al. 1991) throughout their cytoplasm. NOS catalyzes the synthesis of nitric oxide (NO; for review see Mayer 1995), which is a mobile, membrane-permeable molecule that has been implicated as an intercellular signal in numerous physiological processes including vasodilation, synaptic plasticity, modulation of cell excitability, and neurotoxicity (for review see Garthwaite and Boulton 1995). Since the synthesis of NO by NOS has an absolute requirement for elevated [Ca\(^{2+}\)], the regulation of [Ca\(^{2+}\)], in MPCn neurons is expected to play a critical role in regulating the spatial and temporal patterns of NO signaling by these neurons. Indeed, recent findings indicate that the Ca\(^{2+}\)-dependent production of NO can be stimulated both within the LDT (Mitchell et al. 1995) and at the synaptic targets of MPCn neurons in the thalamus (Williams et al. 1997) and the pontine tegmentum (Leonard and Lydic 1997).

Functionally, MPCn neurons are hypothesized to play a pivotal role in generating the electroencephalographic (EEG)-desynchronized states of waking and REM sleep via their projections to the thalamus and medial pontine reticular formation (for review see Steriade and McCarley 1990). They begin to increase their firing rates seconds prior to the expression of these states and then maintain an elevated...
level of tonic firing for the duration of the states (El Mansari et al. 1989; Kayama et al. 1992; Steriade et al. 1990). We have investigated whether physiological rates of repetitive firing engender Ca\(^{2+}\) transients in the somata and proximal dendrites of MPCh neurons.

It has recently been established that excitatory synaptic input to MPCh neurons utilizes a mixture of \(N\)-methyl-D-aspartate (NMDA) and non-NMDA subtypes of excitatory amino acid receptors (Sanchez and Leonard 1994, 1996). NMDA receptor activation can lead to increases in intracellular \([\text{Ca}^{2+}]\) (MacDermott et al. 1986), can trigger Ca\(^{2+}\)-dependent changes in gene expression (Bading et al. 1995; Xia et al. 1996), and can stimulate NO production in cerebellar granule cells (Garthwaite et al. 1988). We, therefore, investigated the possibility that activation of NMDA receptors could elevate somatic \([\text{Ca}^{2+}]\) in MPCh neurons.

Finally, serotonin (5-HT) is an important transmitter in the control of behavioral state (for review see Steriade and McCarley 1990). Its actions in the CNS are mediated by a large policy on humane care and use of laboratory animals. Female guinea pigs as indicated above except that following induction of deep anesthesia, animals were perfused through the heart and superfused with ACSF at room temperature for record-

### METHODS

#### Slice preparation, solutions, and drugs

Brain slices used for sharp electrode recordings (350–400 \(\mu\)m) were prepared according to previously published methods (Leonard and Llinás 1994) in accordance with the National Institutes of Health policy on humane care and use of laboratory animals. Female guinea pigs (175–300 g) were deeply anesthetized with pentobarbital sodium (50–75 mg/kg, ip) and decapitated. Coronal slices were cut in an ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 5 KCl, 1.2 NaH2PO4, 2.7 CaCl2, 3 MgSO4, 26 NaHCO3, 10 glucose and saturated with 95% O2–5% CO2 using a vibrating microtome (Ted Pella Instruments). After at least 1 h of incubation in ACSF at room temperature, slices were submerged in a recording chamber that was perfused at 3–5 mL/min with ACSF maintained at 22 ± 1°C.

5-HT, TTX (Sigma), and NMDA (Research Biochemicals) were dissolved in ACSF for superfusion or in ultrapure water for focal pressure application. Focal application was accomplished with controlled pressure pulses (Picospritzer; General Valve) applied to a patch pipette (1–2 \(\mu\)m opening) positioned just above the slice.

#### Sharp microelectrode recording and imaging

Sharp borosilicate (cat. No. 6030, AM-systems) micropipettes were pulled horizontally (Sutter Instruments, P87) and filled with the potassium salt of either Calcium Green-1 (2–3 mM), Fura-2 (10–14 mM), or Flu-3 (2–3 mM; Molecular Probes) dissolved in 2 M KCl. In some cases, 1–2% biocytin (Sigma) was included for cell visualization following histochemistry (Leonard and Llinás 1994; Sanchez and Leonard 1994). Micropipettes had DC resistances of 80–120 M\(\Omega\). Cells were injected for 10–30 min with −0.2 to −0.4 nA current pulses (2 Hz, 60% duty cycle). Membrane potential was recorded using an Axoclamp-2A (Axon Instruments) operated in bridge mode. Pulses were delivered and voltages were recorded using PCLAMP software (Axon Instruments). Current, voltage, and shutter timing pulses were recorded on a Neurocorder (Neurodata) having a bandwidth of 0–11 KHz/channel.

Fura-2 was excited by a 100-W Hg source that was reduced two to fourfold by neutral density filters and filtered at 380 ± 5 nm. Flu-3 and Calcium Green-1 were excited at 480 ± 20 nm by the Hg or a 75-W Xe source (reduced 2–4 fold). Illumination was limited to acquisition time by shuttering. Neurons were imaged through a 10× objective (Nikon; 0.3 NA) with an intensified (Gensys 2) charge coupled device camera (Dage 72) and acquired at 1–4 Hz with a frame grabber (Data Cube) controlled by software (Ratiosool; Innovision) running on a workstation (Sun). Each image acquired was an average of four to eight video frames. No significant photobleaching or phototoxicity was detected during recordings lasting more than 1 h under these conditions. The slice chamber and micromanipulators were mounted on a three-plate microscope stage so that the image field could be shifted while maintaining cell impalement. Background images were acquired just prior to the experiment from an adjacent image field lacking filled cell processes for subtraction from data images to compensate for tissue autofluorescence and shading. Fluorescence was measured as the average pixel value over selected somatic and proximal dendritic regions from background-subtracted images. Fluorescence versus time data and electrophysiology data were imported into Igor Pro software (Wavemetrics, Lake Oswego, OR) running on a Macintosh computer for off-line analysis. Data are reported as mean ± SE. Comparisons of means were accomplished with paired t-tests corrected for multiple comparisons, where appropriate, using DataDesk Software (Data Description, Ithaca, NY). Intracellular \([\text{Ca}^{2+}]\) was monitored by computing \(\Delta F/F\) from the measured fluorescence according to

\[
\Delta F/F(t) = \frac{F(t) - F_{\text{Baseline}}}{F_{\text{Baseline}}}
\]

Absolute \([\text{Ca}^{2+}]\), was estimated in some cases from \(\Delta F/F\) (cf. Jaffe et al. 1992) using an estimate of resting \([\text{Ca}^{2+}]\), (60 nM), the \(\Delta F/F\)\(_{\text{max}}\), which was estimated to range from 1.3 to 1.5 for Calcium Green-1, and the \(K_w\), which is 235 nM for Calcium Green-1 at room temperature (Eberhard and Erne 1991).

#### Whole-cell recording and imaging

Patch pipettes (4–8 M\(\Omega\)) were fabricated from Corning 7052 glass and filled with a solution containing (in mM) 144 K-glutamate, 0.1 Calcium...
Green-1, 3 MgCl₂, 10 HEPES, 0.3 NaGTP, 4 Na₄ATP. Neurons were visualized for whole-cell recording at 100× magnification by visible-light differential interference contrast optics, using a nivicon tube camera (Dage VE-1000) mounted on a fixed-stage microscope (Olympus BX50WI). Cells for recording were chosen from within the bounds of the LDT nucleus which was identified using a 4× objective. Gigaseals were obtained under visual control using an Axoclamp 2A amplifier (Axon Instruments) operated in continuous voltage-clamp mode to monitor seal resistance with the output filter set at 3 KHz. After establishing the whole-cell recording configuration, cells were filled with Calcium Green-1 by diffusion from the pipette. Previous experiments indicated that solution exchange occurs with a time constant of about 6 min (Sanchez et al. 1998) so imaging experiments were not begun until at least 20 min after the time of breakthrough. The neuron’s electrophysiological properties were characterized in this initial period using the current-clamp mode of the amplifier with the output filter set to 10 KHz. Current and voltage traces were digitized (20–100 KHz) and command pulses were generated with custom software (TI Workbench; Inoue et al. 1998) run on a Macintosh OS computer which controlled an ITC-18 interface (Instrutech). Neurons were imaged through a 40× objective (Olympus LumaPlan Fluor, 0.8 NA) using a cooled, CCD camera equipped with a back-illuminated, EEV57 frame-transfer chip having an imaging area of 512×512 pixels (MicroMax, Roper Scientific). Calcium Green-1 was excited at 480 ± 20 nm with light from a 75-W Xe lamp that was reduced in intensity with a neutral density filter (12% transmission) and shuttered to restrict tissue illumination to the frame acquisition epochs. TI Workbench software also controlled the camera and shutter, allowing precise synchronization of the image sequence with the electrophysiological data. The camera was read-out with a 1 MHz, 14-bit A/D converter. Images were binned on the chip at either 4×4 or 5×5 pixels and acquired every 35–50 ms. Changes in fluorescence were quantified by the average pixel values within regions of interest (ROIs) that were positioned on background-subtracted fluorescence images as described above. For whole-cell experiments, the background was determined from an ROI which was positioned at a location remote from any fluorescent processes.

**Spike shape analysis**

Action potential shape was measured using Igor Pro Software. Spike amplitude was determined as the difference between the peak and the baseline potentials. Because spikes were evoked by 2-ms-duration current pulses, current artifacts sometimes contaminated the rising phase of the action potentials making it impossible to measure spike width. We, therefore, used the time from the spike peak to the time of the maximum repolarization rate as an estimate of spike width. The maximum repolarization rate was used to characterize the speed of spike repolarization.

**Histochemistry**

Slices containing biocytin-filled cells were processed for NADPH-diaphorase as previously reported (Leonard and Llinàs 1994; Sanchez and Leonard 1994). Injected cells were labeled with avidin-Texas Red and NOS-containing cells were labeled by NADPH-diaphorase histochemistry. Injected neurons were imaged with a confocal microscope (Zeiss LSM 310) using a HeNe laser (543 nm) to visualize Texas-Red fluorescence. The transmitted light from the same laser was used to visualize the NADPH-diaphorase reaction product.
**RESULTS**

Repetitive firing evoked by current injection produced somadendritic Ca\(^{2+}\) transients

All neurons studied with sharp electrodes \((n = 21)\) had stable resting potentials \((-55.1 \pm 2.6\) mV), overshooting action potentials, and displayed the characteristic electrophysiological properties of the principal NOS-containing cells found in guinea pig LDT (Fig. 1, \(A_1-A_3\)) which have been termed type II neurons (Leonard and Llinás 1990, 1994). These cells express a prominent sub-threshold A current (Sanchez et al. 1998), which characteristic produces a delay in the return of the membrane potential to baseline following a hyperpolarizing current pulse (Fig. 1A\(_2\)). These cells also lacked low-threshold Ca\(^{2+}\) spikes. Following injection of the indicator dye, somata were readily visible, and in some cases \((n = 4)\), proximal dendrites were also observed with the intensifier and camera gains used to measure somatic fluorescence (Fig. 1B\(_1\), arrows). Direct current injection sufficient to evoke action potentials also produced changes in cell fluorescence in both the soma and proximal dendrites (middle panel Fig. 1, \(B_1\) and \(B_2\)).

To estimate the time course of the fluorescence changes and the relation between steady-state firing and steady-state Ca\(^{2+}\) fluorescence, we utilized a paradigm consisting of 10-s-duration current pulses separated by 50–70 s. This allowed the firing rate to reach a steady value after spike frequency accommodation was complete and also allowed for a sufficient number of fluorescence samples during cell firing since our imaging rate was limited to between 1 and 4 per second. This is illustrated for another type II LDT neuron in Fig. 2. The change in fluorescence \((\Delta F/F)\) was computed from a small region selected over the soma. A stable baseline measure was established after first having electrophysiologically characterized the cell and injecting it with indicator dye. Membrane hyperpolarization produced no measurable change in fluorescence (Fig. 2A, \(-0.05\) nA; 2B, left panel) suggesting that voltage-dependent Ca\(^{2+}\) currents contribute little to the resting Ca\(^{2+}\) levels in these cells, unlike hippocampal pyramidal neurons (Magee et al. 1996). In contrast, a just-suprathreshold depolarization, which produced a firing rate of 0.5 spikes/s (averaged over 10 s), evoked an increase in fluorescence that appeared correlated with individual action potentials (Fig. 2A, \(0.02\) nA; 2B, middle panel). Increasing the strength of the current pulses evoked more spikes and larger increases in \(\Delta F/F\) (Fig. 2A and 2B, right). At spike frequencies higher than the imaging rate, \(\Delta F/F\) appeared to increase and decrease with a smooth time course that was well fit by an exponential function. The decay time constant was measured for different amplitude transients from six cells. Since the time constant did not strongly depend on the amplitude of the transient, the data were pooled. Decay time constants ranged from 2.5 to 9.2 s with an average value of \(5.0 \pm 0.24\) s \((n = 77\) from 6 cells). It was also apparent from these data that in addition to the rapid rise and fall of \(\Delta F/F\) which accompanied current injection, there was a slow increase in \(\Delta F/F\) that built up over the entire sequence of current injections (Fig. 2A). Following termination of the last current pulse, this accumulated signal decayed very slowly. To estimate the time course, the \(\Delta F/F\) signal following the final current pulse was fit with a double exponential function. The average slow time constant was \(134.4 \pm 19.5\) s \((n = 5\) from 5 cells), which was significantly longer than the average fast time constant \((P < 0.0001)\).
Current-evoked Ca\textsuperscript{2+} transients were TTX sensitive

To determine if the Ca\textsuperscript{2+} influx observed during DC injection was related to the genesis of action potentials, we examined the effect of TTX on the evoked changes in fluorescence. Superfusion of the slice with Ringer containing TTX (1 μM) completely abolished action potentials (n = 3) and strongly reduced the current-induced increase in fluorescence (Fig. 3, A and B). Moreover, the relation between the magnitude of the evoked ΔF/F and the strength of the injected current was attenuated by spike blockade over the entire range of currents tested (Fig. 3D). Application of the potassium channel blocker tetraethylammonium (TEA, 20 mM) resulted in the production of Ca\textsuperscript{2+} spikes following DC injection (Fig. 3C). The occurrence of Ca\textsuperscript{2+} spikes restored the magnitude of the current-induced ΔF/F to the levels achieved prior to Na\textsuperscript{+} channel blockade. Indeed, as few as two Ca\textsuperscript{2+} spikes evoked nearly maximal increases in fluorescence (Fig. 3D). These data indicate that the majority of Ca\textsuperscript{2+} signal resulting from the current-evoked increases in ΔF/F resulted from activation of high-voltage activated Ca\textsuperscript{2+} channels.

ΔF/F is related to firing rate

The steady-state relation between the ΔF/F and the firing rate was estimated by comparing the change in ΔF/F to the total number of spikes elicited during each current pulse. The number of spikes evoked during the 10-s current injection was linearly related to current strength over the range of currents studied and is summarized for six type II cells in Fig. 4A. Comparing the peak ΔF/F to the total number of spikes revealed that this relation was well approximated by a straight line for average firing rates below about 2 spikes/s and then began to saturate at higher firing rates (Fig. 4B). A linear fit of the data below 2 spikes/s indicated that cells ranged in sensitivity between 7.5 and 19.2% ΔF/F/spike/s with a mean of 15.6 ± 1.8% ΔF/F/spike/s (n = 6). These data indicate that action potentials were very effective at triggering somatic [Ca\textsuperscript{2+}] transients. Indeed, even low firing rates averaging 1.3 ± 0.27 spikes/s resulted in average increases in ΔF/F of 25.4 ± 0.46% (n = 6).

NMDA receptor activation produced somatic Ca\textsuperscript{2+} transients that were TTX sensitive

Application of NMDA also produced membrane depolarization and triggered increases in somatic [Ca\textsuperscript{2+}]. Both superfusion and local pressure-ejection caused robust increases in somatic ΔF/F (n = 10) which appeared to depend on the generation of action potentials. For example, NMDA produced dose-dependent increases in ΔF/F (Fig. 5, A1–6); however, subthreshold doses produced only marginal increases (Fig. 5, A7–10).
**CA**

**A1** and **A2** and suprathreshold doses produced increases that lagged the depolarization onset, but closely followed spiking onset (See Fig. 5A3). The NMDA-evoked Ca²⁺ transients were mimicked by DC injection (Fig. 3, **B6** and **B7**), suggesting that action potentials were primarily responsible for triggering these Ca²⁺ transients. This was confirmed by comparing the effects of identical NMDA applications before and after TTX blockade of action potentials which resulted in a 65.4 ± 8.6% reduction in the peak ΔF/ΔF₀ (n = 4; compare Fig. 5A6 and B1). Nevertheless, the residual increase in ΔF/ΔF₀ evoked by NMDA following TTX was not completely mimicked by direct depolarization (Fig. 5, **B1** and **B2**), suggesting that a small portion of the increase might be mediated by Ca²⁺ influx through NMDA receptor channels.

**5-HT inhibited current- and NMDA-evoked calcium transients**

Neither superfusion (100 μM; n = 2) nor local pressure-ejection of 5-HT (n = 5) had any detectable effects on baseline ΔF/ΔF₀, although both produced strong membrane hyperpolarization as previously reported (Leonard and Llinás 1994; Luebke et al. 1992). In contrast, even brief 5-HT application effectively and reversibly reduced Ca²⁺ transients evoked by suprathreshold constant-current stimulation (Fig. 6A), apparently by suppressing the number of evoked action potentials. This conclusion was supported by experiments in which the effect of halting steady firing with either 5-HT or DC current was compared. Under these conditions, 5-HT was no more effective at decreasing ΔF/ΔF₀ than was DC (Fig. 6B).

5-HT also effectively reduced somatic Ca²⁺ transients evoked by NMDA (n = 3). By preceding NMDA application with a pulse of 5-HT, the magnitude and time course of the NMDA-evoked ΔF/ΔF₀ was dramatically reduced (Fig. 7A). As with DC, however, the suppressive effect of 5-HT appeared related to membrane hyperpolarization and the reduction in the number of action potentials. Results from experiments in which DC was used to mimic the effect of 5-HT supported this conclusion (Fig. 7B). In this case, two different doses of NMDA were applied at two membrane potentials. At a membrane potential of −70 mV, the NMDA-evoked depolarizations were ineffective at generating somatic Ca²⁺ transients since the smaller dose was subthreshold and the larger dose produced only a single spike (Fig. 7, **B1** and **B2**). However, the same pair of doses resulted in dramatically larger Ca²⁺ transients when delivered from resting potential (−60 mV) since both responses were suprathreshold (Fig. 7, **B3** and **B4**). This effect of steady hyperpolarization was mimicked by 5-HT. When the same pair of NMDA doses was preceded by 5-HT application, which hyperpolarized the membrane by about 5 mV, the Ca²⁺ transients were suppressed. The smaller NMDA dose evoked only a subthreshold depolarization and no Ca²⁺ signal, while the larger dose evoked an intermediate number of spikes and an intermediate Ca²⁺ signal (Fig. 7B5).
5-HT inhibited action potential-evoked calcium transients

The previous data demonstrate that 5-HT reduces activity-dependent calcium signals in LDT neurons by reducing the number of action potentials fired in response to a given stimulus. To determine if 5-HT also reduced the Ca\textsuperscript{2+} transients produced by individual action potentials, we conducted whole-cell recording experiments with simultaneous high-speed imaging in which action potentials were evoked by a train of five brief current pulses (2 ms). Pulses were delivered at 1–10 Hz following a 100-ms hyperpolarizing current pulse which was used to estimate input resistance. Sixteen type II neurons were patched under DIC optics and filled with 100 \mu M Ca-Green-1 dissolved in patch solution (Fig. 8A). Sufficient data concerning spike-evoked changes in \(\Delta F/F\) and spike shape were obtained for six cells from six slices each of which underwent one to three 5-HT applications (total of 13). Since the results from each application were qualitatively similar, the data were pooled. These neurons all had resting potentials more negative than \(-50\) mV and large overshooting action potentials (mean amplitude: \(91.11 \pm 2.0\) mV). As suggested from the results

FIG. 6. Serotonin-inhibited somatic Ca\textsuperscript{2+} transients evoked by suprathreshold membrane depolarization. A: repetitive action potentials resulting from a 10-s duration, 0.2-nA current injection evoked increases in somatic \(\Delta F/F\) (symbols; left). Focal application of 5-HT (200-ms pulse) coincident with the onset of DC current strongly hyperpolarized the membrane which halted the firing and reduced the amplitude of the evoked Ca\textsuperscript{2+} transient (middle). This effect recovered as the membrane conductance activated by 5-HT subsided (right). B: elevated \(\Delta F/F\) produced by the injection of steady depolarizing current (+dc 0.24 nA) and resultant firing was halted, in sequence, by brief, identical, focal applications of 5-HT (5-HT, 5-HT 1). The decrease (dashed lines) in \(\Delta F/F\) produced by 5-HT was no different from the decrease in \(\Delta F/F\) produced by termination of the DC current (DC off). B1: membrane potential change corresponding to the 5-HT 1 application. B2: membrane potential change produced by termination of DC current (DC off in B).
obtained with sharp electrodes, individual action potentials produced significant increases in $\Delta F/F$ measured from the soma and proximal dendrites (Fig. 8B). Each spike produced a transient increase in $\Delta F/F$ which rose more rapidly than it decayed, such that $\Delta F/F$ summated over the time course of the five stimuli. Spike-evoked fluorescence transients were routinely observed in the proximal dendrites and were completely absent when spike-failure occurred (data not shown). These dendritic transients were typically larger and faster decaying than those measured at the soma. Since the data were qualitatively similar for each of the one or two dendritic branches recorded per cell and the recording distances from the soma were similar (24.8 ± 2.1 μm; n = 11), dendritic data were averaged for each cell.

As expected, application of 5-HT produced a reversible membrane hyperpolarization (7.17 ± 1.00 mV) and large decrease in apparent input resistance (51.98 ± 7.12%; n = 13). These membrane changes typically resulted in spike failure and required increased current strength to overcome. Suprathreshold current pulses delivered during 5-HT application revealed that the spike-evoked increases in $\Delta F/F$ were smaller than in control conditions (compare Fig. 8B to 8C). Moreover, this reduction occurred throughout the soma and proximal dendrites as can be seen by comparing images obtained at the peak of the spike-evoked fluorescent transients (dotted line in Fig. 8B) before and during 5-HT application (compare Fig. 8B to 8C). To measure the time course of these effects, we tracked the peak $\Delta F/F$ evoked by five spikes along with the membrane potential and input resistance before, during, and after 5-HT application. We also measured the maximum spike-evoked $\Delta F/F$ elicited from a hyperpolarized membrane potential (−dc; −10.91 ± 1.14 mV) in the absence of 5-HT and from the control potential during 5-HT application (+dc) to examine the effect of membrane potential on the spike-evoked Ca$^{2+}$ transients. Results indicated that the 5-HT effects were reversible and that the inhibition of spike-evoked $\Delta F/F$ evolved with a time course similar to that for $V_m$ and input resistance following 5-HT application (Fig. 8D). The spike-evoked fluorescence transients for each condition are superimposed in Fig. 8E for a somatic and dendritic region (lowercase

FIG. 7. Serotonin-inhibited NMDA evoked somatic Ca$^{2+}$ transients. A: NMDA evoked reliable increases in somatic $\Delta F/F$. Preceding NMDA application with two brief pressure applications of 5-HT had no effect on baseline $\Delta F/F$ but reversibly attenuated NMDA evoked $\Delta F/F$ increases. B: inhibitory effect of 5-HT was mimicked by direct membrane hyperpolarization (baseline potential indicated above $\Delta F/F$ trace). Continuous record of $\Delta F/F$ indicates the relative timing of events (top). Membrane potential changes associated with drug applications 1–5 are shown below (B1–B5). From a baseline potential of −70 mV, a 200-ms-duration pulse of NMDA (B1, NMDA) produced a subthreshold 10 mV depolarization and no measurable change in $\Delta F/F$. A 400-ms pulse (B2, NMDA × 2) produced a larger depolarization and a single spike but only a negligible increase in $\Delta F/F$. From resting potential (−60 mV), identical NMDA pulses (B3 and B4) evoked 3 and 9 spikes, respectively, and correlated increases in $\Delta F/F$. Following a brief 5-HT application (B5), identical NMDA pulses evoked similar membrane depolarizations but resulted in fewer action potentials and accordingly attenuated changes in $\Delta F/F$. Time and voltage calibration in B5 also applies to B1–B4.
letters indicate the corresponding condition in Fig. 8D). These data illustrate the reduction in the spike-evoked Ca\(^{2+}\) transients produced by 5-HT and indicate that this reduction was greater than any change produced by altering baseline potential. Moreover, 5-HT inhibition of the spike-evoked ΔF/ΔF\(_0\) was not associated with gross changes in spike amplitude or shape (Fig. 8E, bottom). Similar changes were observed for each of the other cells studied. The group data show that while there was no statistical difference between spike-evoked changes in ΔF/ΔF measured at baseline and hyperpolarized membrane potentials, 5-HT reduced the spike-evoked ΔF/ΔF to 57 ± 1.4 and 68.7 ± 5.3% of the control values measured at the soma and dendrites, respectively (Fig. 9A). Moreover, even when the membrane was depolarized back to baseline during the 5-HT application, the spike-evoked changes in ΔF/ΔF were significantly smaller than control. The ΔF/ΔF data and statistical differences for these groups are summarized in Table 1.

While the action potentials in the control and 5-HT conditions appeared grossly similar, we examined them in more detail to determine if there were any subtle changes in spike shape that might correspond to the changes in ΔF/ΔF produced by 5-HT. We first examined the possibility that 5-HT produced smaller amplitude action potentials. Membrane hyperpolarization alone produced a small but significant reduction (3.4 mV) in the peak value of the action potential (33.8 ± 1.7 vs. 30.5 ± 2.0 mV; \(n = 20; P < 0.01\)). However, comparing action
FIG. 9. Summary of serotonin actions on spike-evoked Ca$^{2+}$ transients and action potential shape. A: mean (±SE) of spike-evoked Ca$^{2+}$ transients measured in the soma (dark bars) and dendrites (light bars) under different conditions are normalized to the control (control base) condition ($V_m = -57.1 ± 1.2$ mV; $n = 13$). In the control – dc condition, spikes were evoked from a hyperpolarized membrane potential ($V_m = -68.0 ± 1.2$ mV; $n = 8$). In the 5-HT condition, spikes were evoked near the peak of the 5-HT-mediated membrane hyperpolarization ($V_m = -64.3 ± 0.7$ mV; $n = 13$). In the 5-HT + dc condition, the membrane was depolarized back to the baseline potential with DC injection ($V_m = -55.1 ± 1.7$ mV; $n = 7$). In the recovery condition, spikes were evoked after the 5-HT-mediated membrane hyperpolarization had recovered ($V_m = -56.6 ± 0.9$ mV; $n = 11$). See text and Table 1 for details. B: the effect of 5-HT on action potential shape is illustrated for the action potentials shown in Fig. 8D. The peaks and repolarization phases of the first spikes in the trains ($V$) evoked in control – dc (solid line) and 5-HT (dotted line) conditions are illustrated along with their first derivatives ($dV/dt$). Arrows indicate the time from action potential peak to the time of the maximum repolarization rate for each spike. 5-HT shortened this duration and increased the maximum repolarization rate. Solid horizontal line corresponds to $dV/dt = 0$. C: comparison of the mean (±SE) of these 2 spike shape parameters ($dV/dt$) across all conditions normalized to the control baseline condition. Spikes depolarized faster and were shorter in duration in the 5-HT condition compared with the control – dc condition (see text for statistical details). Data from the 5-HT + dc condition was omitted because the action potentials were distorted by the injected current pulse in this condition.

This finding indicates that serotonergic inhibition of the
spike-evoked Ca$^{2+}$ transients could not result from inadequate depolarization of the soma during the action potential.

Since action potentials of MPCh neurons become narrower following Ca$^{2+}$-channel blockade with extracellular Co$^{2+}$ or

FIG. 8. Serotonin inhibits somatic and proximal dendritic Ca$^{2+}$ transients evoked by individual action potentials. A: fluorescence image of a type II LDT neuron filled with Ca-Green 1 (100 μM) by patch electrode (right) is shown with a somatic (1: red) and two dendritic regions of interest (ROIs; 2: yellow; 3: green). $B_1$ and $C_1$: $dF/dF$ values for the corresponding ROIs in A are displayed with membrane potential and injected current (bottom black traces; $B_1$ and $C_1$) as a function of time. The spike-evoked changes in $dF/dF$ ($B_1$, control) were larger than they were in the presence of 5-HT ($C_1$). $B_2$ and $C_2$: pseudocolor $dF/dF$ images of the soma and dendritic region of the neuron shown in A (from region bounded by dotted line) indicating the maximum change in $dF/dF$ following 5 action potentials (time of acquisition is indicated by the dotted vertical line in $B_1$ and $C_1$). The scale to the right gives the relation between color and %$dF/dF$. In the control condition ($C_1$), $dF/dF$ was larger throughout the soma and proximal dendrites than it was in the presence of 5-HT ($C_2$). D: time course of the experiment indicating maximum $dF/dF$ evoked by 5 spikes from ROIs 1–3, membrane potential ($V_m$), injected DC current ($I$), and input resistance ($R_{in}$). At the initial time points ($a$), the membrane was hyperpolarized to near −70 mV with DC current. $V_m$ was returned to −60 mV (b) just before 5-HT application. 5-HT application (30 μM for 2 min beginning at dashed line) hyperpolarized the membrane, decreased input resistance, and reduced spike-evoked $dF/dF$ ($c$, $e$). $dF/dF$ was inhibited even during DC depolarization to −60 mV during the 5-HT application ($d$). Recovery from the inhibition of spike-evoked $dF/dF$ was observed at both −60 mV ($f$) and −70 mV ($e$). E: superimposed traces of spike-evoked $dF/dF$ for the control conditions corresponding the points labeled $a$, $b$, $f$, and $g$ in D (red) and for the 5-HT conditions corresponding to $c$, $d$, and $e$ in D (blue). Somatic traces are on the left and dendritic traces from ROI 2 are on the right. $dF/dF$ was inhibited by application of 5-HT at both membrane potentials tested. Below is shown the first action potentials of each train from conditions $a$ and $b$ (lower left) and from conditions $b$ and $c$ (lower right). Action potentials were generally similar in amplitude and shape following either membrane hyperpolarization or 5-HT application.
play the “type II” electrophysiological properties were NOS-containing since they robustly stained for NADPH-diaphorase (Leonard and Llinàs 1994; Sanchez and Leonard 1994). All of the cells investigated in this study had type II physiological characteristics and therefore were likely to have been NOS-containing. To verify that the studied cells were, in fact, NOS-containing, some neurons were co-injected with biocytin ($n = 5$) and processed by NADPH-diaphorase histochemistry. Cells were injected with biocytin so that they could be visualized after processing using avidin-Texas Red, which is resistant to quenching by the histochemistry. As expected from their location and their physiological properties, all three of the biocytin-injected cells that were recovered were also NADPH-diaphorase containing (Fig. 10, A–C). These data confirm that the described pattern of Ca$^{2+}$ transient regulation was typical of NOS-containing MPCh neurons.

**DISCUSSION**

A principal finding of this study is that TTX-sensitive action potentials are of prime importance in mediating [Ca$^{2+}$] transients evoked by DC injection in NOS-containing LDT neurons. Results from other central neurons indicate that action potentials are variable in their ability to evoke somatic [Ca$^{2+}$] transients. High rates of “simple” spiking by cerebellar Purkinje cells result in relatively small increases in somatic and dendritic [Ca$^{2+}$] unless a Ca$^{2+}$ spike is triggered (Lev-Ram et al. 1992; Midtgaard et al. 1993; Tank et al. 1988). In hippocampal pyramidal cells (Jaffe et al. 1992), action potentials are somewhat more effective at elevating somatic [Ca$^{2+}$]. Our estimates indicate that even moderate firing rates of ~4 spikes/s in MPCh neurons result in increases of ΔF/ΔF which averaged 48.4 ± 3.8% ($n = 6$). Based on the assumptions described in the Methods, this corresponds to a concentration of ~200–250 nM and suggests that even moderate firing rates produce substantial increases in somatic [Ca$^{2+}$] in MPCh neurons. Moreover, two factors suggest this is an underestimate of the peak concentration changes achieved during repetitive firing. First, our maximal imaging rate in the sharp electrode experiments was low, which resulted in undersampling of the transients associated with each action potential. Second, the cytoplasmic concentration of indicator dye was probably high.

**TABLE 1. Comparison of average ΔF/ΔF values across conditions**

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Somatic Mean ± SEM (%ΔF/ΔF)</th>
<th>Dendritic Mean ± SEM (%ΔF/ΔF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control baseline</td>
<td>13</td>
<td>6.77 ± 0.83</td>
<td>16.40 ± 2.37</td>
</tr>
<tr>
<td>Control – dc</td>
<td>8</td>
<td>7.27 ± 0.76</td>
<td>17.77 ± 2.71</td>
</tr>
<tr>
<td>5-HT</td>
<td>13</td>
<td>3.88 ± 0.40</td>
<td>11.27 ± 1.48</td>
</tr>
<tr>
<td>5-HT + dc</td>
<td>7</td>
<td>4.90 ± 0.75</td>
<td>13.93 ± 2.97</td>
</tr>
<tr>
<td>Recovery</td>
<td>11</td>
<td>6.38 ± 0.77</td>
<td>15.85 ± 2.45</td>
</tr>
</tbody>
</table>

Significant differences between conditions are indicated by brackets and apply to both somatic and dendritic measures. Comparisons were made with a paired t-test corrected for multiple comparisons. Total alpha was 0.05 and individual alphas were 0.005. $n$, number of observations.

Cd$^{2+}$ (Leonard and Llinàs 1990), we examined two parameters of spike repolarization: the maximum repolarization rate and the time from the action potential peak to the time of the maximum repolarization rate. Membrane hyperpolarization alone did not produce a statistically meaningful increase in the maximum spike repolarization rate ($-49.2 ± 1.8$ mV/ms; $n = 40$ vs. $-52.0 ± 2.4$ mV/ms; $n = 35$; $P > 0.08$); however, it did significantly shorten the time from the peak to maximum repolarization rate from 728.6 ± 31.6 μs ($n = 40$) to 674.8 ± 42.6 μs ($n = 35$; $P < 0.004$). Application of 5-HT increased the maximum spike repolarization rate to $-55.4 ± 1.8$ mV/ms ($n = 35$) which was significantly faster than observed for spikes produced during membrane hyperpolarization alone ($P < 0.0001$). Moreover, 5-HT reduced the time from the peak to the maximum repolarization rate to 601.0 ± 22.0 μs ($n = 55$), which was significantly lesser than that observed for spikes during membrane hyperpolarization alone ($P < 0.0001$). An example of these effects on spike shape is illustrated in Fig. 9B and is summarized for the entire sample in Fig. 9C. Thus 5-HT resulted in a speeding of spike repolarization and a shortening of the action potential to a degree larger than expected for membrane hyperpolarization alone. These parameters all recovered upon washout of 5-HT.

**Recorded cells were NADPH-diaphorase positive**

Previous studies combining intracellular labeling and histochemistry found that >90% of guinea pig LDT neurons displaying “type II” electrophysiological properties were NOS-containing mesopontine cholinergic (MPCh) neurons. Five cells studied with Ca-Green 1 were co-injected with biocytin and Ca-Green. Following fixation and resectioning, these cells were labeled with avidin-Texas Red and the tissue was processed for NADPH-diaphorase histochemistry. Confocal microscopic images of the 3 recovered Texas-Red labeled cells are shown in A–C (top). Bright-field images of the same areas show that the Texas-Red labeled cells were also NADPH-diaphorase-positive (A–C, bottom). Large arrows, somata; small arrows, dendrites. Data shown in Fig. 7B was obtained from the neuron labeled in A. Scale bar in A and C corresponds to 25 μm. Scale in C applies to both B and C.
enough to significantly buffer entering calcium, thereby diminishing the peaks and slowing the changes in $[Ca^{2+}]_i$. Evidence for buffering by the indicator was suggested by the transient decay time constants, which were long compared with those observed with minimal dye concentrations in other neurons (Helmcchen et al. 1996). This was true even in our whole-cell recording where the indicator concentration was 100 $\mu$M. However, several other factors including the rate of pumping from the cytoplasm and the capacity of the endogenous calcium buffers may also contribute to this longer decay and will need to be determined for these cells. Thus these data support the idea that a relatively large somatic $Ca^{2+}$ influx occurs with each action potential. To our knowledge, the only other brain NOS-expressing central neurons for which spiking has been related to $[Ca^{2+}]_i$ are immature cerebellar granule cells (Connor et al. 1987), which differ from MPCCh neurons morphologically and electrophysiologically. Nevertheless, following strong depolarization produced by raising $[K^+]_o$ to 25 mM, TTX blocked about 50% of the early $[Ca^{2+}]_i$ increase. Thus substantial spike-mediated elevation of somatic $[Ca^{2+}]_i$ may be a general feature of NOS-containing neurons.

In cases where we were able to image the dendrites along with the cell body, we observed action potential-dependent changes in fluorescence. These data indicate the presence of voltage-gated $Ca^{2+}$ channels in the proximal dendrites. It is likely that action potentials generated in the somata propagate into the dendrites to open these channels as described in some other cell types (for review see Johnston et al. 1996), but further work will be necessary to verify where action potentials are initiated in these neurons.

Another finding of this study was that NMDA-receptor activation produced large and rapid somatic $Ca^{2+}$ transients which strongly depended on action potentials. In the presence of action potential blockade by TTX, only small and slow somatic $[Ca^{2+}]_i$ transients were observed even though application of NMDA produced substantial somatic membrane depolarizations. This observation suggests that the major route by which the somatic $[Ca^{2+}]_i$ becomes elevated following NMDA receptor activation is through activation of high-threshold voltage-gated $Ca^{2+}$ channels by action potentials. The $Ca^{2+}$ influx through the NMDA receptors expressed by these cells (Sanchez and Leonard 1994, 1996) was probably not detectable because it was highly localized to the synapses on the soma (Honda and Semba 1995) or in the dendrites, as suggested for pyramidal neurons (Miyakawa et al. 1992), where $Ca^{2+}$ influx through NMDA receptors appears localized to spines (Yuste et al. 1999). $Ca^{2+}$ influx through ion channels can produce very localized concentration changes (Simon and Llinas 1985) and this influx could have important consequences for the spatial control of NO signaling. This topic warrants further investigation.

Another principal finding of this study was that 5-HT inhibited somatic and dendritic $[Ca^{2+}]_i$, transients produced by depolarizing stimuli both by inhibiting action potential production and by inhibiting the increase in $[Ca^{2+}]_i$ produced by individual action potentials. Serotonin has multiple actions on central neurons including the mobilization of $[Ca^{2+}]_i$, which, among other things, can activate $Ca^{2+}$-dependent $K^+$ currents (Uneyama et al. 1993). Our data, however, indicate that 5-HT produced a membrane hyperpolarization without any measurable change in $\Delta F/F$. This finding supports our earlier conclusion that the 5-HT activated $K^+$ current in MPCCh neurons is not $Ca^{2+}$ dependent (Leonard and Llinas 1994). Thus if functional 5-HT$_3$ receptors are present on the somata of MPCCh neurons (Morlik and Ciaranello 1993), they do not directly trigger changes in resting $[Ca^{2+}]_i$. Our findings also indicate that 5-HT inhibits NMDA- and current-evoked increases in somatic $[Ca^{2+}]_i$, by inhibiting action-potential generation. While this is an indirect action on $[Ca^{2+}]_i$, it demonstrates that 5-HT-mediated signals can be cross-coupled to $Ca^{2+}$-dependent processes via the modulation of firing.

Finally, our data demonstrate that 5-HT inhibits about 40% of the action potential-triggered $Ca^{2+}$ transient in the soma and proximal dendrites of type II LDT neurons. This inhibition did not result from membrane hyperpolarization or a reduction in amplitude of the somatic action potential. Rather, the inhibition correlated with a speeding of action potential repolarization and shortening of action potential duration. These action potential changes produced by 5-HT were small but qualitatively similar to the changes in spike shape observed following $Ca^{2+}$-channel blockade with solutions containing low extracellular $Ca^{2+}$ and $Cd^{2+}$ or $Co^{2+}$ (Leonard and Llinas 1990). Considering that 5-HT has been shown to inhibit high-voltage-activated $Ca^{2+}$ channels in several types of neurons (Bayliss et al. 1995, 1997; Foehring 1996; Penington and Kelly 1990), it is likely that the 5-HT effects on action potential shape and spike-evoked $Ca^{2+}$ transients result from inhibition of voltage-gated $Ca^{2+}$ channels. This interpretation is bolstered by previous reports showing that high-threshold $Ca^{2+}$ spikes in type II MPCCh neurons are generated, in part, by $N$- rather than $L$-type $Ca^{2+}$ channels (Takakusaki and Kitai 1997) and that $N$- and $P/Q$-type $Ca^{2+}$ channels are inhibited by 5-HT (Bayliss et al. 1997; Penington et al. 1991), while $L$- (Foehring 1996) and $T$-type (Bayliss et al. 1995) $Ca^{2+}$ channels are not. Nevertheless, other possibilities may also account for or contribute to this inhibition and further experiments will be necessary to fully resolve the underlying mechanisms.

An inhibition of spike-evoked $Ca^{2+}$ transients by 5-HT has recently been reported for hippocampal CA1 pyramidal neurons (Sandler and Ross 1999). In that study, 5-HT hyperpolarized the soma and dendrites but did not reduce the peaks of somatic action potentials (although the peaks of dendritic action potentials were reduced). Since somatic spikes were unchanged, and spike-evoked $Ca^{2+}$-transients were inhibited by $\sim 30\%$ throughout the cell, the authors suggested that the inhibition of spike-evoked $Ca^{2+}$ transients resulted from inhibition of voltage-gated $Ca^{2+}$ channels. In general, our findings agree well with that study. One difference was that in MPCCh neurons, 5-HT produced detectable changes in the shape of somatic action potentials, while in CA1 neurons, the somatic action potentials were reported to be unchanged by 5-HT. One possible explanation for this difference is that different channel types or different relative channel densities contribute to spike repolarization in these two cell types. Indeed, such differences are evident from the effect of blocking $Ca^{2+}$ currents with $Cd^{2+}$ or $Co^{2+}$ on the spike. In type II MPCCh neurons, the action potential becomes narrower (Leonard and Llinás 1990), while in CA1 pyramidal neurons, the action potential broadens (Lancaster and Nicoll 1987; Storm 1987), apparently due to the importance of BK-type $Ca^{2+}$-activated $K^+$ channels in spike repolarization (Shao et al. 1999). Preliminary evidence from mouse MPCCh neurons suggests that BK channels contribute...
little to spike repolarization since Iberiotoxin, which selectively blocks BK-type channels, has negligible effect on action potential repolarization (Tyler et al. 1999). Thus it is possible that 5-HT produces a negligible effect on spike shape in CA1 pyramidal neurons due to a reduction in both the Ca\(^{2+}\) current and the Ca\(^{2+}\)-activated K\(^+\) current which would cancel. Nevertheless, the collective evidence discussed above indicates that a common action of 5-HT is to inhibit high-threshold Ca\(^{2+}\) currents which suppresses spike-evoked increases in [Ca\(^{2+}\)].

Functional implications

Extracellular recordings of neural activity in regions containing mesopontine cholinergic neurons have identified neurons whose firing rates vary with behavioral state in cat and rat (El Mansari et al. 1989; Kayama et al. 1992; Steriade et al. 1990). One population of these neurons has higher firing rates during waking and REM sleep than during slow-wave sleep and another population fires selectively during REM sleep. Evidence that the neurons that display these two firing patterns are cholinergic is mostly indirect; however, a recent study using juxtaneuronal applications of biocytin has provided strong support for this idea in rat (Koyama et al. 1998). These “presumed” cholinergic neurons fire slowly, have broad action potentials (Kayama et al. 1992), and are inhibited by cholinergic agonists in rat (Koyama and Kayama 1993) and cat (Sakai and Koyama 1996). In these ways, they resemble the properties of mature identified mesopontine cholinergic neurons in vitro (Leonard and Llinás 1994). Our finding that somatic [Ca\(^{2+}\)]

\[c_{\text{Ca}^{2+}}\]

is related to the steady firing rate indicates that state-dependent firing rates of mesopontine cholinergic neurons should lead to state-dependent fluctuations of [Ca\(^{2+}\)]. Moreover, our finding that serotonin inhibits spike-evoked somadendritic Ca\(^{2+}\)-transients in these neurons suggests that [Ca\(^{2+}\)]

\[c_{\text{Ca}^{2+}}\]

would become most elevated by firing during REM sleep, when the serotoninergic input to MPCh neurons is expected to be lowest (for review see Portas et al. 2000). Such state-dependent increases in [Ca\(^{2+}\)]

\[c_{\text{Ca}^{2+}}\]

could then function to control Ca\(^{2+}\)-dependent cellular processes such as gene expression and NO synthesis in a state-dependent manner.

Evidence that gene expression varies with behavioral-state in brainstem regions containing mesopontine cholinergic neurons has come from studies examining immunostaining for the product of the immediate early gene cfos, which has been used extensively as a marker of activity in the CNS (Hunt et al. 1987; Morgan et al. 1987; Sagar et al. 1988). Increased numbers of cfos-labeled cells in these regions have been found following periods of carbachol-induced REM sleep in cats (Shirromani et al. 1992; Yamuy et al. 1993) and periods of natural REM rebound in rats (Maloney et al. 1999). While only a fraction of these cfos-labeled cells were cholinergic, the number of cholinergic neurons stained for cfos was also increased, and it was increased in proportion to the percentage of time spent in REM sleep following periods of REM deprivation (Maloney et al. 1999). Since cfos transcription can be stimulated through Ca\(^{2+}\)-dependent signals (Morgan and Curran 1986) arising from activation of voltage-dependent Ca\(^{2+}\) channels in neurons (Bading et al. 1993), our observation that somatic [Ca\(^{2+}\)]

\[c_{\text{Ca}^{2+}}\]

varies as a function of steady firing, supports the hypothesis that state-dependent changes in action potential frequency regulates the expression of cfos, and presumably other genes, in mesopontine cholinergic neurons. This observation also supports the interpretation that increased cfos immunoreactivity reflects changes in action potential frequency in this system. This point was further supported by our observation that both NMDA and 5-HT receptor activation, which might have directly altered intracellular [Ca\(^{2+}\)], had their primary influence on somatic [Ca\(^{2+}\)], by altering firing rate and/or spike-evoked Ca\(^{2+}\) transients.

Functionally, our findings also support the hypothesis that NO production can be triggered by a spike-related increase in somatic [Ca\(^{2+}\)], in MPCh neurons. Although the Ca\(^{2+}\)-dependence of NOS has not been measured in situ, the purified enzyme is inactive at [Ca\(^{2+}\)] <80 nM and fully active at concentrations >400–500 nM (Schmidt et al. 1992). We found that even moderate rates of firing (~4 spikes/s) are estimated to produced increases in average [Ca\(^{2+}\)], sufficient to activate the enzyme. During waking or REM sleep, putative MPCh neurons in rats are reported to fire at rates of ~10 spikes/s (Kayama et al. 1992), which we estimate could produce Ca\(^{2+}\) transients >1 μM. Thus the somatic Ca\(^{2+}\) levels achieved by physiological rates of firing appear sufficient to stimulate somatic NOS activity. This is supported by results from our experiments in which NO was electrochemically detected within the LDT in response to local electrical stimulation in brain slices (Mitchell et al. 1995).

Conclusion

Our findings support the hypothesis that the increased repetitive firing of MPCh neurons associated with the behavioral states of waking and REM sleep evoke significant rises in soma-dendritic [Ca\(^{2+}\)]. Moreover, our data suggest that synaptic activation of NMDA receptors would mainly influence somatic [Ca\(^{2+}\)], by generating action potentials. In addition, we found that 5-HT reduces activity-dependent increases in [Ca\(^{2+}\)], both indirectly, by inhibiting action potential generation, and directly, by inhibiting spike-evoked Ca\(^{2+}\) transients, most likely by inhibiting voltage-gated Ca\(^{2+}\) channels. We therefore predict that the greatest increases in [Ca\(^{2+}\)] occur during REM sleep, when MPCh neurons fire at high rates and when the serotoninergic input to LDT neurons is expected to be lowest.

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