Brief Communication

Cooperative Glutamatergic and Cholinergic Mechanisms Generate Short-Term Modifications of Synaptic Effectiveness in Prepositus Hypoglossi Neurons

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To maintain horizontal eye position on a visual target after a saccade, extraocular motoneurons need a persistent (tonic) neural activity, called "eye-position signal," generated by prepositus hypoglossi (PH) neurons. We have shown previously *in vitro* and *in vivo* that this neural activity depends, among others mechanisms, on the interplay of glutamatergic transmission and cholinergic synaptically triggered depolarization. Here, we used rat sagittal brainstem slices, including PH nucleus and paramedian pontine reticular formation (PPRF). We made intracellular recordings of PH neurons and studied their synaptic activation from PPRF neurons. Train stimulation of the PPRF area evoked a cholinergic-sustained depolarization of PH neurons that outlasted the stimulus. EPSPs evoked in PH neurons by single pulses applied to the PPRF presented a short-term potentiation (STP) after train stimulation. APV (an NMDA-receptor blocker) or chelerythrine (a protein kinase-C inhibitor) had no effect on the sustained depolarization, but they did block the evoked STP, whereas pirenzepine (an M_1 muscarinic antagonist) blocked both the sustained depolarization and the STP of PH neurons. Thus, electrical stimulation of the PPRF area activates both glutamatergic and cholinergic axons terminating in the PH nucleus, the latter producing a sustained depolarization probably involved in the genesis of the persistent neural activity required for eye fixation. M_1-receptor activation seems to evoke a STP of PH neurons via NMDA receptors. Such STP could be needed for the stabilization of the neural network involved in the generation of position signals necessary for eye fixation after a saccade.

Key words: **acetylcholine; glutamate; short-term potentiation; prepositus hypoglossi; oculomotor system; rats**

Introduction

It has been shown in both cats and monkeys that neurons located in the prepositus hypoglossi (PH) nucleus encode pure eye position and functionally related position–velocity and velocity–position signals (Delgado-García et al., 1989; Fukushima et al., 1992; McFarland and Fuchs, 1992; Moschovakis, 1997). Indeed, transient pharmacological inactivations or permanent electrolytic lesions of the PH nucleus indicate that prepositus neural circuits are necessary for the generation of eye position signals subsequent to on- and off-directed saccades (Cheron and Godaux, 1987; Arnold et al., 1999; Moreno-López et al., 2002). PH neurons receive eye velocity signals from excitatory burst neurons (EBN) located in the paramedian pontine reticular formation (PPRF) (Igusa et al., 1980), and they project monosynaptically on extraocular motoneurons and other brainstem and cerebellar structures related to eye movements (McCrea and Baker, 1985). As a result, it has been suggested that PH neurons perform the neural integration of eye velocity signals into eye position motor commands in the horizontal plane (Robinson, 1981; Delgado-García et al., 1989; Moschovakis, 1997).

It has been shown recently that the sustained activity present in PH neurons during eye fixations is the result, at least partially, of synaptic events evoked by EBN, located in the PPRF, and cholinergic neurons, located either in the pontomesencephalic region and/or in the PH nucleus (Navarro-López et al., 2004). These synaptic effects were mediated by AMPA-kainate and M_1 muscarinic receptors located in PH neurons. Here, we show that train stimulation of the PPRF (mimicking eye velocity signals) evokes a short-term potentiation (STP) that follows the sustained activity of PH neurons. In this case, the activation of NMDA receptors (NMDAR) and M_1 muscarinic receptors is apparently responsible for the short-lasting potentiation of PH neurons. We also provide evidence that the interplay between NMDA and M_1 receptors seems to be mediated intracellularly through intermediate steps including the activation of protein kinase C (PKC) (Salter and Kalia, 2004). Thus, more than one synaptic mechanism seems to be involved in the generation of eye position signals characterizing the firing of PH neurons.

Materials and Methods

Animals. Experiments were performed in 25–35 rats (50–75 g) raised in the Salamanca University Animal House. Experiments were performed according to the European Union directive (609/86/EU) for the use of laboratory animals in acute experiments.

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Preparation of slices. Animals were anesthetized deeply with halothane gas and decapitated. The brain was excised and immersed rapidly in oxygenated ice-cold (4–6°C) artificial CSF (ACSF) with sucrose (234 mM) replacing the NaCl (117 mM) to maintain osmolarity. Brainstem sagittal slices (350 μm thick) were cut in cold oxygenated Ringer's solution using a Vibratome-S1000 (Technical Products International, O'Fallon, MO) and placed in an incubation chamber, where they were maintained for ~2 h at room temperature. Additional details of this *in vitro* preparation have been described previously (Yajeya et al., 2000).

In vitro *recordings.* For recordings, a single slice containing PH nucleus and rostral PPRF was transferred to an interface recording chamber (BSC-HT and BSC-BU; Harvard Apparatus, Holliston, MA) and perfused continuously with ACSF comprising the following (in mM): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_4, and 11 glucose. The ACSF was bubbled with carbogen gas (95%O_2–5%CO_2) and maintained at 30 ± 2°C.

Intracellular records from PH neurons were obtained with borosilicate glass microelectrodes (140–180 MΩ; World Precision Instruments, Sarasota, FL) filled with a potassium acetate solution (3 M) and connected to the head stage of an intracellular recording amplifier (VF180; Biologic, Claix, France). Micropipette tips were directed to the rostral third of the PH nucleus, where neurons carrying eye position signals are located (Delgado-García et al., 1989; Navarro-López et al., 2004). Only data from neurons that had stable resting potential with absolute values greater than −60 mV in the absence of DC holding currents and that presented overshooting action potentials were collected for analysis. Spike amplitude and afterhyperpolarizing potentials were measured relative to threshold.

Synaptic potentials were elicited orthodromically by stimulating the ipsilateral PPRF using a monopolar stainless-steel electrode (2 MΩ of effective resistance; World Precision Instruments) and a programmable stimulator (Master-8; AMPI, Jerusalem, Israel). Single, cathodal, square-wave pulses of 100–200 μs duration and 100–500 μA intensity were adjusted to subthreshold values for orthodromic spike generation. Postsynaptic potentials were characterized according to their amplitude (as a function of the resting potential) and latency. When needed, the same stimulating pulses were also presented in trains (100 ms) at 50–200 Hz.

Identification of stimulating and recording sites. Recorded neurons were identified following procedures described previously (Navarro-López et al., 2004). Briefly, selected neurons were stained by the intracellular injection of biocytin diluted in a 2 M potassium acetate solution (McDonald, 1992) using positive current pulses of 0.2 nA for 6 min. Slices were fixed and cut in sections (45 μm) using a freezing microtome (HM400R; Microm, Heidelberg, Germany). Sections were incubated with avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA). 3,3′-Diaminobenzidine was used as chromogen for visualization of the biocytin complex. Alternate sections were counterstained with cresyl violet. Neurons were reconstructed from serial sections using a camera lucida (Nikon Labophot; Nikon, Kawasaki, Japan).

Drugs. All chemicals used in this study were applied by superfusion in the ACSF. The chemicals used were atropine sulfate (a nonspecific antagonist of cholinergic receptors), 6-cyano-nitroquinoxaline-2,3-dione (CNQX; a potent, competitive AMPA-kainate receptor antagonist), and 2-amino-5-phosphonovalerate (APV; a specific blocker of NMDAR) from Sigma (St. Louis, MO), pirenzepine (a selective blocker of M_1 muscarinic receptors) from Biogen Científica (Madrid, Spain), and chelerythrine chloride (specific and potent, cell-permeable inhibitor of PKC) from Alomone Labs (Jerusalem, Israel).

Data storage and statistical analysis. Data were acquired and stored, as analog signal, on a videocassette, using a modified video recorder (Physiorec-3; Cibertec, Madrid, Spain). Off-line data acquisition and analysis were performed with the help of a Cambridge Electronic Design (Cambridge, UK) 1401 interface between the tape recorder and a computer, using the Mini Analysis program, version 5.2.1 (Synaptosoft, Decatur, GA). Unless otherwise indicated, the electrophysiological data are always expressed as mean ± SEM, and *n* represents the number of averaged neurons. Synaptic potentials were averaged (≥5) before quantitative analysis. Statistical analysis of collected data was performed using a paired Student's *t* test and, when necessary, one-way ANOVA. Statistical significance was determined at a level of $p \leq 0.05$.

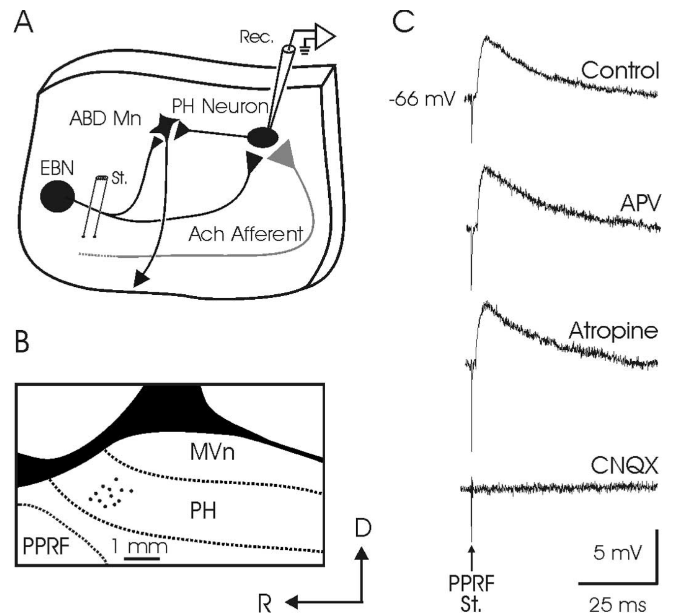

Figure 1. Neural circuits and connectivity. ***A***, A diagram illustrating neural circuits present in the sagittal brainstem slice used here. EBN are located in the PPRF, rostrally to abducens nucleus motoneurons (ABD Mn), and project monosynaptically on PH neurons. Stimuli (St.) applied to the PPRF also activate cholinergic neurons and/or axons. Rec., Recording electrode. ***B***, Location of some ($n = 12$) biocytin-injected neurons to illustrate the recording area. R, Rostral; D, dorsal; MVn, medial vestibular nucleus. ***C***, Glutamatergic nature of PPRF synaptic contacts on PH neurons. Top, EPSP evoked in a PH neuron by a single subthreshold stimulus (St.; 100 μs, 200 μA) applied to the PPRF. The EPSP was not affected by atropine sulfate (1 μM) or APV (50 μM), but the application of CNQX (10 μM) completely removed the evoked synaptic potential.

Results

Characterization of PH neurons and their response to PPRF stimulation

This study comprises 42 intracellular recordings from PH, selected because of their resting potential (less than or equal to −60 mV) and monosynaptic activation from the PPRF. PH neurons were identified electrophysiologically by the absence of spontaneous firing at resting membrane potential and the presence of a biphasic afterhyperpolarization in their action potential when depolarized (Navarro-López et al., 2004). The location of selected PH neurons ($n = 12$) filled with biocytin is illustrated in Figure 1*B*. The morphology and initial axonal trajectories of labeled PH neurons suggest that they could be considered "principal cells" (McCrea and Baker, 1985) (i.e., a type of PH cell projecting to many brainstem sites related to the oculomotor system, including the abducens nucleus).

Single subthreshold stimulations of the PPRF evoked monosynaptic EPSPs in PH neurons in all cases ($n = 42$). The EPSPs presented a mean latency of 2.57 ± 0.30 ms, a rise time of 6.5 ± 2.9 ms, a decay time of 20.8 ± 8.7 ms, and a duration of 71.6 ± 23.8 ms (Fig. 1*C*, top). EPSPs evoked in PH neurons by PPRF stimulation were not modified in amplitude or latency by slice superfusion with atropine sulfate (1–3 μM) or APV (50 μM) but were removed completely by the application of CNQX (10–12 μM) (Fig. 1*C*). These results indicate that the EPSP evoked in PH neurons by PPRF stimulation was mediated by glutamate acting exclusively on AMPA-kainate receptors, at least when stimulus rate was set at ≤0.2 Hz.

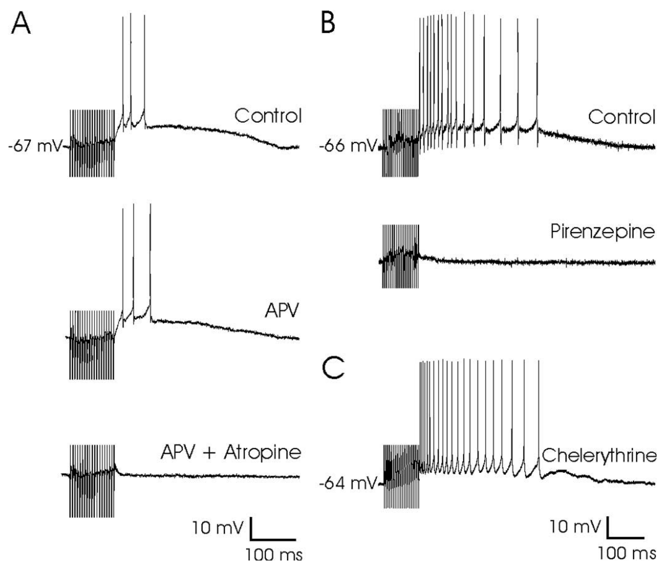

Figure 2. Differential effects of train stimulation of PPRF on PH neurons in presence of glutamatergic and/or cholinergic drugs. ***A***, The top record (Control) illustrates the effect of a PPRF train (100 ms, 200 Hz, 200 μA) on a PH cell. Note the large and sustained depolarization recorded after the end of the train. The middle record shows that the application of APV (50 μM) to the bathing solution did not affect the posttrain activation of the PH neuron. In contrast, this sustained depolarization of the PH neuron was impossible to evoke in the presence of atropine sulfate (1 μM). ***B***, Top, Another example of sustained depolarization, including a lasting burst of action potentials, evoked in a PH neuron by train stimulation (100 ms, 200 Hz, 450 μA) of the PPRF. Superfusion with pirenzepine (bottom; 0.5 μM) completely removed the evoked post-train depolarization. ***C***, The sustained depolarization of PH neurons after train stimulation (100 ms, 200 Hz, 425 μA) of the PPRF was not affected by chelerythrine (2.5 μM). The resting membrane potential for the illustrated neurons is indicated. Calibration in ***C*** also applies to ***B***.

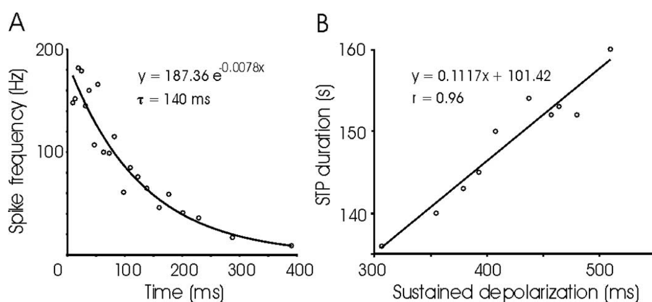

Figure 3. Plots of PH neuron responses after train stimulation. ***A***, Exponential decay of burst firing evoked in PH neurons after train stimulation of PPRF. The illustrated data correspond to a single recording collected from a PH neuron. The burst of action potentials was evoked after a train of stimuli (100 ms, 200 Hz, 400 μA) applied to the PPRF ($r = 0.979$; $p < 0.01$). ***B***, Plot of STP duration (in seconds) against sustained cholinergic depolarization duration (in milliseconds). Each point corresponds to the responses of one neuron ($n = 10$).

Differential effects of train stimulation of PPRF on PH neurons in presence of glutamatergic and/or cholinergic drugs

Train stimulation (200 Hz, 100 ms) of the PPRF evoked a sustained depolarization of PH neurons that exceeded the end of the train by up to 400–500 ms (Fig. 2). This persisting depolarization was large enough to evoke a burst of action potentials that reached peak frequencies of 150–200 spikes/s. The frequency of the burst of action potentials evoked by PPRF train stimulation decayed with time, with a time constant of 140 ± 15 ms (range, 70–210; $n = 11$; $p < 0.01$) (Fig. 3*A*) [i.e., a value similar to the time constants of cat abducens motoneurons (90–197 ms) (Delgado-García et al., 1986) and "position-velocity" PH neurons recorded in behaving cats (46–371 ms) (Delgado-García et al., 1989)].

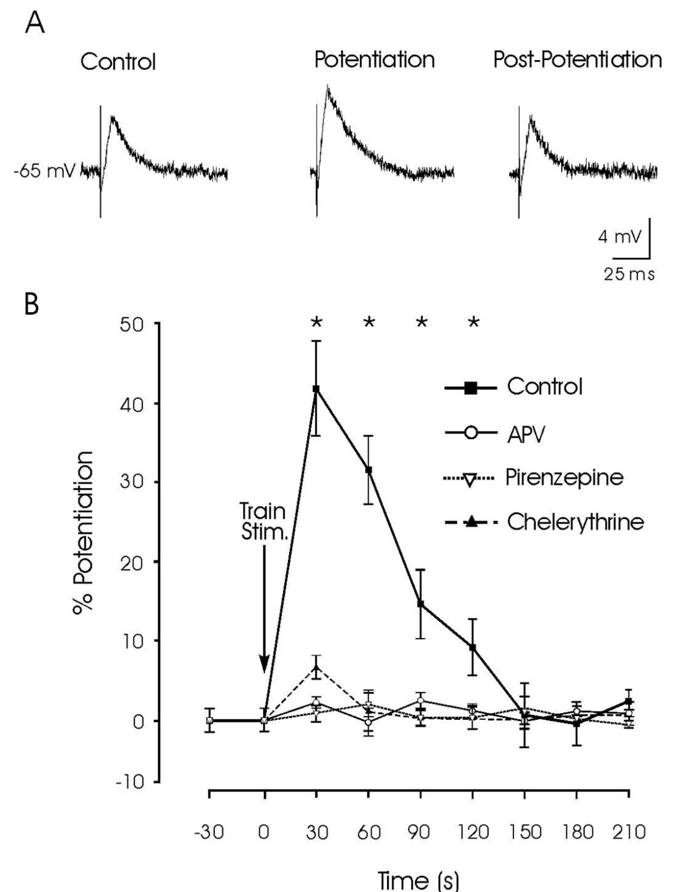

Figure 4. STP of PH neurons after train stimulation of PPRF. ***A***, EPSPs evoked in a PH neuron by single subthreshold stimuli applied to the PPRF 30 s before (Control), and 30 s (Potentiation) and 180 s (Post-Potentiation) after train stimulation (100 ms, 200 Hz, 250 μA; arrow) of PPRF. ***B***, Quantitative analysis of the synaptic potentiation evoked in PH neurons ($n = 15$) by a train of stimuli (Train Stim.) presented to the ipsilateral PPRF. Potentiation was determined as the percentage increase in the amplitude of the evoked EPSP. Data represent mean percentage ± SD, computed every 30 s. Note that the STP was present at significant values (asterisks) for ~120 s ($p \leq 0.01$). Single pulses applied to the PPRF were presented at a rate of 0.2 Hz. STP was not evoked in the presence of APV (50 μM; $n = 8$), pirenzepine (0.5 μM; $n = 6$), or chelerythrine (2.5 μM; $n = 5$) superfused to the bathing solution.

The sustained depolarization was the probable result of M_1-muscarinic-receptor activation, because the effect was removed by atropine sulfate (1 μM; $n = 7$) (Fig. 2*A*) and by pirenzepine (0.5 μM; $n = 6$) (Fig. 2*B*). However, superfusion with APV (50 μM; $n = 8$) or chelerythrine (2.5 μM; $n = 6$) had no effect on sustained depolarization or on action potentials firing (Fig. 2*A*, *C*, respectively). Thus, the monosynaptic effects of PPRF on PH were mediated by the activation of AMPA-kainate receptors, whereas the sustained depolarization was caused by the activation of M1 cholinergic receptors.

STP of PH neurons after train stimulation of PPRF

Train stimulation of the PPRF also produced an STP of EPSPs evoked in PH neurons by single pulses presented to that reticular formation zone. We compared the amplitude of EPSPs evoked in PH neurons ($n = 15$) by single pulses applied to the PPRF before and after the application of a train of stimuli (200 Hz, 100 ms, 200–300 μA) to the same PPRF area (Fig. 4*A*). Data are represented as the percentage increase with respect to control EPSPs,

using the following equation: potentiation percentage = [(experimental EPSP amplitude/control EPSP amplitude) − 1] × 100. Compared with control values, the train of stimuli produced a significant ($p \leq 0.01$) increase in EPSP amplitude lasting for 120 s, reaching a maximum increase (41.7 ± 6.0%; $p < 0.001$) 30 s after the train (Fig. 4*B*). The potentiation decreased exponentially with time, with a time constant of 79.8 ± 9.2 s. Durations of the sustained depolarization (Fig. 2) and that of the STP (Fig. 4) were related linearly ($p < 0.01$) (Fig. 3*B*). Bath superfusion with APV (50 μM; $n = 8$) completely removed the EPSP potentiation evoked in PH neurons by train stimulation of the PPRF (Fig. 4*B*), suggesting an NMDA dependence. Blocking the sustained depolarization with pirenzepine (0.5 μM; $n = 6$) or atropine sulfate (1 μM; $n = 7$) (data not shown) also blocked the STP. Such results sustained the interdependence of NMDA and M_1 muscarinic receptors. Finally, chelerythrine (2.5 μM; $n = 5$) also blocked the STP, which means that it is mediated by the activation of PKC.

Discussion

Interaction between muscarinic and glutamatergic receptors located in PH neurons

It is known that train stimulation (>30 Hz) is able to activate cholinergic axons (Moises et al., 1995; Faber and Sah, 2002) and that cholinergic axons originated in the pontomesencephalic reticular formation projects to the PH nucleus (Carpenter et al., 1987; Semba et al., 1990). It can therefore be suggested that cholinergic axons projecting onto PH neurons were activated during train stimulation of the PPRF (Navarro-López et al., 2004). However, it is also possible that cholinergic neurons located in PH (Tighilet and Lacour, 1998) are activated by collateral axons of the ipsilateral and contralateral circuits involved in the integration of eye position signals from eye velocity motor commands (Aksay et al., 2003). The presence of an M_1 muscarinic cholinergic modulation of glutamatergic (NMDA) synapses has been studied at the striatum (Calabresi et al., 1998) and the hippocampus (Marino et al., 1998; Fernández de Sevilla et al., 2002; Sur et al., 2003). The activation of G-protein-coupled receptors, such as the M_1 muscarinic receptor, potentiates NMDA-evoked responses in hippocampal neurons (Marino et al., 1998; Sur et al., 2003). This potentiation is blocked by pharmacological inhibition of endogenous PKC (Lu et al., 1999). In its turn, PKC seems to enhance NMDAR-mediated synaptic currents in neurons (Lu et al., 1999; Salter and Kalia, 2004). As shown here for PH neurons, the activation of M_1 receptor enhances NMDAR-mediated synaptic currents via PKC.

The presence of positive and negative feedbacks in premotor extraocular circuits, particularly those generating eye position signals, have been suggested (Escudero et al., 1992; Aksay et al., 2003). The level of synchronization between neurons present in those circuits could give some information regarding the stability of the neuronal integrator (Tegnér et al., 2002; Aksay et al., 2003). It has been shown that the ratio between synaptic currents generated by NMDA versus AMPA receptors plays an important role in the generation of the persistent activity necessary for eye fixation (Tegnér et al., 2002). Thus, when the ratio is large, the degree of synchronization in the neural integrator circuit is low, and network stability is increased (Aksay et al., 2003). As experimental support for these theoretical contentions, the two mechanisms proposed here (sustained depolarization and STP), involving AMPA-kainate and NMDA glutamatergic receptors, are dependent on the coincident activation of M_1 muscarinic receptors, as further indicated by their linear relationship (Fig. 3*B*).

Persistent activity and STP are both present in PH neurons

As proposed recently (Navarro-López et al., 2004) and confirmed here, train stimulation of the PPRF, with stimulus parameters similar to those evoked in PPRF neurons during normal saccades in behaving mammals, is able to produce a sustained depolarization of PH neurons outlasting the train by up to 500 ms. This sustained depolarization is dependent both on the activation of AMPA-kainate receptors by EBN (Igusa et al., 1980) and on cholinergic axons terminating on the same PH neurons and acting on M_1 muscarinic receptors. These results, collected from brainstem slices, have been confirmed in behaving cats by the local (at the PH nucleus) injection of different cholinergic drugs (Navarro-López et al., 2004). Here, we also report an STP (lasting for ~120 s) evoked in PH neurons by train stimulation of the PPRF. In this case, the potentiation seems to be mediated by NMDAR, because it was easily blocked by superfusion with APV, indicating a postsynaptic origin. Additional proof that this STP has a postsynaptic origin is that both pirenzepine and chelerythrine were able to block it when applied to the bathing solution.

Although the sustained depolarization triggered in PH neurons by PPRF stimulation (i.e., by EBN) (Igusa et al., 1980) carrying eye velocity signals can be described as a synaptic mechanism involved in the generation of the neural persisting activity (Aksay et al., 2003; Major and Tank, 2004; Navarro-López et al., 2004) necessary for eye positions of fixation, the role of the STP described here is of a difficult interpretation within oculomotor system performance. The involvement of NMDAR of the PH in the generation of eye position signals has been demonstrated previously in the alert cat (Cheron et al., 1992; Mettens et al., 1994). On the basis of *in vivo* and *in vitro* experiments, the NMDA-related STP mechanism seems to be crucial for persistent activity in the PH. This is somewhat paradoxical, because STP, mainly in the form of posttetanic potentiation, was first described in motor networks, although, in recent years, it has been related more with cortical network functioning (Nadim and Manor, 2000). Nevertheless, the reported STP could act as a mechanism for high-pass filtering in oculomotor function, facilitating the synchronous activation of (postsynaptic) PH neurons, increasing signal-to-noise ratio (Lisman, 1997), and canceling out the disturbing effects of low rate firing of EBN. STP could still be useful in facilitating the action of error signals addressed to correct aimed versus achieved eye positions (Schultz and Dickinson, 2000). In this regard, Shen (1989) proposed a model of oculomotor integration based on potentiation phenomena at the synaptic level. If we assume an opposite action (i.e., short-term depression) evoked by contralateral inhibitory burst neurons (Hikosaka et al., 1980), the *in vivo* interplay of short-term synaptic facilitation and depression would help network stability and maintenance (Nadim and Manor, 2000). In contrast, the sustained activity that follows train stimulation of the PPRF seems to be related more with the generation of eye position signals. Indeed, the amplitude and duration of the evoked EPSPs are related linearly to train frequency (Navarro-López et al., 2004), and, as shown here, the time constant of the decaying firing (Fig. 3*A*) is similar to the time constant of PH and abducens neurons and of the oculomotor plant (Robinson, 1981; Delgado-García et al., 1989; Fukushima et al., 1992; Moschovakis, 1997).

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