Effects of Pedunculopontine Nucleus (PPN) Stimulation on Caudal Pontine Reticular Formation (PnC) Neurons In Vitro

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Received 9 August 2001; accepted in final form 24 January 2002

INTRODUCTION

The pedunculopontine nucleus (PPN), as the cholinergic arm of the reticular activating system (RAS), has been implicated in the modulation of sleep-wake states, the startle response (SR), and of posture and locomotion (reviewed in Garcia-Rill 1991; Reese et al. 1995; Steriade and McCarley 1990). Electrical stimulation of the PPN can induce widespread effects, both ascending and descending, such as cortical electroencephalographic (EEG) desynchronization (Moruzzi and Magoun 1949) [better referred to as synchronization of fast cortical rhythms (Steriade et al. 1996)], changes in muscle tone (Lai and Siegel 1990), and the recruitment of stepping (Garcia-Rill et al. 1983, 1986, 1987; Garcia-Rill and Skinner 1987a,b, 1988, 1991). The PPN therefore appears to be involved in the ascending control of transitions in state from slow-wave sleep to either waking or REM sleep, and in descending functions that involve changes in muscle activity from standing (extensor activation), to the SR (flexor activation, extensor inhibition), to the atonia of REM sleep (flexor and extensor inhibition), and to locomotion (flexor-extensor alternation). PPN neurons are known to increase their firing rates during synchronization of fast rhythms in waking and REM sleep [tonically in waking, bursting during REM sleep, and reduced activity during slow-wave sleep (Steriade and McCarley 1990; Steriade et al. 1990)] and to show both tonic and rhythmic activity in relation to either the duration of stepping episodes, or the rhythmic alternation of locomotor movements (Garcia-Rill et al. 1983; Garcia-Rill and Skinner 1988). The PPN sends diffuse, mostly cholinergic, projections throughout the pontine reticular formation (Garcia-Rill 1991; Garcia-Rill and Skinner 1987b; Garcia-Rill et al. 1986; Jones 1990; Mitani et al. 1988; Rye et al. 1988; Sembra et al. 1990; Shiromani et al. 1988). In turn, the induction of REM sleep has been proposed to be facilitated by pontine reticular neurons (Jouvet 1975; Yamamoto et al. 1990).

During REM sleep, the release of acetylcholine in the pontine reticular formation is augmented (Kodama et al. 1990; Leonard and Lydic 1997), and neurons in this region are depolarized (Ito and McCarley 1984). Injections of cholinergic agonists into the pontine reticular formation have been found to depolarize pontine reticular formation neurons (Greene et al. 1989) and to induce REM sleep whether injected into anterodorsal pons (Baghdoyan et al. 1987; Yamamoto et al. 1990), or into more posterior pontine regions, although with lowered effectiveness (Baghdoyan et al. 1987). Electrical stimulation of the PPN increases the release of acetylcholine in the pontine reticular formation (Lydic and Baghdoyan 1993), as well as enhances REM sleep (Thakkar et al. 1996). Interest-
ingly, the parameters of PPN stimulation used to elicit acetylcholine release in the pontine reticular formation are similar to those used for inducing locomotion [i.e., continuous 0.5-ms pulses at 50 Hz (Lydic and Baghdoyan 1993) vs. continuous 0.5-ms pulses at 20–60 Hz (Garcia-Rill 1991; Garcia-Rill et al. 1987), respectively], but different from those used to induce suppression of muscle tone [i.e., short trains of 0.2-ms pulses at 100 Hz (Lai and Siegel 1990)]. These results would at first sight appear contradictory, unless one postulated, among other options, that there is a stimulation frequency-dependent effect at play. A recent study reported the presence, in the decerebrate cat caudal pontine reticular formation, of extracellularly and intracellularly recorded neurons that showed such frequency-dependent responses following PPN stimulation (Garcia-Rill et al. 2001). That study showed that short (1-s) trains of medium frequency (60-Hz) stimulation induced prolonged responses (>12 s) in caudal pontine neurons, whereas low (10-Hz) or high (100-Hz) frequency trains induced much briefer responses, if any. However, to perform a more thorough investigation of the responses and potential mechanisms mediating these responses, intracellular recordings in vitro became essential. The present studies were undertaken to determine the nature of the responses of rat caudal pontine reticular neurons recorded in vitro following PPN stimulation using various frequencies of stimulation. Preliminary findings have been reported in abstract form (Homma et al. 2000).

Methods

Subjects

Adult timed-pregnant Sprague-Dawley rats (280–350 g) were used and the litters culled to 10. When the pups were 12–21 days old, individual pups were anesthetized using ±2-(2-chlorophenyl)-2-(methylamino)cyclohexanone (70 mg/kg im) until tail pinch and corneal reflexes were absent, then they were rapidly decapitated. The individual pups were anesthetized using 2-(2-chlorophenyl)-2-(methylamino)cyclohexanone (70 mg/kg im) until tail pinch and corneal reflexes were absent, then they were rapidly decapitated. The present studies were undertaken to determine the nature of the responses of rat caudal pontine reticular neurons recorded in vitro following PPN stimulation using various frequencies of stimulation. Preliminary findings have been reported in abstract form (Homma et al. 2000).

Recording procedures

The recording chamber allowed the slice to be suspended on a nylon mesh so that oxygenated ACSF could flow all around the slice. The gravity-fed ACSF flowed through a sleeve of circulating warmed water so that the temperature of the ACSF in the chamber was 30 ± 1°C. The outflow was removed by suction and flow adjusted to 2–3 ml/min. Microelectrodes were pulled in a Sutter Instruments (Novato, CA) puller using Omega-Dot, thin-wall borosilicate glass and filled with 3 M K+ acetate and 1% biocytin, with a resistance of 70–100 MΩ. Signals were amplified with an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA) in these current-clamp recordings. Neurons were impaled and allowed to stabilize for about 5 min before testing. Neurons that showed a stable resting membrane potential (RMP) less than or equal to –55 mV and action potentials ≥40 mV and that had stable, long-term recordings were accepted for data analysis. PnC neurons at the ages studied appeared to be small in size, as evidenced by their high-input resistance (Rin) and morphology. The RMPs were verified and adjusted when the electrode was withdrawn at the end of recordings (usually only 1–2 mV difference, sometimes >5 mV especially after biocytin injection). In bridge mode, a series of hyperpolarizing and depolarizing current steps of 0.1 nA at RMP were applied to determine several membrane properties (see following text). These current steps also allowed the calculation of a preliminary current-voltage (I-V) curve during the linear range of voltage deflections using SuperScope software (GW Instruments, Somerville, MA). In some neurons, we also calculated an I-V curve using ramp stimulation, usually from –80 to +30 mV to reveal potential bistable properties. Ramp stimulation I-V curves were calculated before, during, and after tetrodotoxin (TTX) superfusion (see following text). The properties measured included membrane input resistance (Rin) determined using hyperpolarizing 300-ms duration pulses of 0.1–0.3 nA applied at RMP), action potential amplitude and threshold (determined by the beginning of the sodium spike to its peak in action potentials occurring spontaneously at RMP or, if no spontaneous activity was evident, by depolarizing the membrane until individual action potentials were induced, i.e., at action potential threshold), action potential duration at threshold (determined as the duration of the action potential at half-amplitude in spikes recorded at action potential threshold), afterhyperpolarization (AHP) amplitude (determined from action potential threshold to the peak of the AHP in individually occurring spikes) and AHP duration (determined from action potential threshold to the return to prespike membrane potential in individually occurring spikes).

Stimulation procedures

Electrical stimulation of the PPN was carried out using a bipolar concentric electrode (100 μm diam, 100 KΩ resistance) applying currents of 100–500 μA in amplitude using pulses of 0.2–0.5 ms duration, at frequencies of 10–90 Hz, individually and in trains of various durations, usually 1 s. The location of the stimulating electrode was confirmed using NADPH diaphorase histochemistry as described below. In the studies described, all stimulating electrode sites were found within the region of NADPH diaphorase-positive (NADPHd+) cells.

Neuroactive agents were applied via a manifold with six perfusion ports, hence multiple gravity-fed solutions could be applied for pharmacological characterization of neuronal properties. The concentrations of the superfused neuroactive agents in ACSF were as follows: carbachol (CAR; 5 μM), kynurenic acid (KYN; 300 μM), (R,S)-2-(2-chlorophenyl)-2-carboxyphencylglycine (MCPG; 300 μM), nifedipine (NIF; 10 μM), scopolamine (SCOP; 10–100 μM), and tetrodotoxin (TTX; 0.3 μM). Direct effects of these agents on recorded PnC neurons were confirmed before, during, and after wash out/recovery from TTX superfusion. Ramp stimulation for I-V curves was carried out during these three conditions. The concentrations of these agents were adjusted so that effects were evident using superfusion times of 1–2 min. CAR was also used for micropressure application using a higher concentration (30 μM) adjusted to elicit a response following 2–5 puffs applied to the surface of the tissue when the pipette was <100 μm from the recording microelectrode. The micropressure system was set at 30 psi, 50-ms duration puffs, and pipette resistance was designed for application of about 100 pl/puff. The micropressure pi-
Histological procedures

At the end of the recording period, each neuron was injected with biocytin using intracellular depolarizing pulses adjusted to elicit a train of action potentials (about 0.5–1.0 nA) of 500 ms duration at 1 Hz for 10–15 min. Such injections yielded well-filled neurons. All of the slices were processed for NADPH diaphorase histochemistry for selective labeling of cholinergic mesopontine (PPN) neurons around stimulation sites. Briefly, slices were fixed in 4% buffered paraformaldehyde for 1–2 h, cryoprotected in 20% sucrose, and cut in a cryostat at 50 μm. Sections were incubated in 1 mg/ml NADPH and 0.1 mg/ml nitroblue tetrazolium in PBS at 37°C for 30–60 min (Garcia-Rill and Skinner 1987a,b, 1988; Skinner and Garcia-Rill 1984). For intracellularly labeled PnC neurons, biocytin immunocytochemistry was carried out preceding NADPH diaphorase histochemistry using a Vector ABC kit using the PAP method with diaminobenzidine as the chromogen. Sections were mounted on gelatin-coated slides and coverslipped with Eukitt (Calibrated Inst., Hawthorne, NY) for brightfield optics.

Statistical procedures

STATISTICAL MODELING. For comparison of data between the different groups in each experiment, measures were tested using one-factor, two-factor, or multifactor ANOVA to conclude whether any of the factors had a significant effect on the magnitude of the variable and also whether the interaction of the factors significantly affects the variable. Differences were considered significant at values of $P \leq 0.05$. If a statistical significance was present, a post hoc test (Scheffe) was used to compare between groups. Each of the measures of intrinsic membrane properties and responses to low versus medium versus high frequency of stimulation were compared using two-factor ANOVA (e.g., response amplitude and stimulation frequency). Comparisons of duration of effect that involved repeated observations on the same neurons, e.g., responses to neuroactive agents over time, were carried out using repeated measures ANOVA since each time sampled could be considered a different “treatment” on each cell. When statistical significance was evident, the post hoc test (Scheffe) was carried out to determine differences between groups of cells across treatment (e.g., duration or frequency of response).

POWER ANALYSIS. Considering, for example, the analysis of AHP amplitude between two groups (prolonged response (PR) vs. nonprolonged response (NPR) cells, Fig. 2C), with an $n = 49$ for NPR and $n = 91$ for PR cells, we had in excess of a two standard error difference in group means. Even with an $n$ of about 15 cells per group, we had in excess of 80% power to detect a difference at the 5% level of significance.

RESULTS

Localization

We recorded a total of 140 cells, which met the criteria of stable RMP less than or equal to −55 mV and action potential ≥40 mV. These neurons were localized in the posterior part of the oral pontine reticular formation (PhO) and throughout the caudal pontine reticular formation (PnC) at the anterior edge of the gigantocellular tegmental field. The cells studied were found between the lateral edge of the midline raphe medially, extending laterally to the medialmost edge of the 7th nerve. Figure 1 is a drawing of a representative histological section of one of the semihorizontal slices used. The localization of a selected sample of the neurons recorded that were well filled with biocytin reflects the distribution described. While only one neuron per side per slice was injected with biocytin, recordings were also carried out in noninjected cells in the vicinity of the injected neurons but still within the boundaries described. Functionally, the region studied appeared to be well posterior and medial to the pontine inhibitory area (Baghdoyan et al. 1984; Yamamoto et al. 1990), to be dorsal to the trapezoid body, and overlap with the distribution of giant PnC neurons known to mediate the SR (Davis 1984; Koch 1999; Swedlow et al. 1992). The region sampled was intended to be equivalent to that sampled in our previous studies in the decerebrate cat PnC (Garcia-Rill et al. 2001).

Electrophysiological properties

We studied a total of 140 PnC neurons, of which 91 (65%) showed PRs and 49 (35%) did not show PRs (NPR). The definition of a PR requires explanation. In our previous study using extracellular recordings in the decerebrate cat brain stem, the PR was defined as the duration of the train of action potentials induced in PnC neurons following PPN stimulation from the beginning of the response until firing had ceased for
1 s (Garcia-Rill et al. 2001). The present intracellular studies revealed that PPN stimulation induced a long-lasting depolarization in PnC neurons on which was superimposed a train of action potentials. For comparative purposes, the duration of the PR herein still is defined as the duration of the action potential train until firing had ceased for 1 s. However, the underlying, and more important, mechanism at play is obviously the much longer-lasting depolarization induced by PPN stimulation. In the sample recordings that follow, the prestimulation membrane potential is denoted by a dotted line to facilitate detection of the depolarization induced in PR cells. We will first describe the general electrophysiological characteristics of PnC cells across age, then compare the properties of PR and NPR cells, and, finally, turn to detailing the features of the PRs.

EFFECTS OF AGE. Of the cells recorded, 67/140 were studied in slices from pups younger than 16 days old and 73/140 in slices from pups 16–21 days old. That is, we compared cells recorded during the first half of the developmental window studied to those recorded during the second half. The rationale behind this division is related to the known change at around 15 days of age in the descending control of locomotor and swimming movements in the rat (Beckoff 1979; Iwahara et al. 1991). For example, if a spinal cord transection is made before 15 days of age, rats recover the capacity for spontaneous locomotion, whereas they do not recover such capacity if the transection is performed after 15 days (Stelzen et al. 1975; Weber and Stelzner 1977). There were no statistically significant differences between PnC cells in these age groups on the basis of mean ± SD RMP (−62 ± 7 mV vs. −62 ± 6 mV), action potential threshold (−46 ± 9 mV vs. −48 ± 8 mV), action potential amplitude (51 ± 8 mV vs. 53 ± 10 mV), action potential duration at half-amplitude (1.0 ± 0.4 ms vs. 0.7 ± 0.4 ms), AHP amplitude (17 ± 4 mV vs. 18 ± 4 mV) or \( R_{in} \) (105 ± 42 MΩ vs. 99 ± 47 MΩ), regardless of response type. However, there was a significant decrease across these ages in AHP duration for all cells (140 ± 68 ms before vs. 116 ± 58 ms after day 16; \( P < 0.02 \)).

<table>
<thead>
<tr>
<th>Property</th>
<th>PR Cells</th>
<th>NPR Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR duration, s (1 s, 400 μA, 60 Hz)</td>
<td>14 ± 4</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>Resting membrane potential, mV</td>
<td>−62 ± 6</td>
<td>−61 ± 7</td>
</tr>
<tr>
<td>Membrane input resistance, MΩ</td>
<td>108 ± 62</td>
<td>130 ± 79</td>
</tr>
<tr>
<td>Action potential threshold, mV</td>
<td>−46 ± 9</td>
<td>−47 ± 9</td>
</tr>
<tr>
<td>Action potential amplitude, mV</td>
<td>50 ± 9</td>
<td>51 ± 9</td>
</tr>
<tr>
<td>Action potential duration, ms (1/2 amp.)</td>
<td>0.9 ± 0.4</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>AHP duration</td>
<td>131 ± 60</td>
<td>132 ± 75</td>
</tr>
<tr>
<td>AHP amplitude</td>
<td>19 ± 3*</td>
<td>15 ± 4</td>
</tr>
</tbody>
</table>

Values are means ± SD. Number of PR cells is 91 and NPR cells is 49. PR, prolonged response; NPR, nonprolonged response; PnC, caudal pontine reticular nucleus. \( * P < 0.01 \).

TABLE 1. Electrophysiological properties of PR and NPR neurons in PnC

Figure 2A shows a PR cell with a lower amplitude AHP along with a more typical PR cell (Fig. 2B) with a higher amplitude AHP. The distribution of PR and NPR cells of all ages plotted in terms of AHP amplitude versus AHP duration are shown in Fig. 2C (it should be stressed that AHP duration was always measured in individually occurring action potentials at RMP or if no spontaneous activity was evident, after depolarizing the membrane potential until individual action potentials were induced, therefore the durations shown in this figure include AHPs in the range of −45 to −65 mV). As stated above, AHP amplitude was statistically different between these response types (NPR 15 ± 3 mV vs. PR 19 ± 3 mV, \( P < 0.01 \)), but AHP duration was not when neurons of all ages were included (Table 1). Both PR and NPR cells showed “A type” (slow repolarization, or sag, after the end of hyperpolarizing pulses, not shown) or “LTS type” (burst of action potentials on a hump after the end of hyperpolarizing pulses) currents (Fig. 2B, arrow). Of the PR cells recorded, 52% showed an A type current, while 14% showed an LTS type current. NPR cells showed a similar proportion of cells with A type (43%) and LTS type (14%) currents. Another current observed in PnC cells was \( I_h \) (a depolarizing current activated by hyperpolarization) and is evident as a time-dependent sag during the last hyperpolarizing step in Fig. 2A, but not in the cell in Fig. 2B. This current was present in 36% of PR cells and 38% of NPR cells. It should be noted that these cell types and their respective currents have been described previously in the pontine reticular formation, and the currents identified pharmacologically (Greene et al. 1989; Ito and McCleary 1984; Steriade and McCarley 1990; Stevens et al. 1992); however, no such pharmacological confirmation was undertaken in the present study.

In PR type PnC neurons, PPN stimulation using single pulses was found to induce short-latency excitatory postsynaptic potentials that decreased in latency and increased in amplitude, and elicited action potentials, as stimulating current levels were increased. The mean ± SD latency to the beginning of the excitatory postsynaptic potential following PPN stimulation using single pulses was 6.1 ± 1.4 ms for the cells tested (\( n = 13 \)). The mean threshold for inducing an excitatory postsynaptic response in both types of PnC neurons following single pulse PPN stimulation was 311 ± 96 μA. The responses of PnC neurons following stimulation of the PPN were similar to those previously reported for descending PPN projections to pontomedullary locomotor areas (Garcia-Rill and Skinner 1987a,b).

PROLONGED RESPONSES (PRs). If trains of stimuli were used, the brief, single action potential responses described above became PRs in 91/140, or 65%, of PnC neurons (Fig. 2D, top).
PPN stimulation (using trains of 1-s at 60 Hz) was applied. Calibration bar vertical 10 mV, horizontal 1 s. Dashed lines added to show prestimulation membrane potential.

and the PR began during, at the end, or within 1 s of the membrane potential being depolarized during the stimulus train evident in PnC neurons with PRs. In some PnC cells (39%), the membrane potential was depolarized for longer duration (1-s) by longer duration (100-ms) trains at 60 Hz, but depolarized for longer (50–100 ms) by shorter duration AHP (left side, depolarized to ~50 mV to induce a single action potential), and nonadapting firing frequency during depolarizing pulses (right side). This particular PR cell had a prominent “LTS” (burst of action potentials on a hump after the end of hyperpolarizing pulses, diagonal arrow after the rebound burst) currents. It should be noted, however, that both PR and NPR cells could also exhibit a prominent “A” current. Calibration bar for A and B, vertical 10 mV, horizontal 50 ms. C: graph of AHP amplitude vs. duration in PR (●, n = 91) and NPR (○, n = 49) PnC neurons recorded at all ages (12–21 days). There was no statistically significant difference between PR and NPR cells in terms of AHP duration (although numerical decreases across age were greater in NPR than PR cells, see text); however, AHP amplitude was significantly greater in PR cells (see text). D: example of a PR cell (12 day) that responded following PPN stimulation (1-s train of 0.5-ms pulses of 500-μA amplitude at 60 Hz) showing a depolarization from the prestimulation RMP (~62 mV) and prolonged action potential train (top record). Example of a NPR cell (18 day) that was hyperpolarized from RMP (~60 mV) following identical stimulation applied to the PPN (bottom record). Calibration bar vertical 10 mV, horizontal 1 s. Dashed lines added to show prestimulation membrane potential.

The PR (operationally described as the duration of the train of action potentials induced) was comparable to results previously described in PnC neurons in the decerebrate cat (Garcia-Rill et al. 2001). However, the duration of the depolarization underlying these action potentials varied considerably, typically lasting <30 s. A maximum stimulus train duration of 1 s was selected for testing because stimulation for longer durations is known to recruit locomotion in decerebrate animals (Garcia-Rill 1991; Garcia-Rill et al. 1987, 1988; Skinner and Garcia-Rill 1984), while 1-s trains elicit only brief spinal cord activation. This also allowed comparison with previous results on PnC neurons in decerebrate animals (Garcia-Rill et al. 2001). Figure 3A shows the responses of a PnC neuron following administration of trains of increasing duration. In general, the membrane potential was depolarized briefly (1–2 s) by short-duration (100-ms) trains at 60 Hz, but depolarized for longer durations (>5–10 s) by longer duration (1-s) trains (Fig. 3A). The mean ± SD amplitude of the depolarization induced by PPN stimulation (using trains of 1 s at 60 Hz) was 4.8 ± 1.3 mV (n = 24). Two types of predepolarization activity were evident in PnC neurons with PRs. In some PnC cells (39%), the membrane potential was depolarized during the stimulus train and the PR began during, at the end, or within 1 s of the termination of the train (Fig. 3B). In the other PnC neurons with PRs (43%), the membrane potential was hyperpolarized during or at the end of the stimulation train (Fig. 3A and C). That is, the PR could be elicited following depolarization or hyperpolarization induced by the stimulation. The remaining 18% of cells showed a poststimulation membrane potential (preceding the PR) equal to the prestimulation membrane potential. As evident in the figures provided (e.g., Figs. 3 and 4), there was a delay between the end of the stimulus train and the beginning of the PR in many neurons. In a group of PR neurons (n = 20) tested with identical stimulation parameters, the latency of the PR was similar using trains of 10 Hz (0.3 ± 0.5 s), 30 Hz (0.6 ± 0.8 s), and 60 Hz (0.5 ± 0.6 s), but increased significantly (P < 0.05) when using trains of 90 Hz (1.3 ± 1.1 s). It should be noted that the increase in latency was not due to a difference in the membrane response preceding the PR, i.e., a higher amplitude hyperpolarization or other factor. There was no obvious difference in electrophysiological properties between neurons that responded within or immediately following the stimulation train (e.g., Fig. 2D), compared with those that responded after some delay (e.g., Fig. 3A–C). Even in the same cell, the latency of the PR could differ somewhat across repeated trials (Fig. 3C).
PRs were stimulation frequency dependent such that low frequencies (10 Hz) of stimulation produced lower amplitude, short-lasting membrane depolarizations, but, as stimulation frequency was increased (30 Hz), the depolarization became prolonged and a maximal duration PR was evident at 60 and 90 Hz (Fig. 3B). However, it is evident that the underlying depolarization observed after 60-Hz stimulation was typically of higher amplitude than after 90-Hz stimulation, despite the similar duration of action potential trains. Delivery of intracellular hyperpolarizing pulses failed to reset the PR, whether the current step was of sufficient amplitude to return the membrane potential to the resting level (Fig. 3C, middle, −60 mV) or to significantly more negative membrane potentials (Fig. 3C, bottom, −80 mV). Such an effect provided the suggestion that PRs may not be calcium dependent (see following text).

PRs were also stimulus amplitude and membrane potential dependent. Figure 4A shows that stimulation at increasing amplitudes using trains of the same frequency (60 Hz in this case) induced maximal duration PRs at 400 μA (14 ± 10 s) compared with 200 or 300 μA. The underlying depolarization also increased in amplitude (compare change in amplitude of depolarization in Fig. 4A, top vs. middle vs. bottom). This representative recording shows the complex nature of the responses of PnC neurons following PPN stimulation. At low-amplitude current levels (Fig. 4A, top), the membrane potential was at first depolarized significantly (along with a couple of action potentials) and was followed by a lower amplitude but persisting depolarization. That is, there may be at least two components to the PRs, a brief, higher amplitude depolarization (early phase), and a long-lasting, lower amplitude persisting depolarization (late phase). Higher amplitude current levels induced trains of action potentials that obscured the initial depolarization (early phase), but it was clear that, as current levels increased, the amplitude of the late phase of the depolarization increased. Figure 4A, top, shows that stimulation using 200-μA pulses induced a small but consistent late phase depolarization. Figure 4A, middle, shows that the amplitude of the late phase depolarization increased. An expanded portion of this record 2–3 s after the beginning of the PR reveals the considerable depolarization induced, an effect that persisted as a prolonged depolarization after the action potentials had ceased. The expanded record in Fig. 4A, bottom, shows no further significant increase in the amplitude of the depolarization occurred when using 400-μA pulses. In general, higher amplitude currents induced shorter latency, increased amplitude and increased duration responses, including action potential trains and underlying early and late phase depolarizations.

Figure 4B shows the effects of membrane potential on the underlying depolarization induced in PnC neurons following stimulation of the PPN using trains of stimuli. In this case,
progressively higher amplitude initial depolarizations were induced as the holding potential was decreased from $-1100^268$ mV to $-1100^256$ mV to $-1100^251$ mV to $-1100^249$ mV, whereas the persistent depolarization did not appear to change significantly. These effects suggest that potassium channels and/or metabotropic receptors might be involved in the underlying depolarizations (initial and/or prolonged) of the PR (see following text). By definition, in this case, a PR (defined narrowly as the duration of the train of action potentials, to allow comparison with previous extracellular studies) was induced by PPN stimulation when the holding potential was greater than $-1100^250$ mV. In the population of PnC neurons recorded, the effects of PPN stimulation were tested at RMP and at more depolarized levels using current injection. The range of membrane potentials in which PRs could be elicited following PPN stimulation was $-1100^265$ to $-1100^245$ mV, although most cells showed responses in the range of $-55$ to $-45$ mV. In most cells, further depolarization induced tonic activity, precluding testing for PRs at potentials less than $-45$ mV.

Figure 5A is a graph of the mean duration of PRs induced by specific frequencies of stimulation tested using identical stimulation parameters in a group of 20 PR cells (0.5 ms duration pulses of $400-\mu A$ amplitude each using a 1-s train). The duration of the PR, as stated above, was determined to be from the first action potential following the depolarization to the last action potential without a 1-s cessation of firing. The mean ± SD duration of the PR induced at 10 Hz was $3 ± 3$ s, at 30 Hz it increased to $5 ± 4$ s, whereas the mean duration of PRs induced by trains of 60 Hz was $14 ± 10$ s, and at 90 Hz it was $14 ± 15$ s. The durations of these PRs were statistically different such that the mean duration at 30 Hz was longer than that at 10 Hz ($P < 0.01$), and that at 60 Hz was longer than that at 30 Hz ($P < 0.01$). However, there was no significant difference between the mean durations of PRs induced by 60 versus 90 Hz; i.e., there was a plateau effect such that PR durations did not keep increasing significantly with stimulation frequency beyond 60 Hz. Although the mean duration of PRs induced by 60 versus 90 Hz trains was not statistically different, the PR durations of individual neurons using 90-Hz trains compared with 60-Hz trains were found to decrease in 6/20 cells, to increase in 8/20 and remain the same in 6/20. That is, the trend was not toward increasing PR duration at frequencies >60 Hz.

There was a difference between the effects of trains of different frequencies and the firing frequency induced in PnC neurons during the PRs. Figure 5B is a graph of the mean firing frequency induced during the 2nd second of the PR (activity during the 1st second of the PR varied considerably from
subsequent activity, therefore the 2nd second was considered to be a steady-state firing frequency. The mean ± SD of the firing frequency of PnC neurons following stimulation using trains of 10 Hz (3 ± 3/s) increased following 30-Hz trains (5 ± 4/s, \( P < 0.01 \)), then increased markedly following 60-Hz trains (10 ± 3/s, \( P < 0.01 \)). This effect appeared to peak at 60 Hz since firing frequency decreased using 90-Hz trains (8 ± 3/s, \( P < 0.01 \)). That is, the maximal firing frequency induced in PnC neurons during PRs following PPN stimulation was in the range of 10 Hz, and that was effected best when using trains of 60 Hz. Firing frequencies using 90-Hz trains were lower in 15/20 cells, the same in 2/20 cells and higher in 3/20 cells. That is, the trend was toward decreasing maximal PnC firing frequencies at stimulation rates > 60 Hz.

A prominent feature of PRs was the presence of changes in \( R_{\text{in}} \) during PRs. Figure 6A shows a representative PnC neuron with a PPN stimulation-induced (1 s duration 60-Hz train of 400 \( \mu \)A, 0.5-ms pulses) PR. The amplitude of hyperpolarizing pulses applied intracellularly to measure conductance are evident before PPN stimulation (Fig. 4A, bottom, left side). After the stimulus, the membrane potential was depolarized slightly and action potentials were elicited. The amplitude of hyperpolarizing pulses decreased in amplitude during the first few seconds of the PR, indicative of a decrease in \( R_{\text{in}} \). However, the membrane potential was manually restored by current injection (Fig. 6A, bottom, underline), and the amplitude of the hyperpolarizing pulses increased in amplitude after the membrane potential had been restored by current injection, indicating an increase in \( R_{\text{in}} \). Figure 6B is a graph of the \( R_{\text{in}} \) observed before PPN stimulation (Pre-STIM) compared with the \( R_{\text{in}} \) observed in the same cells after the membrane potential had been restored manually (PR). That is, the \( R_{\text{in}} \) of each cell before PPN stimulation (such as that shown in Fig. 6A, bottom, before the stimulation artifacts) is compared with that during the restored membrane potential (such as that shown in Fig. 6A, bottom, during the underlined segment). In the cells tested (\( n = 12 \)), the input resistance increased by an average of 18 ± 13 MΩ during the PR (statistically significant increase from 88 ± 25 MΩ to 106 ± 30 MΩ, \( P < 0.01 \)).

Pharmacological properties

We then studied the possible nature of the mechanism(s) involved in generating PRs. One potential mechanism that is known to produce responses greatly outlasting the activation of inputs is that of plateau potentials. Plateau potentials are intrinsic membrane properties such that prolonged depolarization can be induced by brief depolarizing steps, which can be reset by hyperpolarizing steps and are usually calcium channel dependent (Hultborn and Kiehn 1992). PPN stimulation-induced PRs in PnC neurons, however, could not be elicited by depolarizing pulses (0.3 nA, 100 ms at 2 Hz) applied intracellularly to measure conductance. The dashed line indicates the peak response of the hyperpolarizing pulses applied before stimulation. Calibration bars, vertical 10 mV, horizontal 500 ms. B: graph of the input resistance (MΩ) at the peak of the hyperpolarizing pulses applied to the PnC neurons \( (n = 12) \) tested before (Pre-STIM) PPN stimulation and during the PPN stimulation-induced PR but after the membrane potential had been restored (as in the underlined period in the bottom record in A). Note that input resistance increased significantly (** \( P < 0.01 \)) in all 12 neurons tested during the PR with compensated membrane potential.
larizing steps (Fig. 2, A and B), or reset, once elicited, using hyperpolarizing steps (Fig. 3C, n/H1100591). Moreover, superfusion with the calcium channel blocker nifedipine (10μM) failed to block PPN stimulation–induced PRs (n/H110057, not shown). A final test for the presence of plateau potential properties was the determination of a negative slope on the I-V curve, characteristic of bistable properties (Kim and Chandler 1995).

Figure 7A shows the PR of a PnC neuron following PPN stimulation using a 1-s long, 60-Hz train of pulses of 500μA in amplitude. Ramp stimulation was induced, in this case between −75 and −20 mV, before, during, and after recovery from TTX superfusion (0.3μM; Fig. 7B). The I-V curves during two cycles of ramp stimulation after TTX were linear and overlapped considerably (Fig. 7C), indicative of the absence of bistable properties (i.e., no negative slope in the I-V curve) in seven PnC cells with PRs tested in this manner. It should be noted that, since TTX superfusion blocked sodium channels and the generation of action potentials, PPN stimulation-induced depolarization and PRs in PnC neurons were absent, in keeping with their synaptic nature.

Since many PPN neurons are cholinergic, we tested the effects of cholinergic agents on the manifestation of PRs in PnC neurons. Superfusion (30μM) of the muscarinic cholinergic antagonist scopolamine (SCOP) reduced or blocked the underlying depolarization and PR induced by PPN stimulation (1-s train of 60 Hz at 400-μA amplitude) in 5/22 cells tested (Fig. 8A) but was ineffective in reducing the PR (tonic firing) or underlying depolarization in 17/22 cells. It is not clear why SCOP was ineffective in modulating PRs in some neurons, since the cells tested probably had access to the SCOP at the concentration used. One possibility is that some PRs are not mediated by cholinergic mechanisms. On the other hand, superfusion (5μM) or micropressure application (30μM), of the cholinergic agonist carbachol (CAR) on PR neurons was found to induce long-lasting depolarization in most PnC neurons. Figure 8B shows the responses of a PnC neuron following PPN stimulation (a PR) and after CAR superfusion (5μM; depolarization and prolonged activation), along with blockade of the CAR-induced effect by superfusion (30μM) of the muscarinic receptor blocker scopolamine (SCOP) 2 min before CAR superfusion. A total of 33 PnC neurons was tested using CAR, 24 PR type cells, and 9 NPR type cells. Interestingly, 20/24 PR and 5/9 NPR cells were depolarized by CAR (as in Fig. 8B), while two cells of each type were hyperpolarized by CAR and two cells of each type showed no response. Since some NPR cells were depolarized by CAR but showed no PRs, the cholinergic receptors were incapable of inducing PRs or the effects of CAR on NPR neurons were indirect. It is not clear why CAR did not affect two of the PR cells tested, whether CAR had insufficient access to the cells being recorded, or whether some PRs were due to noncholinergic mechanisms.
Interestingly, some PnC neurons were hyperpolarized by CAR (2 of each type), in keeping with known cholinergic inhibition in this region (see following text). For example, Fig. 10 (middle) shows the hyperpolarization induced in a PnC neuron by PPN stimulation, an effect matched by CAR superfusion (5 μM). The two NPR neurons that were hyperpolarized by CAR had relatively low \( R_{in} \) (63 ± 11 MΩ) compared with PR neurons that were depolarized by CAR (95 ± 41 MΩ), or cells of both types that showed no response to CAR (105 ± 23 MΩ).

Since a subpopulation of cholinergic PPN neurons also contain glutamate as a co-transmitter (Lai et al. 1993), we tested the possible roles of ionotropic and metabotropic glutamate receptors on PnC neurons showing PPN stimulation-induced PRs. The nonspecific ionotropic glutamate receptor antagonist kynurenic acid (300 μM) failed to block PPN stimulation-induced PRs in five cells tested, as did the metabotropic glutamate receptor antagonist MCPPG (300 μM; \( n = 4 \); not shown).

To test the potential mechanism for the increased resistance/decreased conductance, we used BaCl₂ superfusion (0.5–2 mM) to test the possibility that it was due to closure of potassium channels. Figure 9A shows the effects of BaCl₂ superfusion (1 mM) on the manifestation of the PR in a PnC neuron. BaCl₂ superfusion reduced or completely blocked the depolarization induced by PPN stimulation without affecting baseline membrane potential. During BaCl₂ superfusion and wash out, the amplitude of the AHP was reduced and AHP duration increased, an effect that was completely reversed by 5 min after wash out. While it is problematic to measure AHP amplitude during high-frequency firing, since this would tend to yield a lower amplitude AHP than after spontaneous/individual spikes, the effects of BaCl₂ on AHP amplitude are all the more impressive. Representative recordings before, during, on wash out, and after recovery from BaCl₂ are shown in Fig. 9B. AHP amplitude during the control PR, which were reduced by the high firing frequency, was decreased during BaCl₂ superfusion even though isolated spikes were being measured. The amplitude of the AHP began to recover during wash out when individual spikes were measured. The reduction in mean ± SD AHP amplitude from control (19 ± 1 mV, artificially low due to repetitive firing), during BaCl₂ superfusion (15 ± 2 mV, \( P < 0.01 \)) and during wash out (19 ± 2 mV, individual spikes) are shown in graphed form in Fig. 9C.

**Anatomical characteristics**

The present studies include only limited morphometric analysis of somatic size in well-injected neurons. These cells were measured as follows: dendrites were truncated at the base, the long and short axes of the cell body measured for each neuron, and long versus short axis ratio calculated. The area of each cell was also measured using an image analysis system with National Institutes of Health software. There were no statistically significant differences between PR and NPR cells in terms of long versus short axis ratio (35 PR cells 1.86 ± 0.51 vs. 20 NPR cells 1.98 ± 0.75), or in terms of cell area (35 PR cells 451 ± 190 μm² vs. 20 PR cells 393 ± 155 μm²). There was a large variability in cell area measures, suggesting the presence of different cell sizes within each population. Of course, recordings were carried out over an age range during which there is considerable growth, which may have contributed to the lack of anatomical differentiation of PnC neurons correlating with physiological responses. When the measured neurons were divided according to age, PR cells <16 days (\( n = 18 \)) had a mean area of 420 ± 209 μm² compared with those ≥16 days, which had an area of 484 ± 165 μm² (\( n = 17 \); NS). The difference across age was greater for NPR neurons (<16 days, \( n = 10 \), 346 ± 129 μm² vs. ≥16 days, \( n = 10 \), 442 ± 173 μm²; NS). Although there was a numerical increase across age for both cell types, the difference was not statistically different. The variability in cell sizes suggests the presence of groups of cells of different mean areas. There were also no statistically significant differences between PR and NPR cell area across age. For illustrative purposes, we have included reproductions of injected PR and NPR cells in Fig. 10. In this case, the PR neuron (328 μm²) was typical of injected PR and most NPR neurons. The NPR neuron shown was hyperpolarized by PPN stimulation and was larger than most other NPR neurons (625 μm²).

![Figure 8](http://jn.physiology.org/)

**FIG. 8.** Effects of cholinergic agents on PR neurons. A: PPN stimulation induced a PR in this PnC neuron (17 day) with a −65-mV RMP (control, top record). However, the PR was blocked by superfusion of the muscarinic cholinergic antagonist scopolamine (SCOP; 30 μM) 3 min before this trial (middle record), an effect that recovered following wash out of SCOP (bottom record). Note that both the action potential train of the PR and the underlying depolarization were blocked or decreased by SCOP. Calibration bar for A and top record in B, vertical 10 mV, horizontal 1 s. B: PPN stimulation induced a PR in another PnC neuron (17 day) with a −65-mV RMP (control, top record). Micropressure application of the cholinergic agonist CAR (30 μM; arrow, middle record) induced a prolonged depolarization in this PnC neuron that recovered after wash out (not shown). The membrane potential was held at −70 mV because SCOP generally induced a hyperpolarization on most PnC neurons tested. Also note that the sampling rate is responsible for the variable action potential amplitude in this longer record. However, if carbachol (CAR; 30 μM) micropressure application (arrow) was preceded by SCOP (30 μM) 3 min prior to the start of the bottom record, the CAR effect was blocked by the SCOP pretreatment. Calibration bar for middle and bottom records in B, vertical 10 mV, horizontal 2 s.

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DISCUSSION

Implications of major results

The implications of the main findings described herein are as follows. 1) PPN stimulation using medium frequency stimulation (60 Hz) induced PRs with longer duration in a large population (65%) of small-medium PnC neurons compared with stimulation at lower frequencies. These results are similar to those previously described in the decerebrate cat and suggest the presence of a frequency-dependent effect of PPN projections to PnC neurons (Garcia-Rill et al. 2001).

2) PPN stimulation at about 60 Hz was the most effective frequency range for eliciting the maximum firing rates (about 10 Hz) in PR cells following PPN stimulation (compared with 10, 30, or 90 Hz). This effect indicates that the maximal firing frequency induced following PPN stimulation was at frequencies known best to induce locomotion, suggesting a possible explanation for the long-known, but little understood need to use such frequencies when stimulating the mesopontine region to induce locomotion (Garcia-Rill 1991; Shik et al. 1966). In addition, 10 Hz is in the range of frequencies (5–20 Hz) required for electrical stimulation of the medioventral medulla to induce locomotion (Kinjo et al. 1990). However, additional study is need to verify that activity in some PnC neurons at a, presumably maximal, rate of 10 Hz is correlated with recruiting locomotor, or other types of motor activity. Interestingly, this is the mean frequency of physiological tremor, the upper limit of individual movements, and is thought to originate as a descending command (reviewed in Llinas 2001). This command is thought to act as a cueing function for synchronizing motoneurons, to provide inertia for overcoming friction and viscosity in muscles, and as a control system for binding inputs and outputs in time (Llinas 2001).

3) The developmental decrease in AHP duration across age may be simply an indication of maturing mechanisms that will allow neurons to respond at faster firing rates. However, the selective decrease in AHP duration in NPR but not in PR cells suggests that PR cells have a stable, maximal firing rate early in development, which is only marginally changed with maturation. Given the range of the durations of the AHP (90–130 ms), firing rates would be expected at 8–12 Hz. 4) PRs were voltage dependent, being present at a range between −65 and −45 mV (see following text). 5) PR cells did not show a...
FIG. 10. Hypothetical role of descending PPN projections to PnC and their ultimate effects on the spinal cord. The records in the top half of this figure (PnC) include an example (17 day) of the 65% of PnC neurons with a PR following PPN stimulation (PnC, top); and of 1 of the few NPR PnC neurons (18 day) found to be hyperpolarized following PPN stimulation (PnC, bottom) using identical stimulation parameters (0.5-ms pulses, 60 Hz, 300 μA, 1-s trains). Drawings of the 2 cells shown are on the left side (PnC, PR cell, NPR cell). Please note that PR and most NPR cells were similar in size and morphology to the PR cell shown here, while only a few NPR cells were as large as the one shown here. The relatively small (328 μm²) PR neuron, by definition, exhibited a prolonged response following PPN stimulation using a 1-s train of 300-μA pulses delivered at 60 Hz (PnC, top left). The same cell was depolarized following a 1-min application of CAR (5 μM; PnC, top right). The larger (625 μm²) NPR neuron (PnC, bottom) showed hyperpolarization following PPN stimulation using the same parameters (PnC, bottom left) and also was hyperpolarized following a 1-min application of CAR (5 μM; PnC, bottom right). The records on the bottom half of this figure (Spinal Cord) are electromyograms (EMG) of hindlimb antagonist muscles (extensor above, flexor below) showing alternating activity (locomotion) in response to PPN stimulation in the decerebrate animal using 60-Hz pulses slowly ramped up to threshold and then applied continuously (bottom underline labeled “60 Hz Cont.”). The locomotion recruited was interrupted by a brief, high-frequency train (thick bar labeled “100 Hz, 300 ms”) of stimuli to the PPN, leading to decreased extensor and increased flexor muscle tone. Ultimately, these transient effects were overcome by the continued 60-Hz stimulation. The hypothetical Push-Pull mechanism proposed suggests that PPN stimulation at the appropriate frequencies (~60 Hz) for recruiting locomotion activates large numbers of PnC neurons (presumably interneurons that ultimately activate reticulospinal elements) via cholinergic receptors to drive these cells into a new state (Push), while inhibiting other PnC neurons that normally inhibit muscle tone (Pull). Presumably (and less certain given the paucity of evidence), when a sudden, high-frequency stimulus is applied to the PPN (e.g., a startling stimulus), there is preferential activation of pathways that inhibit extension and activate flexion, such as during the SR. Much additional evidence is needed to support this suggestion, although the findings described herein lend some support to this hypothesis. Calibration bars, PnC, PR, and NPR cell bar 100 μm; top left records PPN stimulation 10 mV and 1 s; top right records CAR superfusion 10 mV and 20 s; Spinal Cord, bottom EMG records 500 μA, 1 s.
negative slope in the $I-V$ curve, induction of the PR by depolarizing pulses or resetting of the PR by hyperpolarizing pulses. In addition, the calcium channel blocker nifedipine failed to alter the manifestation of PRs, so that these responses are unlikely to be plateau potentials (Hultborn and Kiehn 1992). However, PRs could be reduced or blocked in some neurons by the muscarinic antagonist SCOP in neurons that were also depolarized by superfusion of the muscarinic agonist CAR, suggesting that a muscarinic receptor is involved in generating some PRs. However, the effects of SCOP were not evident in a number of PnC neurons tested, indicating that there may be multiple mechanisms, as yet unidentified, involved in the generation of PRs. 7) Some PnC neurons were hyperpolarized by CAR superfusion, suggesting that descending PPN projections have differential effects on certain populations of PnC neurons. 8) Although some PPN neurons also release glutamic acid, PRs induced in PnC cells by PPN stimulation were not reduced or blocked by ionotropic or metabotropic glutamate antagonists, suggesting that PRs are not glutamate dependent. 9) PRs may have multiple components, an early phase of higher amplitude followed by a late phase during which there was an increase in input resistance, suggesting that PPN stimulation may lead to the closure of some channels in PnC neurons with PRs. The finding that PRs were reduced by membrane hyperpolarization, and blocked by BaCl$_2$ superfusion, indicates that PRs may be mediated by the closure of potassium channels. These results are in keeping with findings indicating that muscarinic agonists can depolarize the membrane in certain neurons, leading to lowered spike frequency accommodation and loss of the slowly decaying portion of the AHP, changes that greatly enhance the level of neuronal excitability (Washburn and Moises 1992). Voltage-clamp analysis of these effects showed that the muscarinic-induced depolarization resulted from inhibition of voltage-activated and voltage-insensitive potassium leak currents (Womble and Moises 1992). These results suggest that PPN projections to PnC may activate muscarinic receptors that block potassium channels and lead to depolarization and higher firing frequencies. Additional testing is required to determine the potential contributions of voltage-activated versus leak $K^+$ currents in the manifestation of PRs in PnC neurons. Moreover, we do not know whether the early and late phases of the PR are differentially controlled. For example, nicotinic and muscarinic activation of pontine neurons has been reported previously (Stevens et al. 1992). Some PnC neurons may resemble thalamic cells, which, in response to PPN stimulation, show a nicotinic receptor-induced decrease in $R_{\text{in}}$, followed by a muscarinic receptor-induced increase in $R_{\text{in}}$ (Curro-Dossi et al. 1991). However, pharmacological studies need to be performed to determine whether early and late phases might be modulated differentially.

**Functional implications**

The PPN sends widespread projections throughout the pontomedullary reticular formation (Reese et al. 1995; Rye 1997; Scarnati and Florio 1997), including the anterior pontine region (PnO) (Mitani et al. 1988). Injections of cholinergic agonists into a region called the pontine inhibitory area induce a REM sleep-like state (Baghdoyan et al. 1984; Yamamoto et al. 1990), an effect thought to be mediated via muscarinic $M_2R$ receptors (Curro-Dossi et al. 1991). 1990), an effect thought to be mediated via muscarinic $M_2R$ receptors (Curro-Dossi et al. 1991). 1990). 1990), an effect thought to be mediated via muscarinic $M_2R$ receptors (Curro-Dossi et al. 1991).

Lesion of this area can produce REM sleep without atonia (Henley and Morrison 1974; Jouvet 1975), but these lesions may also damage neurons/axons responsible for REM sleep initiation and maintenance (Sanford et al. 1994). Comprehensive electrophysiological studies on pontine reticular neurons have been performed by McCarley (reviewed in Steriade and McCarley 1990), who found LTS burst and non-bursting neurons, some with a transient outward A current (similar to those reported herein for the PnC). These neurons receive excitatory cholinergic input from the lateral pontocerebellar nucleus (LDT) (Imon et al. 1996) and are depolarized by nicotinic and muscarinic agonists (Gerber et al. 1991; Stevens et al. 1993), although some cells are hyperpolarized by muscarinic agonists (Greene et al. 1989).

Descending PPN projections to the posterior pontine region (PnC) may be involved in, for example, modulation of the startle response (SR) (see comprehensive reviews on the SR for more information: Davis 1984; Koch 1999; Swerdlow et al. 1992). The pathway for the SR, which includes giant neurons in the PnC, has been worked out (Davis 1984). These giant cells make up only about 1% of PnC neurons (Koch 1999), but when lesioned by excitotoxic agents injected into this region, the amplitude of the SR is reduced (Koch et al. 1992). PPN lesions have been reported to reduce prepulse inhibition of the SR (Swerdlow and Geyer 1993), and auditory-responsive PnC neurons appear to be inhibited by cholinergic agonists (Koch et al. 1993), suggesting that the PPN modulates SR gating. On the other hand, stimulation of the cat PPN using trains of pulses produce multisynaptic, prolonged responses in PnC cells that do not show auditory responses or project to the spinal cord, i.e., they appeared to be interneurons (Garcia-Rill et al. 2001). Moreover, the present study showed that rat PnC neurons recorded in vitro showed similar excitatory PRs in many PnC neurons, while relatively few PnC neurons were inhibited, following PPN stimulation and/or CAR superfusion. This suggests that ascending PPN projections activated by medium frequencies of stimulation may inhibit few PnC cells (perhaps some SR-related neurons with spinal projections), while activating large numbers of PnC cells (possibly interneurons, as suggested by the absence of reticulospinal projections in cat PR cells) to induce PRs, thereby eliciting a lasting change in state in these cells.

The slice preparation, however, is limited in its inability to identify reticulospinal versus nonreticulospinal neurons or interneurons. Therefore we could not determine which PnC neurons are descending output neurons or interneurons. Moreover, our electrophysiological or morphological analyses do not allow us to determine which PnC neurons will develop into giant neurons, since both PR and NPR neurons included those having low $R_{\text{in}}$ ($>1$ SD below the mean $R_{\text{in}}$) and large cells ($>1$ SD above the mean cell area). All that can be concluded is that large numbers of PnC neurons are activated to produce PRs while only few are hyperpolarized/inhibited by PPN outputs. If this proves to be the case given further experimentation, descending PPN projections may exercise a “push” toward locomotion (PRs in PnC neurons) as well as a “pull” away from decreased muscle tone (inhibition of some PnC neurons), such as that observed in the SR (Koch et al. 1992, 1993), to promote stepping. In a similar fashion, ascending cholinergic projections “push” fast cortical rhythms by prolonged activation of thalamocortical relay neurons, while they
“pull” away from slow cortical rhythms by hyperpolarizing GABAergic thalamic reticular neurons responsible for inducing slow waves, thereby promoting waking (Steriade et al. 1990; Steriade and McCarley 1990). A model of this hypothesis is provided in Fig. 10.

In general, it is well accepted that the RAS, while controlling sleep/wake cycles and arousal, also modulates posture and locomotion, perhaps related to the execution of fight-or-flight responses. On the one hand, stimulation of various points in the posterior mesencephalon is known to induce controlled locomotion on a treadmill in the decerebrate cat and rat (Shik et al. 1966; Skinner and Garcia-Rill 1984). Such locomotion-inducing sites include the PPN (reviewed in Garcia-Rill 1991). Their stimulation induces short-latency excitation of hindlimb motoneurons at disynaptic latencies (Degtyarenko et al. 1998; Scarnati and Florio 1997). On the other hand, stimulation of the PPN has been found to suppress muscle tone but induce stepping (i.e., initial suppression followed by activation) (Lai and Siegel 1990). We proposed a firing frequency-dependent mechanism that can explain all of these observations, demonstrating that 1) using amplitudes that are slowly ramped up at medium frequencies of stimulation (40–60 Hz), reticulospinal pathways for locomotion are “recruited” to produce alternation of flexor and extensor hindlimb nerves, and 2) using sudden-onset trains of high frequencies (>90 Hz), locomotion can be inhibited and muscle tone suppressed (Garcia-Rill et al. 1996). The initial effect may be equated with a "reset" function, which interrupts ongoing activity, allowing the motor system to trigger or recruit subsequent activity without interference (Garcia-Rill 1991; Reese et al. 1995). Further research is required before more conclusive statements can be made regarding the frequency dependency of such complicated functions; however, this type of control system would have great survival value when involved in fight versus flight responses.

We are very grateful for the valuable comments and suggestions made by Dr. Kevin D. Phelan.

This work was supported by National Institute of Neurological Disorders and Stroke Grant NS-20246.

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