Development and Serotonergic Modulation of NMDA Bursting in Rat Trigeminal Motoneurons

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INTRODUCTION

Excitatory amino acid neurotransmission, and in particular, N-methyl-D-aspartate (NMDA) receptor activation, are critical for the production and maintenance of oral-motor activity and control of trigeminal motoneuronal output both in vivo (Chandler 1989; Katakura and Chandler 1990) and in vitro (Appenteng et al. 1995; Kim and Chandler 1995; Kogo et al. 1996; Trueblood et al. 1996). For instance, direct application of NMDA antagonists to guinea pig trigeminal motoneurons during cortically induced rhythmical jaw movements suppresses rhythmical motoneuronal discharge (Katakura and Chandler 1990). Furthermore, using the isolated neonatal brain stem preparation, which preserves the circuitry for rhythmical oral-motor activity, bath application of NMDA receptor antagonists blocks the ability to induce rhythmical oral-motor activity in response to bath application of the excitatory amino acid agonist AMPA (Kogo et al. 1996), indicating a critical role for NMDA receptors in rhythmical oral-motor activity.

NMDA receptors play important roles in diverse functions such as neuronal development, learning and memory, cell death, and motor control (Bliss and Collingridge 1993; Mori and Mishina 1995; Schmidt et al. 1998). The importance of NMDA receptors in these behaviors most likely results from the NMDA receptor channels high permeability to Ca$^{2+}$ and voltage-dependent block by external Mg$^{2+}$ ions (Mayer and Westbrook 1987). With respect to rhythmical motor behavior, the Mg$^{2+}$ block in combination with activation of repolarizing conductances endows the neuron with bistable properties (the ability to switch between 2 stable membrane potentials) and, therefore, the capability of generating maintained bursting behavior (Hochman et al. 1994; Kim and Chandler 1995; Schmidt et al. 1998; Sigvardt et al. 1985; Tell and Jean 1993). Additionally, Ca$^{2+}$ entry through voltage-gated L-type Ca$^{2+}$ channels is critical for NMDA oscillations in adult turtle spinal motoneurons (Guertin and Hounsgaard 1998) and may serve a similar role in other vertebrate species as well.

Rhythmical oral-motor activity such as sucking and chewing is thought to be under control of a network of brain stem neurons, typically referred to as a central pattern generator (CPG) (reviewed in Goldberg and Chandler 1990; Lund et al. 1998). However, the fact that trigeminal motoneurons exhibit nonlinear intrinsic membrane characteristics imparted by the presence of NMDA receptors (Kim and Chandler 1995) and that these receptors are functional on trigeminal motoneurons during oral-motor behaviors (Katakura and Chandler 1990), suggest that the final rhythmical motoneuronal output is deter-
dined by a complex interaction between rhythmically occurring trigeminal premotor neurones and NMDA receptor-mediated intrinsic motoneuronal membrane oscillations (Kim and Chandler 1995). Similar views for the contribution of NMDA receptors to rhythmical network function in rat, lamprey, and tadpole locomotion have been put forward (Guertin and Houngaard 1998; Hochman et al. 1994; Schmidt et al. 1998; Scrymgeour-Wedderburn et al. 1997; Wallen and Grillner 1987). Presently, there is a paucity of information on the development of the circuitry underlying oral-motor rhythmical activity and, in particular, the properties of the trigeminal motoneurones and NMDA receptors during the transition from rhythmical sucking behavior to adult-like mastication (Turman et al. 1999), a behavior that begins around P12 in the rat (Westneat and Hall 1992). This information is essential, at a minimum, for a complete understanding of the cellular factors controlling oral-motor behaviors.

NMDA receptors undergo changes in the expression of NR2A and NR2B subunits during development (Kirson et al. 1999; Moyer et al. 1994; Watanabe et al. 1993). These changes in the receptor subunits are thought to control the sensitivity of the receptor channel to Mg$^{2+}$ block (Kuner and Schoepfer 1996), and hence the magnitude of the negative going slices of a neonatal rat brain stem (1–30 days). Rats were anesthetized by halothane inhalation (Halocarbon Laboratories, River Edge, NJ). The brain was removed and placed in oxygenated ice-cold cutting solution. Coronal sections (250–300 μM) through the trigeminal motor nucleus were obtained and then transferred into a holding chamber in incubation solution (see following text) at 37°C for 40 min.

**Solutions**

Solutions were bubbled with 95% O$_2$–5% CO$_2$ and maintained at pH 7.25–7.30 (22–24°C). Cutting solution was composed of the following (in mM): 126 NaCl, 3 KCl, 1.25 NaH$_2$PO$_4$, 26 NaHCO$_3$, 10 glucose, 1 CaCl$_2$, 5 MgCl$_2$, and 4 lactic acid. The recording solution consisted of the following (in mM): 124 NaCl, 3 KCl, 1.25 NaH$_2$PO$_4$, 26 NaHCO$_3$, 10 glucose, 2 CaCl$_2$, and 2 MgCl$_2$. Incubation solution was identical to recording solution except for the addition of 4 mM lactic acid. To isolate NMDA currents during voltage-clamp experiments, the bathing solution contained tetrodotoxin (TTX) [0.5 μM; Sigma (St. Louis, MO)] to block Na$^+$ currents; CdCl$_2$ (100 μM; Sigma) to block voltage-gated Ca$^{2+}$ currents; 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX; 10 μM; RBI, Natick, MA) to block non-NMDA receptors; bicuculline methiodide (10 μM; Sigma) and strychnine hydrochloride (5 μM; Sigma) to block GABA$_A$ and glycine Cl$^-$–mediated currents, respectively; and glycine (5 μM; Sigma) to saturate the glycine-binding sites on NMDA receptors.

Normal pipette solution contained (in mM) 115 K-gluconate, 9 NaCl, 25 KCl, 1 MgCl$_2$, 10 HEPES buffer, 0.2 EGTA, 3 K$_2$-ATP, and 1 Na-GTP, with a pH of 7.25–7.30 and osmolarity of 280–290 mM. To isolate NMDA currents, we used a modified pipette solution containing (in mM) 110 CsF, 20 TEA, 10 HEPES buffer, 2 MgCl$_2$, 1 CaCl$_2$, and 10 BAPTA, pH of 7.25–7.30 (osmolarity, 280–290 mM). To investigate the modulation of NMDA-induced currents by 5-HT, we used a modified pipette solution containing (in mM) 108 Cs-MeSO$_3$H, 12 CsCl, 10 HEPES buffer, 0.2 EGTA, 20 TEA, 1 MgCl$_2$, 2 K$_2$-ATP, and 0.3 GTP-Tris salt. Lucifer yellow (0.1%, Sigma) was added to small volumes of electrode solution for fluorescent viewing in initial experiments.

**Drug application**

Drugs were bath applied at the following concentrations: NMDA (50 μM; RBI), d,l-2-amino-5-phosphonovaleric acid (APV; 10 μM; Sigma), and nimodipine (10 μM; RBI).

**Identification of the trigeminal motor nucleus**

The trigeminal motor nuclei were identified bilaterally in the coronal slice under low magnification (×5) as an opaque, pale oval region medial to the trigeminal principal sensory nucleus and ventral lateral to the peri-aqueductal gray and central canal (Chandler et al. 1994). In early experiments, retrograde labeling of TMNs from microinjections of Texas red (10%, Molecular Probes, Eugene, OR) into the masseter or mylohyoid jaw muscles was used to confirm that the area recorded was the trigeminal motor nucleus.

**Whole cell recording**

Slices were perfused with oxygenated recording solution (3 ml/min) while secured in a recording well mounted on a Zeiss fixed stage Axioskop microscope equipped with bright-field, fluorescence, and Nomarski optics in combination with infrared video microscopy for enhanced resolution of individual neurones. Patch recordings were obtained with the use of an Axopatch 1D (Axon Instruments, Foster City, CA) in concert with pCLAMP acquisition software (Version 8.0.2, Axon Instruments) operating on a Pentium-based personal computer. Signals were digitized on-line and filtered to 2 kHz (voltage clamp) or 5 kHz (current clamp). Patch pipettes were fabricated from conventional thin-wall glass (1.5 mm OD, 0.86 mm ID; Warner Instrument, Hamden, CT), pulled on a Brown/Flaming P-97 micropipette puller (Sutter Instruments, Novato, CA) and had bath resistances of 3–5 MΩ. Signals were grounded by a 3 M KCl agar bridge.
electrode (Ag/AgCl wire) mounted in the recording well. Liquid junction potentials were measured between the pipette and bath solutions and varied between 9 mV (normal pipette solution) and 7 mV (modified pipette solutions) and were corrected off-line. Cell capacitance (C_m) for each trigeminal motoneuron recorded in voltage clamp was determined from the integral of capacity current in response to 15-ms hyperpolarizing voltage commands or directly obtained by the pClamp software during the experiment. Uncompensated series resistance (R_s) was calculated from the decay time constant (tau) of the transient and was usually less than 20 MΩ. Sixty to 80% compensation was routinely employed.

Data analysis

Current- and voltage-clamp data were analyzed in Clampfit 8.0 (Axon Instruments). NMDA-induced bursting characteristics, such as time of burst onset and termination, intraburst spike frequency, cycle time, and burst duration were measured in Datapac III 1.61 (Run Technologies, Irvine, CA). Burst duration was defined as the mean time from burst onset to termination in three or more consecutive bursts. Burst cycle time was defined as the mean time from burst onset to the next onset for three or more consecutive cycles. Postburst afterhyperpolarizing potential (AHP) was measured as the difference between the voltage level at the termination of the last spike in the burst and the peak of the trough immediately following a burst.

Isolation of NMDA currents

The NMDA current (I_NMDA) was obtained in selected experiments by digital subtraction of the current traces obtained by applying voltage ramps in the absence (control) and presence of bath applied NMDA in 30 μM or 1 mM external Mg^{2+} using the dedicated solution described in the preceding text. Averages from at least three consecutive responses to ramps were obtained. The I_NMDA-V relation for each cell was then obtained for each of those Mg^{2+} concentrations. The protocol is shown in Fig. 3. Briefly, the patched neuron was held at −80 mV then stepped up to +30 mV for 1.5 s to inactivate any transient voltage-gated currents. Subsequently, a ramp voltage command (0.13 mV/msec) was brought to −100 mV and then returned to −80 mV. The protocol was initiated in control ACSF and then again after obtaining a steady-state response to bath application of NMDA (50 μM). The currents recorded in control were subtracted from those recorded in the presence of NMDA to obtain the I_NMDA. The subtracted currents for each Mg^{2+} concentration were then normalized to the maximal outward current at +30 mV. The I_NMDA-V relationship was then obtained and reduced to 41 membrane potential points and plotted (Fig. 3).

SigmaPlot 4.0 (Jandel Scientific, San Rafael, CA), Excel (Microsoft, Redmond, WA), and StatView 5.0 (SAS Institute, Cary, NC) were used for additional analyses. Values are expressed as means ± SE. Significant differences were tested with Student’s paired and unpaired t-tests. A significance level of P ≤ 0.05 was used in all tests unless otherwise stated.

RESULTS

The data presented in this paper are based on patch-clamp recordings from 203 rat trigeminal motoneurons (TMNs) ages P1–P10 obtained from brain stem slice preparations. The criteria for inclusion into the database were resting potential greater than −50 mV, action potential amplitude exceeding 80 mV and input resistance at least 100 MΩ. Prior to NMDA application, all neurons exhibited continuous spike discharge when artificially depolarized above spike threshold and were silent at potentials below threshold.

NMDA-induced burst discharge

Previously, we showed that bath application of NMDA produced rhythmic membrane potential fluctuations and bursting in adolescent guinea pig trigeminal motoneurons (Kim and Chandler 1995). To determine if such conditional bursting is present in neonatal rat TMNs, the effects of NMDA application were examined. A typical example of NMDA application is shown in Fig. 1 taken from a P9 neuron. As shown, bath application depolarized the neuron and increased input resistance from 104 to 130 MΩ prior to spike onset (Fig. 1A). Once threshold was reached (−50 mV), continuous spiking occurred (Fig. 1A2) that could be transformed into bursting behavior by membrane hyperpolarization with extrinsic current application (Fig. 1A3). In this example, the onset of burst discharge started around −63 mV. This behavior was observed in 67/108 neurons examined (62%). Extrinsic polarization of the membrane potential in the absence of NMDA never produced bursting. In seven P8–P10 neurons, the precise range of membrane potentials where bursting discharge was initiated and terminated was measured and was between −64.1 ± 2.1 and −76.9 ± 2.8 mV. In the presence of TTX, the rhythmic bursting behavior was replaced by rhythmically occurring plateau potentials (Fig. 2A, 1 and 2) that were blocked by the specific NMDA antagonist APV (10 μM; Fig. 2A3). These data indicate that the bursting behavior in the presence of NMDA resulted from specific activation of NMDA receptors and is not dependent on circuit activity as previously demonstrated in...
Development of NMDA burst discharges

To determine if NMDA-induced bursting is developmentally regulated, the effects of bath application of NMDA on P1–P10 TMNs were examined. In 21 neurons examined prior to P8, only 2 neurons showed bursting activity in response to NMDA application. However, in nonbursting neurons, NMDA produced membrane depolarization and continuous spike discharge, as shown in Fig. 1B1 for a P2 neuron. In contrast to that observed in neurons older than P7, alterations of the membrane potential within the same potential range did not produce bursting (Fig. 1B, 2 and 3). In P1–P4 neurons, which rarely showed bursting, the mean membrane depolarization in response to 50 μM NMDA application prior to spike threshold was 14.5 ± 1.3 mV, n = 15; while that for the P8–P10 group, which usually showed bursting, was 15.0 ± 0.6 mV, n = 45. Table 1 shows a summary of the effects of NMDA application on membrane potential and R_{mp} for P1–P4 and P8–P10 groups. The data indicate that the lack of bursting in P1–P4 neurons must not reside in the lack of functional NMDA receptors but rather to a change in the underlying ionic mechanism and/or membrane properties responsible for NMDA bursting.

Development of NMDA receptor Mg^{2+} sensitivity

NMDA-induced bursting is critically dependent on the presence of a region of negative slope conductance (NSC) in the steady-state total membrane current I_{tot}-V relationship (Kim and Chandler 1995; Schmidt et al. 1998). In guinea pig TMNs in response to NMDA, bursting behavior results from the voltage-dependent Mg^{2+} block and unblock of the receptor ionophore in combination with repolarizing outward currents (Kim and Chandler 1995). In nominal Mg^{2+} solutions, which remove the NSC in the steady-state I_{tot}-V relationship, bursting in guinea TMNs is not possible (Kim and Chandler 1995). An example of the dependence on extracellular Mg^{2+} for NMDA-induced bursting in rat TMNs is shown in Fig. 2B, 1–3. Reduction of the extracellular Mg^{2+} from 1 mM to 30 μM transformed the bursting pattern into continuous spike discharge that was easily reversed on washout.

To determine if the development of NMDA bursting is dependent on changes in the affinity of NMDA receptors for Mg^{2+} and development of a voltage-dependent Mg^{2+} block, in voltage clamp we isolated the NMDA current and examined the effects of two different Mg^{2+} concentrations on the underlying normalized I_{NMDA}-V (see METHODS) relationship in P2–P4 and P8–P10 groups. Composite normalized I_{NMDA}-V relationships for these two groups are shown in Fig. 3, A and B. Although the reversal potentials for both groups were similar (approximately 0 mV), it is apparent that in the young group NMDA receptors have already developed a sensitivity to extracellular Mg^{2+}; at voltages negative to around −25 mV, increasing Mg^{2+} reduced the I_{NMDA}. However, the voltage dependence of the Mg^{2+} block was less apparent in the young compared with the older group, indicating that the efficacy of depolarization for relieving the Mg^{2+} block was less in the young group. Thus in the P2–P4 group, depolarization from −100 to −50 mV produced a small increase in normalized I_{NMDA} in 1 mM Mg^{2+} compared with the P8–10 group. These developmental changes in the voltage-dependent Mg^{2+} block

Table 1. Effects of NMDA and 5-HT on passive membrane properties of TMNs

<table>
<thead>
<tr>
<th>Property</th>
<th>Age</th>
<th>Control</th>
<th>NMDA</th>
<th>Control</th>
<th>5-HT</th>
</tr>
</thead>
<tbody>
<tr>
<td>R_{mp}, MΩ</td>
<td>P1–P4</td>
<td>130.6 ± 11.0 (5)</td>
<td>179.4 ± 20.2 (5)</td>
<td>137.4 ± 32.5 (4)</td>
<td>191.1 ± 45.5 (4)</td>
</tr>
<tr>
<td>V_{m}, mV</td>
<td>P1–P4</td>
<td>−61.7 ± 1.1 (15)</td>
<td>−47.2 ± 0.9 (15)</td>
<td>−61.2 ± 1.6 (9)</td>
<td>−48.0 ± 1.8 (9)</td>
</tr>
<tr>
<td></td>
<td>P8–P10</td>
<td>123.2 ± 10.5 (6)</td>
<td>153.0 ± 13.9 (6)</td>
<td>110.6 ± 9.8 (5)</td>
<td>148.5 ± 20.5 (5)</td>
</tr>
<tr>
<td></td>
<td>P8–P10</td>
<td>−63.8 ± 0.8 (45)</td>
<td>−48.8 ± 0.6 (45)</td>
<td>−67.6 ± 1.3 (5)</td>
<td>−50.4 ± 2.6 (5)</td>
</tr>
</tbody>
</table>

Values are means ± SE. n values are in parentheses. NMDA, N-methyl-d-aspartate; 5-HT, serotonin; TMN, trigeminal motoneuron.
 Role of calcium ions in the generation of NMDA bursting

In turtle spinal motoneurons, rhythmic NMDA bursting depends on L-type calcium channel activation (Guertin and Hounsgaard 1998). To determine the role of calcium entry for initiation and maintenance of NMDA-induced rhythmical bursting in rat TMNs, we performed a series of experiments that either blocked voltage-gated calcium channels (VGCC), reduced extracellular calcium, or maintained the intracellular [Ca\textsuperscript{2+}] to low levels by chelation with 10 mM BAPTA during NMDA application.

To investigate the possibility of L-type Ca\textsuperscript{2+} channel participation in NMDA bursting, we first examined the effects of nimodipine (10 μM), an L-type calcium channel blocker, on NMDA bursting (Fig. 4A, 1 and 2). Although nimodipine altered burst duration and intraburst spike frequency (Table 2), maintained bursting was still present. In fact, when Cd\textsuperscript{2+}, a general VGCC blocker was used, NMDA bursting was still evident in spite of the change in burst characteristics (Fig. 4B, 1 and 2, and Table 2). These data suggest that VGCC contribute to shaping NMDA burst characteristics but are not necessary for initiation and maintenance of NMDA-induced rhythmical burst discharge.

Although calcium entry through VGCC was not essential for maintained NMDA bursting, calcium does permeate NMDA channels (Mayer and Westbrook 1987), and this could have been sufficient for NMDA bursting. To test this possibility, the following experiments were performed. First, in the presence of Cd\textsuperscript{2+} to block VGCC, the effects of reduced [Ca\textsubscript{ext}] on NMDA bursting were examined. Figure 4C shows that during these conditions, only continuous spike discharge was observed (n = 9).

To determine whether a change in intracellular [Ca\textsuperscript{2+}], as opposed to Ca\textsuperscript{2+} as a charge carrier, is necessary for NMDA burst generation, BAPTA was applied directly through the pipette (10 mM) to chelate the intracellular free calcium to low levels. Figure 5, A and B, shows a typical example observed in 5/6 neurons. NMDA was applied within the first 5 min of whole cell recording to induce maintained burst discharge (Fig. 5A). Prior to applying NMDA, a short pulse (3 ms) elicited a single spike with a subsequent AHP (inset, BAPTA 4°). After

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Modulation of NMDA-mediated bursting by serotonin

We examined whether 5-HT could enable NMDA bursting in the P1–P4 group because 5-HT receptor activation is necessary for NMDA bursting in tadpole spinal motoneurons (Scrymeour-Wedderburn et al. 1997) and can directly induce rhythmical membrane potential oscillations in trigeminal motoneurons (Del Negro et al. 1999; Hsiao et al. 1998) as well as modulate rhythmical TMN discharge during cortically induced rhythmical jaw movements in vivo (Katakura and Chandler 1990).

To exclude the possibility that 5-HT directly produces bistability and bursting in rat TMNs (Del Negro et al. 1999; Hsiao et al. 1998), we examined the effects of 5-HT on TMN membrane properties in both current and voltage clamp. Figure 6 shows the effects of bath application of 5-HT on membrane potential in current clamp (Fig. 6A) and total membrane current in voltage clamp (Fig. 6C). Typically, in current clamp, after 10 μM 5-HT the membrane potential depolarized and produced continuous spiking. In contrast to that observed in adult guinea pig trigeminal motoneurons (Hsiao et al. 1998), rhythmical burst discharge was never observed. In the presence of TTX, 5-HT depolarized the neuron (not shown) and, based on current pulse application, increased input resistance (Fig. 6B).

### Table 2. Drug effects on burst cycle characteristics

<table>
<thead>
<tr>
<th>Drug</th>
<th>Intraburst Spike Frequency</th>
<th>Burst Cycle Time</th>
<th>Burst Duration</th>
<th>Postburst AHP</th>
</tr>
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<tbody>
<tr>
<td>Nimodipine</td>
<td>↑ 26.8 ± 16.4% (2/3)</td>
<td>↑ 34.2 ± 16.4% (3/3)</td>
<td>↓ 32.7% (1/3)</td>
<td>↑ 49.6% (1/3)</td>
</tr>
<tr>
<td>Cd2⁺</td>
<td>↓ 9.7% (1/3)</td>
<td>NC (2/2)</td>
<td>NC (1/3)</td>
<td>NC (3/3)</td>
</tr>
<tr>
<td>5-HT</td>
<td>↑ 44.3 ± 7.3% (3/5)</td>
<td>↑ 170.9 ± 67.8% (5/5)</td>
<td>↑ 127.1 ± 41.5% (5/5)</td>
<td>↑ 51.0 ± 10.6% (5/5)</td>
</tr>
<tr>
<td></td>
<td>NC (2/5)</td>
<td>NC (2/5)</td>
<td>NC (3/5)</td>
<td>NC (3/5)</td>
</tr>
</tbody>
</table>

Parentheses indicate number of neurons that showed a significant change in response to drug compared to total examined. Arrows indicate the percentage increase or decrease from control for those cells that exhibited a significant change. Values are expressed as means ± SE. NC, no change. AHP, afterhyperpolarization.

### Figure 5. Maintenance of a requisite level of intracellular Ca²⁺ is essential for NMDA bursting. A: intracellular bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA) diffusion after 8 min did not eliminate NMDA bursting or the slow afterhyperpolarization (AHP) following an evoked spike (3-ms pulse; inset, —). B: BAPTA diffusion after 30 min eliminated the slow AHP following an evoked spike (inset, •••) and suppressed burst discharge indicating a role for intracellular Ca²⁺ in maintenance of NMDA bursting.

![Figure 5](http://jn.physiology.org/)

### Figure 6. Effects of 5-HT on trigeminal motoneurons. A: current-clamp record showing membrane depolarization and spiking in response to serotonin (5-HT) from a P4 neuron (10 μM). B: membrane potential response to extrinsic current pulse application (V-I relationship) before (•) and during 5-HT application (○). Note the increase in steady-state R_{inp} (slope) in the presence of 5-HT. The intersection of the curves indicate estimated reversal potential of approximately −90 mV. C: voltage-clamp record showing inward current in response to 5-HT application from a P3 neuron. Holding potential −50 mV. D: steady-state V-I relationship of total membrane current obtained in response to ramp voltage command before (•) and during (○) 5-HT application. Note the presence of outward rectification but lack of a NSC for both traces. Inset shows I_{test} obtained by subtraction of control current from current obtained during 5-HT application.
with an apparent reversal potential of approximately \(-90 \text{ mV}\).

Table 1 summarizes the effects of 5-HT on membrane properties for P1–P4 and P8–P10 groups. In voltage clamp, 5-HT produced inward current (Fig. 6C). Figure 6D shows the steady-state $I_{\text{tot}}$-$V$ relationship derived from ramp commands (see METHODS), before and during 5-HT application. The inset shows the $I_{5,\text{HT}}$ obtained from digital subtraction. It is apparent that 5-HT produced an inward current throughout the voltage range examined and, more importantly, did not induce any prominent NSC in the steady-state $I_{\text{tot}}$-$V$ relationship. Furthermore, the $I_{5,\text{HT}}$ had an extrapolated reversal potential negative to \(-80 \text{ mV}\) indicating that the increase in input resistance and depolarization resulted from a reduction of a leakage $K^+$ current (Hsiao et al. 1997).

The effects of 5-HT on NMDA bursting in a P2 and P8 neuron are shown in Fig. 7. In the absence of NMDA, 5-HT did not induce any rhythmical bursting activity in either age group (Fig. 7, A1 and B1). In the P2 neuron, NMDA application produced continuous spike discharge (Fig. 7A2). However, in the presence of 5-HT, this discharge was transformed into a robust, rhythmical bursting activity (9/12 neurons; Fig. 7A3). Figure 7C shows the $I_{\text{tot}}$-$V$ relationship prior to and during 5-HT application in the presence of NMDA for this neuron. Prior to 5-HT application, NMDA did not induce an NSC. However, after addition of 5-HT, a distinct NSC was observed in 4/5 neurons; a necessary condition for bursting. In the P8 neuron, which exhibited NMDA bursting (Fig. 7B2), simultaneous application of 5-HT increased intraburst spike frequency and prolonged burst duration (Fig. 7B3, Table 2). Figure 7D shows the $I_{\text{tot}}$-$V$ relationship obtained from voltage clamp in the presence of NMDA before and during simultaneous 5-HT application. As shown, 5-HT shifted the current downward, increased $R_{\text{in}}$ and enhanced the NSC region. These changes most likely account for the increase in burst duration (Fig. 7B3). This was observed in 4/5 neurons. These data demonstrate that neurons younger than P8 are, in fact, capable of maintained NMDA bursting and bistability in the presence of 5-HT.

The synergistic effect of 5-HT and NMDA that resulted in the induction of bursting could have resulted from a direct voltage-dependent block and unblock of the NMDA channel by 5-HT, similar to that caused by Mg$^{2+}$ (Chesnoy-Marchais and Barthe 1996). To test for this possibility, Mg$^{2+}$ was removed from the bath (Fig. 8A2) during NMDA/5-HT induced bursting in a P10 neuron (Fig. 8A1). During these conditions, rhythmical bursting was eliminated and maintained membrane depolarization was produced ($n = 3$). This was tested more directly in voltage clamp from P8–P10 neurons. A representative example is shown in Fig. 8B, 1 and 2. In 0 Mg$^{2+}$ solutions, NMDA did not produce a region of NSC in the $I_{\text{tot}}$-$V$ relationship. After the addition of 5-HT, the $I_{\text{tot}}$-$V$ relationship was shifted downward without induction of an NSC ($n = 6$), indicating that 5-HT per se does not produce a voltage-dependent block of the NMDA channel (Chesnoy-Marchais and Barthe 1996) that could have created a condition for bistability.

The preceding data suggest that in addition to the increase in total membrane current and input resistance produced directly by 5-HT, 5-HT also enables NMDA bursting in P1–P4 neurons through enhancement of the NSC region in the steady-state $I_{\text{NMDA}}$-$V$, presumably by alterations in the Mg$^{2+}$ block of the NMDA channel. To test this directly, we isolated the $I_{\text{NMDA}}$ and examined the effects of 5-HT on the steady-state $I_{\text{NMDA}}$-$V$ relationship in 1 mM Mg$^{2+}$ in the P1–P4 group. Figure 9A
FIG. 9. 5-HT enhances NSC region of $I_{\text{NMDA}}-V$ relationship. A: normalized $I-V$ relationship for NMDA current prior to (○) and during 5-HT (10 μM) application (●) for a P2 neuron. Inset: command protocol. V, command potential. B: composite $I-V$ relationship for 4 neurons (P1–P4). Note the increase in $I_{\text{NMDA}}$ within the voltage range of approximately −60 to −20 mV. C: the ratio of the normalized peak $I_{\text{NMDA}}$ (arrow in B) to NMDA current measured at −100 mV ($I_{\text{NMDA}}$) is plotted as a histogram for both NMDA only conditions (○) and NMDA plus 5-HT (●). A value of 1 indicates the lack of an NSC. *, statistical difference, $n = 4, P \leq 0.03$.

shows an example from one neuron. Prior to 5-HT application the normalized $I_{\text{NMDA}}-V$ relationship showed a very small region of NSC. In the presence of 5-HT, this region was enhanced most prominently between −60 and −40 mV, suggesting a reduction in the voltage-dependent block by Mg$^{2+}$ at depolarized potentials. These effects are summarized in Fig. 9B ($n = 4$) and are quantified in the histogram in Fig. 9C where the ratio of peak $I_{\text{NMDA}}$ to $I_{\text{NMDA}}$ recorded at −100 mV prior to and during 5-HT application are depicted.

DISCUSSION

The present study demonstrates that NMDA-induced bursting is developmentally regulated within a narrow time window; TMNs less than P5 seldom exhibit NMDA bursting and this was coincident with the lack of a prominent voltage-dependent Mg$^{2+}$ block of the NMDA iontophore channel. However, in the presence of 5-HT receptor activation, NMDA bursting was initiated. The “enabling” of the NMDA receptor/iontophore by 5-HT for ages less than P5 most likely resulted, in part, from enhancement of the negative slope conductance region of the total membrane current steady-state $I_{\text{NMDA}}-V$ relationship and from an increase in net inward membrane current due to the reduction of a leakage conductance in the presence of 5-HT. Because trigeminal motoneurons, and oral-motor activity in general, are dependent on NMDA receptor activation for production of rhythmic discharge (Kataoka and Chandler 1990; Kogo et al. 1996) and are subject to 5-HT modulation (Chandler et al. 1985; Hsiao et al. 1997), these findings become significant for understanding the factors controlling rhythmic sucking and the transition to adult-like masticatory behaviors in trigeminal motoneurons.

Development of NMDA-induced bursting activity

Bath application of NMDA produces membrane oscillations and rhythmic burst discharge in different types of neurons (Schmidt et al. 1998). The bistable behavior is generally attributed to the production of a negative slope conductance region in the steady-state $I-V$ relationship in combination with voltage- and time-dependent repolarizing conductances (e.g., Kim and Chandler 1995; Schmidt et al. 1998).

In the presence of synaptic blockade of rapid excitatory and inhibitory synaptic transmission, bath application of NMDA did not induce consistent bursting behavior until approximately P8 in the present study. Prior to that, NMDA application produced membrane depolarization and continuous spike discharge, providing physiological evidence for the anatomical observation (Turman et al. 1999) that functional synaptic and/or extra-synaptic receptors for NMDA are present on trigeminal motoneurons at birth. However, the inability to produce bursting most likely relates to the lack of a sufficiently developed NSC in the steady-state $I_{\text{NMDA}}-V$ relationship. This is in contrast to that reported for neurons within the nucleus tractus solitari where bursting, albeit irregularly occurring, was observed at birth in conjunction with an NSC (Vincent et al. 1996). It is difficult to compare our data to theirs because they studied NMDA currents in the absence of synaptic blockade and the possibility of NMDA receptor modulation by background synaptic input, and a contribution from rhythm-generating networks, cannot be excluded.

The absence of rhythmic NMDA-induced bursting and a prominent NSC in P1–P4 neurons most likely resulted from insufficient development of a voltage-dependent Mg$^{2+}$ block of the NMDA receptor channel. As shown in the present study and others (e.g., Kim and Chandler 1995; Tell and Jean 1993), NMDA bursting is critically dependent on the presence of Mg$^{2+}$ in the bath. Although the $I_{\text{NMDA}}-V$ relationship for P2–P4 neurons showed sensitivity to changes in extracellular Mg$^{2+}$ at hyperpolarized voltages, in the presence of 1 mM Mg$^{2+}$ very little voltage dependence was observed as indicated by the small NSC in the steady-state $I_{\text{NMDA}}-V$ relationship. In contrast, animals older than P7 exhibited a prominent NSC that provides the basis for bistability. These differences most likely contributed to the lack of bursting observed in the younger age group neurons. Although the exact postnatal time period may differ, similar developmental changes in the sensitivity to Mg$^{2+}$ and the NMDA voltage-dependent Mg$^{2+}$ block have been reported in neocortex (Burgard and Hablitz 1994), tractus solitari (Nabekura et al. 1994) and hippocampal CA1 pyramidal neurons (Ben-Ari et al. 1988; Kirson et al. 1999), although, a lack of a postnatal developmental change in the voltage-
dependent Mg$^{2+}$ block has been reported (Kirson and Yaari 1996; Kleckner and Dingledine 1991).

The present study did not address the mechanism(s) for the change in voltage-dependent block by Mg$^{2+}$ of the NMDA receptor. However, given that the expression profile of the different NMDA receptor subunits changes in trigeminal neurons during development (Turman et al. 1999) and that in heterologous systems, recombinant NMDA receptors containing NR2A or NR2B subunits (Kuner and Schoepfer 1996; Monyer et al. 1994) are more strongly blocked by Mg$^{2+}$ than those containing NR2C or NR2D, the developmental change in the voltage-dependent block by Mg$^{2+}$ of the NMDA receptor channel in trigeminal motoneurons could be due to alterations in the expression of NMDA receptor subunits. Alternatively, developmental regulation of the Mg$^{2+}$ block by intracellular messengers such as protein kinase C (PKC) (Ben-Ari et al. 1992; Chen and Huang 1992; Lan et al. 2001) must also be considered.

Intracellular calcium requirement for NMDA bursting

The present study provides evidence that NMDA bursting is dependent on intracellular messenger activity and in particular maintenance of a requisite level of intracellular calcium concentration. A previous study on spinal motoneurons of adult turtle, in vitro, showed that NMDA bursting depended on L-type Ca$^{2+}$ channel activation (Guertin and Houngsaard 1998). In the present study, L-type channel blockers or Cd$^{2+}$, which blocks all trigeminal motoneuronal HVA Ca$^{2+}$ currents (unpublished observation), did not block NMDA oscillations or the underlying plateau potentials during the bursts but did increase intraburst spike frequency and burst duration in the majority of neurons tested. This suggests that Ca$^{2+}$, as a charge carrier per se through VGCCs, is not necessary for NMDA bursting but changes in [Ca$_i$] can alter NMDA burst cycle characteristics. This is not unexpected because TMNs posses a calcium-dependent K$^+$ conductance that is responsible for the slow AHP following each action potential and contributes to regulation of spike discharge (Chandler et al. 1994; Inoue et al. 1999). However, when Ca$^{2+}$ entry through both VGCC and NMDA channels was substantially reduced by simultaneous application of both Ca$^{2+}$ and low external Ca$^{2+}$ media or when BAPTA was present in the pipette to buffer intracellular Ca$^{2+}$ to low levels, bursting was not possible, suggesting that [Ca$_i$] acting as an intracellular messenger is necessary for NMDA bursting. This, of course, is reminiscent of the requirement for intracellular Ca$^{2+}$ for some forms of LTP (Bliss and Collingridge 1993).

Serotonin enables NMDA bursting

In the presence of 5-HT robust NMDA-induced bursting was observed in most neurons, including those less than P8. Interestingly, in contrast to what was demonstrated in guinea pig TMNs where serotonin directly induces bistable bursting behavior (Hsiao et al. 1998), serotonin application in the absence of NMDA produced only tonic depolarization and an increase in $R_{dep}$; thus eliminating the possibility that 5-HT directly induced nonlinear conductance changes, which could have supported bistability. A similar “enabling” function by 5-HT for NMDA oscillations was described in Xenopus spinal cord neurons where, in the absence of 5-HT receptor activation, NMDA bursting was not possible (Scrymgeour-Wedderburn et al. 1997). Similarly, in rat spinal motoneurons NMDA oscillations are dependent on endogenous serotonergic receptor activity (Maclean et al. 1998).

The present data indicate that the mechanism(s) for “enabling” of NMDA bursting by 5-HT in trigeminal motoneurons at birth is due, partly, to a reduction in the Mg$^{2+}$ block at depolarized potentials, thus leading to enhancement of the NSC in the steady-state $I_{tot}$-V. Based on current-clamp analysis, this was proposed for the “enabling” of NMDA oscillations by 5-HT in Xenopus motoneurons (Scrymgeour-Wedderburn et al. 1997). In the present study, in animals less than P5, the $I_{tot}$-V relationship showed either a very small or no NSC in the presence of NMDA. After 5-HT application, the $I_{tot}$-V relationship was shifted downward due to the reduction in leakage current and exhibited a prominent NSC (Fig. 7C). It is unlikely that the NSC in the $I_{tot}$-V relationship resulted from activation of L-type Cu$^{2+}$ channels by 5-HT, as demonstrated in guinea pig trigeminal motoneurons (Hsiao et al. 1998), because in the absence of NMDA an NSC was not observed after 5-HT. Furthermore in the absence of external Mg$^{2+}$ but in the presence of 5-HT, NMDA bursting was not possible. This excludes the possibility that 5-HT directly effected the NMDA ionophore to produce a voltage-dependent block of the NMDA receptor in a manner analogous to Mg$^{2+}$ as shown previously in embryonic spinal neurons (Chesnay-Marchais and Barthe 1996).

A number of reports indicate that 5-HT modulates NMDA receptor function (Blank et al. 1996; Nederbragt et al. 1986; Trueblood et al. 1996), yet the precise mechanism for the effect is not clear. However, in Xenopus oocytes it was shown that 5-HT$_3$ receptors potentiate NMDA responses via a PKC-dependent process (Blank et al. 1996). Because the Mg$^{2+}$ block of the NMDA receptor channel in trigeminal sensory neurons is reduced by PKC (Chen and Huang 1992) and 5-HT couples to PKC in trigeminal motoneurons (Inoue et al. 1999), it is possible that a similar intracellular transduction mechanism is responsible for the increase in the NSC in the $I_{NMDA}$-V and thus enabling of NMDA bursting by 5-HT in TMNs as well.

Physiological implications

NMDA receptors have been implicated in a number of behavioral activities such as synaptic plasticity and learning during development (Bliss and Collingridge 1993; Mori and Mishina 1995) and excitotoxic brain damage (Diemer et al. 1997), among others. Less attention has been placed on NMDA receptor participation in rhythmic motor activities such as synaptic integration during ongoing rhythmical activity (Kim and Chandler 1995). For instance in Xenopus, it was proposed that the NMDA oscilla-
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REFERENCES


