Ph.D. Thesis

Long-Term Potentiation of Intrinsic Excitability in Hypoglossal and Trigeminal Motoneurons

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Dedicated to my husband and parents

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ABSTRACT

Synaptic plasticity is an intrinsic and conserved feature of neuronal activity that has been most extensively studied in the context of learning and memory. However, the intracellular mechanisms underlying plasticity at motor nuclei, influencing motor behavior, are less well studied. Rhythmical oral-motor activities are produced in response to action potentials mostly generated by hypoglossal and trigeminal motoneurons (HMNs and TMNs). The processes that determine the firing behavior of motoneurons are therefore important in understanding the transformation of neural activity to motor behavior. Activity-dependent plasticity, referred to as long-term potentiation (LTP), in the central nervous system and recently in motor systems has been the subject of many studies. However, little is known about the presence of plasticity in the hypoglossal system and the main responsible pathway controlling the LTP in trigeminal system. Therefore, the present study was aimed at addressing these issues by using brainstem-spinal cord preparation and whole-cell patch clamp techniques in neonatal rats. In the present study the mechanisms of LTP of intrinsic excitability (LTP-IE) in HMNs and TMNs, and its dependence to calcium influx were investigated.

Experiments were performed on neonatal (1-4 days) rats using the extracellular recording and the whole-cell patch-clamp recording to assess the intrinsic excitability of motoneurons. Intrinsic response properties were examined by applying an induction pulse at high frequency firing with ionotropic transmission blocked.

There was no increase in amplitude of the extracellular response from hypoglossal motor branch and intrinsic excitability of single HMN in response to the brief period of positive current injection. These results indicate that hypoglossal motor system shows no LTP-IE while trigeminal motor system does. I also found that an intracellular calcium

increase during the induction protocol is necessary for the induction of LTP-IE in TMNs. The increase in excitability after induction depends on protein kinase activation. LTP-IE is calcium dependent and protein kinase A is a main regulator among other protein kinases.

Abbreviations:

LTP-IE	long-term potentiation of intrinsic excitability
HMNs	hypoglossal motoneurons
TMNs	trigeminal motoneurons
APs	action potentials
HFS	high frequency stimulation
PKA	protein kinase A
PKC	protein kinase C
CaMKII	calcium/calmodulin-dependent protein kinase II

INTRODUCTION

Synaptic plasticity is an intrinsic and conserved feature of neuronal activity that facilitates the adaptation of organisms to changing environments and circumstances. Plasticity in the nervous system has been most extensively studied in the context of learning and memory (Petit et al., 1989). Examples include long-term potentiation of intrinsic excitability (LTP-IE) in LV visual cortical neurons (Cudmore and Turrigiano, 2004), LTP in the rabbit hippocampus (Bliss and Lomo, 1973), and more recently in the respiratory system of mammals, including the demonstration of long-term facilitation in rat respiratory motoneurons (Bocchiaro et al., 2003; Fuller et al., 2000). While there is an extensive body of knowledge on neuronal plasticity involved in the mammalian hippocampus and LV, less is known about the subcellular mechanisms underlying plasticity at the level of motor pools.

Motoneurons (MNs) produce behavior by integrating and transforming patterns of premotoneuronal activity into commands for skeletal muscle contractions. MNs change dramatically during the developmental process. Changes in intrinsic membrane properties (e.g., action potential and afterhyperpolarization duration) are associated with sharp reductions in MNs excitability (Adachi et al., 2010). Parallel maturation of neurotransmitter systems that modulate MNs excitability (either by altering membrane and synaptic properties or MNs responses to synaptic inputs), however, is hypothesized as critical for maintaining MNs excitability during development and, as behavioral repertoire increases, for mediating changes in excitability that are specific to a given arousal state or behavior (Rekling et al., 2000).

Postsynaptic calcium influx is a critical trigger for many forms of neural plasticity including LTP (Abbott and Nelson, 2000; Chen et al., 2007; Cudmore and Turrigiano, 2004; Malenka and Nicoll, 1999). A number of calcium-dependent protein kinases participate in the

signal transduction cascades that lead to LTP, i.e., Protein kinase A (PKA), Ca^{+2} dependent protein kinase II (CaMKII) and protein kinase C (PKC) (Jacobs and Meyer, 1997; Llano et al., 1994). PKA activation is an essential component of LTP-IE in LV visual cortical neurons, while dopamine D1/5 receptor-mediated LTP-IE requires PKC to mediate modulation of voltage-dependent ion channels in PFC pyramidal layer V/VI neurons (Chen et al., 2007; Cudmore and Turrigiano, 2004). Studies of LTP in these neurons have identified that many intracellular mechanisms are conserved (Pittenger and Kandel, 2003), so it is arguable that the same molecules participate in the induction and maintenance of LTP in mammalian motor pools.

Hypoglossal MNs (HMNs) and trigeminal MNs (TMNs) participate in multiple reflex and rhythmic motor behaviors, including suckling, chewing, swallowing, and respiration (Bartlett et al., 1990; Miller, 2002). Past studies from my laboratory have focused on elucidating the underlying mechanisms responsible for the LTP-IE in TMNs (Okamoto et al., 2010). To this end, I have utilized brainstem transection techniques in neonatal rat brainstem *in vitro* to determine that the activity of the motoneuron population which is recorded from the motor branch of the trigeminal nerve, exhibited LTP-IE. Additionally, on testing with the whole-cell patch clamp method, TMNs exhibited a significant increase in excitability with a leftward shift in *F-I* curves generated with depolarizing current injections.

Although MNs are not involved in rhythm generation, their pattern of action potentials (APs) determines the strength and timing of muscle contractions that, when convolved with masticatory mechanics, determines the rhythmical jaw movements. Clearly, changes in their excitability are functionally important. How neurons integrate synaptic inputs to generate APs from interneurons to MNs is of great importance and of considerable interest (Bourque and Kolta, 2001; Kolta, 1997). Action potential firing and subsequent calcium influx have the effect of activating various second messengers. Establishment of the

cellular basis of the intrinsic plasticity of the hypoglossal and trigeminal system is of considerable interest. However, there is little evidence for plasticity of HMNs and TMNs. In the present study, I examined the plasticity of intrinsic excitability in HMNs, TMNs and role of calcium-dependent protein kinases, among the number of potential mediators, on calcium-dependent plasticity.

MATERIALS AND METHODS

In vitro Brainstem-Spinal Cord Experiment on hypoglossal motor branch

Preparation of tissue

45 Neonatal (1-4 days old) Sprague-Dawley (SD) rats (Nihon SLC Co. Ltd., Shizuoka, Japan) were deeply anesthetized with halothane (Flouthane®, Takeda, Osaka, Japan) and the brainstem-spinal cord were isolated as described (Kogo et al., 2002; Yamanishi et al., 2008). Briefly, the cerebellum was removed, and the brainstem-spinal cord was transected (Fig. 1A). 800 μm coronal sections were cut at the pontomedullary and medulla-spinal cord junction including the hypoglossal motor nucleus and hypoglossal motor nerve (Fig. 1A). The preparation was pinned down with the ventral surface upward in the recording chamber, bathed in and continuously perfused, via a tube placed just above the preparation, with an artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 3.0 KCl, 1.5 $CaCl_2$, 1.0 $MgSO_4$, 25 $NaHCO_3$, 0.5 NaH_2PO_4 and 30 D-glucose equilibrated with 95% O_2 and 5% CO_2 at 25-27 °C.

Electrophysiological techniques

Motoneuron population discharges were recorded from the hypoglossal rootlet with the use of glass electrodes filled with ACSF. Glass electrodes were fabricated from borosilicate glass capillary tubing (1.5 mm outer diameter, 1.12 mm inner diameter) using a micropipette puller (P-87 Sutter instrument Company, Novato, CA). Signals from the recording were amplified and high-pass-filtered (50 Hz) with a differential amplifier (DAM-50, World Precision Instruments, Sarasota, FL, USA). The amplified signals were digitized and analyzed with a PowerLab version (ver.) 4/30 and Chart ver. 7.3.5 software

(ADInstruments, Colorado Springs, CO, USA). The raw signals were rectified, smoothed using a time constant of 0.001 s, and then processed to determine the integrated area of the rectified extracellular response (Tsuji et al., 2011).

Whole-cell patch-clamp Experiment on HMNs and TMNs

Preparation of tissue

Coronal slices from 40 neonatal SD rats (1-4 days old) (Nihon SLC Co. Ltd., Shizuoka, Japan) were cut according to a protocol described in previous reports (Enomoto et al., 2006; Okamoto et al., 2010). Briefly animals were rapidly anesthetized with halothane (Flouthane®, Takeda, Osaka, Japan), decapitated and then brains were quickly removed and immersed in oxygenated (95% O_2-5% CO_2) ice-cold cutting ACSF of the following composition (in mM): 126 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 Glucose, 1 $CaCl_2$, 5 $MgCl_2$, and 4 Lactic acid (Wu et al., 2005). The brainstem was immersed in the agarose and glued by its rostral end to the platform of a chamber and covered with ice-cold cutting ACSF. Slices (300 μm) were cut on a vibrating slicer (LinearSlicer PRO 7, Japan), and placed at room temperature in an oxygenated incubation ACSF of the following composition (in mM): 126 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 Glucose, 1 $CaCl_2$, 5 $MgCl_2$ and 8 Lactic acid (Wu et al., 2005). The slices were incubated at 37 °C for 40 to 50 min and then maintained at room temperature (22-24 °C). The hypoglossal motor nucleus was identified bilaterally in coronal slices under low magnification (5x) as a well-defined area located ventral to the central canal just lateral to the middle line. HMNs were distinguished based on their location and electrophysiological characteristics (Viana et al., 1993). The trigeminal motor nucleus was also identified bilaterally in coronal slices under low magnification (5x) as an oval grayish region located immediately rostral to the facial nerve. TMNs were distinguished based on their location and size (Chandler et al., 1994).

Electrophysiological techniques

Patch electrodes were fabricated from borosilicate glass capillary tubing using a micropipette puller (P-87 Sutter instrument Company, CA). The patch pipettes were wrapped near the tip with silicon agent (SYLGARD; Dow Corning, Midland, MI) to reduce capacitance. Whole-cell current recordings were performed using a Multiclamp 700B, (Molecular Devices, Foster city, CA) in concert with pclamp acquisition software (ver. 9.2, Molecular Devices). Signals were grounded (Ag/AgCl wire) using a 3-M KCl agar bridge. After the establishment of a gigaohm seal, the whole-cell configuration was obtained by brief suction. Cells with resting potential <-55mV were discarded. Electrode resistances were 3 to 5 MΩ when filled with the intracellular solution of the following composition (in mM): 115 K-gluconate, 25 KCl, 9 NaCl, 10 HEPES, 0.2 EGTA, 1 $MgCl_2$, 3 K_2-ATP, and 1 Na-GTP, pH 7.25 with KOH, with osmolarity adjusted to 280 to 290 mOsm. Slices were perfused with oxygenated ACSF (2-3 ml/min) and visualized by infrared differential interference contrast microscopy (Stuart et al., 1993).

Chemical application

All drugs were purchased from Sigma (St. Louis, MO), dissolved in distilled water or dimethylsulfoxide (DMSO) and bath-applied except, were it mentioned. Experiments were performed in the presence of 2-amino-5-phosphonovaleric acid (D-APV, 50 μM), 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX, 20 μM), bicuculline methiodide (BIC, 20 μM), and strychnine (Strych, 5 μM) to block N-methyl-D-aspartate (NMDA), amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainite, $GABA_A$, and glycinergic receptor-mediated currents, respectively. Following drugs were applied on each steps where it mentioned; Calcium chelator, bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA, 10 mM, in the pipette), membrane-permeable broad-spectrum protein kinase inhibitor, H-7 (100 μM), PKA inhibitor, H-89 dihydrochloride hydrate (100 μM in the pipette), PKC inhibitor, chelerythrine (10 μM), calcium/calmodulin-dependent protein kinase II (CaMKII) inhibitor,

KN-62 (10 μM), an adenylyl cyclase activator, forskolin (50 μM), PKC activator, phorbol 12-myristate 13-acetate (PMA, 1 μM).

Data acquisition and analysis

Data were collected and analyzed with a combination of software packages [Chart software (ver. 7.3.5 ADInstruments), Clampfit (ver. 9.2, Molecular Device), StatView (SAS Institute, Cary, NC), and Microsoft Excel]. Results are reported as mean ±SE, unless otherwise indicated. Comparisons of mean values between groups were performed with Student`s t-test with a level of significance of $P<0.05$.

All experimental procedures were approved by the Osaka University Graduate School of Dentistry Intramural Animal Care and Use Committee in accordance with the guidelines of NIH, USA, and all efforts were made to minimize the number of animal used.

RESULT

Extracellular recording from hypoglossal motor branch

In the brainstem-spinal cord preparations, spontaneous inspiratory activity (0.11 ± 0.02 Hz) was recorded from the hypoglossal motor nerve in thin blocks of brainstem (800 μm) (Fig. 1A). Swallowing activity was induced by applying electrical stimulation (4-5 V) to vagal afferent nerve using suction electrodes (Fig. 1B). Two discharge patterns of the hypoglossal motor system (respiratory activity and swallowing activity) were characterized in *in vitro* brainstem preparations, as previously reported (Yamanishi et al., 2010).

Next, the intrinsic plasticity of the HMN population was examined. To prevent synaptic input, excitatory and inhibitory ionotropic transmission were blocked using D-APV (50 μM), CNQX (20 μM), BIC (20 μM), and strychnine (5 μM). A fixed 500-ms test pulse (every 30 seconds) was injected into HMNs. Focal stimulation (400-500 μV) was delivered via a concentric bipolar electrode. The exposed tip of the stimulator was positioned on the motor nucleus. The extracellular responses of the hypoglossal motor branches were measured (Fig. 2A). An electrical stimulus of 1.5 to 2x the size of the test pulse (500 ms) (every 5 s for 5 min) was injected to stimulate induction. After 5 min of stimulation with the induction pulse, the extracellular response of motor branch was recorded by a test pulse again (Fig. 2B). I then compared the amplitude of the resultant HMN population response after induction with that of the pre-induction response. To quantify the change in the extracellular response before and after induction, amplitude of the extracellular response was measured as the averaged integrated area. There was no significant change in the amplitude of integrated area before and after induction in hypoglossal motor branch (Fig. 2C)

Whole- cell patch-clamp recording

I examined whether a brief period of high-frequency stimulus (HFS) could produce intrinsic excitability in a single motoneuron. To test this hypothesis, electrophysiological recordings were performed on HMNs and TMNs from rat brain slices using whole-cell patch-clamp recording after blocking excitatory and inhibitory ionotropic transmission. Membrane input resistance (R_{inp}) was monitored during the recording. A fixed 500-ms test pulse (every 30 seconds) was injected through the patch pipette to evoke four to five APs (Fig. 3A, 4A), and then a brief period of HFS (20 - 25 Hz for 500 ms every 5 s for 5 min) was induced by current application as an induction pulse through the patch pipette (Cudmore and Turrigiano, 2004; Desai et al., 2006). After 5 min of stimulation with the induction pulse, spike firings were elicited by the same test pulse as used before the induction (Fig. 3B, 4B) and number of APs was compared with that before induction. There was no significant difference in the number of APs in HMNs before (Fig. 3C) and after induction (Fig. 3D) while there was a significant increase in the number of APs in TMNs (Fig. 4C, D). This increase in intrinsic excitability in TMNs remained elevated for >20 min. In control recordings from both motoneurons, the responses to the test pulse remained constant (Fig. 3C, 4C). Increase in the resultant firing rate (with firing rate calculated in steady-state at the end of the step pulse) was observed in TMNs group with no change in HMNs group (Fig. 5A, B). In addition, I determined whether induction increased excitability across a range of suprathreshold voltages. The frequency-current relationship was examined in neurons with and without induction. 500-ms current steps of increasing intensity were delivered (Fig. 6A, C). Injection of a series of DC current steps (50 pA increments, 500 ms) through the patch pipette revealed the *F-I* curve. *F-I* curves for both recordings and both MNs were obtained. At each suprathreshold current, the post-induction frequency of HMNs was similar to that pre-induction (Fig. 6B) while it was larger in TMNs which resulted in a leftward shift in the *F-I* curve (Fig. 6D). No

remarkable change in firing rate was observed in control recordings. As shown in the previous report (Okamoto et al., 2010), firing characteristics of a single TMN were measured after blocking excitatory and inhibitory ionotropic transmission. TMNs exhibited a significant increase in excitability generated by the induction with depolarizing current injections.

LTP-IE is calcium dependent

Different behavior of two MNs in response to the same induction protocol justified this step. In this step, I aimed to study about the signal transduction pathway of LTP-IE in trigeminal motor system.

My results suggested that the observed potentiation of excitability could involve changes in voltage-dependent conductance that activate around the threshold and affect repetitive spiking characteristics. Postsynaptic calcium influx is a critical trigger for many forms of neural plasticity including LTP and LTP-IE (Abbott and Nelson, 2000; Chen et al., 2007; Cudmore and Turrigiano, 2004; Malenka and Nicoll, 1999). Many forms of calcium-dependent kinase participate in the signal transduction cascades that lead to LTP and LTD (Chen et al., 2007; Cudmore and Turrigiano, 2004). To find out whether calcium influx during the induction protocol is essential, I limited calcium influx by washing in ACSF with 0 mM Ca^{2+} during the induction period. This prevented the long-lasting increase in intrinsic excitability (Fig. 7A). Temporally, more excitable effect due to a reduction in Ca^{2+}-dependent K^+ current such as $I_{K\text{-}Ca}$ by washing in 0 Ca^{2+} ACSF was predictable in TMNs. This increase in excitability was transient, and was returned by washing out with normal ACSF. In addition, the dependence on calcium influx during the induction protocol was examined by buffering the intracellular calcium with the calcium chelator BAPTA in the recording pipette (10 mM). Then the change of firing rate which normally induced by the induction protocol was recorded. This blocked the increase in firing rate (Fig. 7B). For both the 0 mM Ca^{2+} and

BAPTA experiments, the induction protocol induced no significant change in the firing rate (Fig. 7C). These data suggest that a rise in intracellular calcium during the induction protocol is necessary for the induction of LTP-IE.

LTP-IE is protein kinase dependent

The cAMP-dependent activation of PKA appeared to contribute to the neural firing activity of TMNs, and through activation of PKA, PKC inhibit Ca^{2+}-activated K^{+} channels (Inoue et al., 1999). The roles that calcium currents and the related second messenger cascade play in control of the neural plasticity involved in trigeminal motoneuronal excitability were assessed. Among the large number of potential mediators of calcium-dependent plasticity, I chose to examine the role of three different calcium-dependent kinases, cAMP-dependent PKA, PKC, and CaMKII. I determined whether H-7, a membrane-permeable broad-spectrum protein kinase inhibitor, could block LTP-IE in TMNs. Micromolar concentrations of H-7 block PKA, PKC, CaMKII, and cGMP-dependent protein kinase (Hidaka et al., 1984; Malinow et al., 1989). When 100 μM H-7 was bath applied during the induction period, it prevented LTP-IE (Fig. 8A). H-7 prevented the increase in spike rate (Fig. 8B). These results strongly suggest that the increase in excitability after induction depends on protein kinase activation in TMNs.

To specifically characterize which protein kinases are necessary for LTP-IE, selective inhibitors of PKA, PKC, and CaMKII were tested. Inclusion of 100 μM H-89, a specific PKA inhibitor (Chijiwa et al., 1990; Cudmore and Turrigiano, 2004), in the pipette blocked the increase in firing rate (Fig. 9A, B) induced by the induction protocol. Thirty minutes (including 5min induction period) bath application of the PKC inhibitor, 10 μM chelerythrine (Harmati et al., 2011; Jimenez-Rivera et al., 2012; Yang et al., 2004), produced a small reduction of the increase in firing rate. In contrast, 30 min (including 5min induction) bath application of the PKC inhibitor chelerythrine (10 μM) could not prevent the increase in

firing rate (Fig. 9C). To determine if LTP-IE depends on CaMKII activation, additional experiments were performed in which the CaMKII inhibitor KN-62 (10 μM) (Alix et al., 2002; Tokumitsu et al., 1990; Xu et al., 2004) was bath-applied for 30min (including 5min induction). KN-62 could not prevent LTP-IE, as well (Fig. 9D). These data suggest that CaMKII activation is not essential for the induction of LTP-IE.

PKA activation is a necessary component for LTP-IE

To assess the role of PKA activation for LTP-IE, forskolin (forsk) (Cudmore and Turrigiano, 2004) an adenylyl cyclase activator was used, to directly elevated cAMP and activate PKA. A 10-min bath application of forsk (50 μM) caused a long-lasting increase in excitability that closely resembled that produced by the induction protocol (Fig. 10A). In addition, forsk caused a shift to the left of the *F-I* curve (Fig. 10B). These data indicate that elevation of cAMP is sufficient to mimic the increase in excitability that follows the induction protocol. Forsk application also occluded stimulation-induced LTP-IE. When the induction protocol was run after the forsk-induced increase in excitability, there was no additional increase in excitability (Fig. 10C). The ability of PKA inhibitors to block, and forsk to mimic and occlude LTP-IE, suggest that LTP-IE is induced via a PKA-dependent mechanism.

PKC activation

To reveal the PKC role in LTP-IE, phorbol 12-myristate 13-acetate (PMA) (Huang et al., 2011; O'Reilly et al., 1997; Qian et al., 1997) was applied to activate PKC. A 10-min bath application of PMA (1 μM) increased the spike rate (Fig. 11A) but this increase was significantly smaller than that with forsk (Fig. 11B)

DISCUSSION

To my knowledge, this study is the first to examine the intrinsic excitability of neonatal HMNs by extracellular and whole-cell patch clamp recording *in vitro* using brainstem preparations.

Previous findings from my laboratory (Okamoto et al., 2010) indicate that TMNs show LTP-IE in both extracellular and whole-cell patch-clamp recordings and this intrinsic plasticity did not require synaptic activation and was directly induced by postsynaptic depolarization. Although HMNs and TMNs are both involved in oral-motor activities, they showed different intrinsic behaviors in response to the same positive induction current. My data indicate that applying the HFS to HMNs during neonatal 1-4 days old could not increase the motoneuronal excitability, and so HMNs do not show LTP-IE.

Stimulation influences

From snails to flies to humans, behavioral adaptations are affected by the pattern of stimuli. For example, in Drosophila, the cellular machinery responsible for memory consolidation is improved by spaced, i.e., episodic, training compared to massed training paradigms (Tully et al., 1994). In Aplysia, short, intermediate, or long-term memory for sensitization can be elicited by different patterns of tail shocks (Sutton et al., 2002).

In vivo studies show that episodic, but not continuous, hypoxia evokes long-term facilitation (LTF) in genioglossus muscle, the principal tongue protrude muscle, in the neonatal rats (McKay et al., 2004). In neonatal rat medullary slices generating respiratory rhythm, episodic but not continuous application of a serotonin (5-HT $_{2A}$) receptor agonist evokes an in vitro form of LTF, i.e., persistent increases of hypoglossal motor output, due to

an increase in AMPA-mediated inspiratory drive currents in hypoglossal motoneurons (Bocchiaro and Feldman, 2004). These studies identified that pattern of stimuli in inducing LTP in hypoglossal motor system is crucial.

Developmental considerations

TMNs controls jaw related movements such as suckling and chewing. The transition from suckling to chewing occurs gradually over a period that can vary between species. In rats, the first masticatory movements appear around post-natal (P) day 12, and the adult patternis established between P18-P21. This transformation occurs in a context of impressive morphological, physiological, and neural maturation (Westneat and Hall, 1992). In this transition period applying the HFS stimulation could increase the TMNs excitability and exhibit the LTP-IE. HMNs participate in multiple reflex and rhythmic motor behaviors that must be functional at birth, including swallowing, and respiration (Bartlett et al., 1990; Miller, 2002). Therefore, modulation of HMNs excitability is important to ensure appropriate output to tongue muscles for execution of these motor behaviors. Basic mechanisms required for respiratory pattern generation are operational at 13-18 days of fetal rats (Greer et al., 1992). Swallowing is also observable in the developing fetus by the 11^{th} week of gestation which is essential for the regulation of amniotic fluid (Bu'Lock et al., 1990). Respiratory regulation during early postnatal development is different from that in adulthood. For example, in the fetus the response to a single episode of hypoxia is ventilator depression, whereas in the neonate, ventilation initially increases, then is depressed and in mature adults ventilation increases (Mortola, 1999; Waters and Gozal, 2003).

Taken together, pattern of stimuli at the proper developmental stage in hypoglossal motor system is likely important and should be considered. Similar modulation of the excitability of other types of motoneurons would represent an important of behavioral adaptation of movements to challenges affecting motor performance (Rekling et al., 2000).

Understanding details of the signal transduction and plasticity mechanism in TMNs and HMNs may be helpful in understanding and perhaps treating pathological conditions such as bruxism, clenching, dysphagia and sleep apnea disorder.

Mechanisms of LTP-IE in TMNs

Protein kinase A (PKA) is activated when calcium/calmodulin activates adenylyl cyclase and increases intracellular cyclic adenosine monophosphate (cAMP) levels. It has been proposed that increase in intracellular calcium-evoked somatic spikes may initially provide a rapid priming mechanism and lead to subsequent activation of Ca^{+2} dependent protein kinase II (CaMKII) and protein kinase C (PKC) and thereby mediate LTP (Jacobs and Meyer, 1997; Llano et al., 1994). PKA activation is an essential component of LTP-IE in LV visual cortical neurons, while dopamine D1/5 receptor-mediated LTP-IE requires PKC to mediate modulation of voltage-dependent ion channels in PFC pyramidal layer V/VI neurons (Chen et al., 2007; Cudmore and Turrigiano, 2004). In mammalian hippocampus, serotonin activates adenylyl cyclase through its G-protein-coupled receptor; leading to an increase in intracellular cAMP, which itself activates PKA. Activated PKA can translocate into the nucleus and phosphorylate cAMP-response-element binding protein (CREB), a transcription factor that regulates the expression of genes involved in long-term increases in synaptic efficacy.

In accordance with previous results (Abbott and Nelson, 2000; Chen et al., 2007; Cudmore and Turrigiano, 2004; Malenka and Nicoll, 1999), I have shown that LTP-IE in TMNs is also calcium dependent, as limiting influx with nominally 0 mM calcium ACSF, or buffering intracellular calcium with BAPTA, both prevent LTP-IE. Calcium influx is essential for neuronal transmitter release (Katz and Miledi, 1967). Calcium entry shapes the amplitude and duration of regenerative APs (Baccaglini and Spitzer, 1977; Llinas and Yarom, 1981; Wong and Prince, 1978). In addition, the gating of several conductances is regulated by

changes in the intracellular free calcium concentration (Blatz and Magleby, 1986; Ewald et al., 1985). Which biomechanical pathways are activated by Ca^{2+} and are required for translating the Ca^{2+} signal into an increase in synaptic strength, are of great importance.

I found that LTP-IE, like LTP, is dependent on protein kinase activation during the induction period, as it could be prevented by including the broad-spectrum kinase inhibitor, H-7 during the induction period. In contrast to many forms of LTP, however, LTP-IE could still be induced in the presence of PKC and CaMKII inhibitors, suggesting that these kinases do not play a necessary role in its induction. My data suggest that the critical kinase is PKA because the specific PKA inhibitor H-89 prevented LTP-IE, whereas activating adenylyl cyclase with forsk both mimicked and occluded firing-induced LTP-IE. PKA is not the only effector of cAMP action in neurons (Kopperud et al., 2003), so directly raising cAMP with forsk could have downstream effects on neuronal properties that are independent of PKA activation. The ability of H-7 and H-89 to completely block LTP-IE, however, suggests that PKA activation is a necessary component of this signal transduction cascade.

In a previous study, cAMP-dependent activation of PKA appeared to contribute to the 5-HT-induced neural firing activity of TMNs, and through activation of PKA, PKC might inhibit Ca^{2+}-activated K^+ channels (Inoue et al., 1999). PKA is known to phosphorylate and downregulate K^+ channels, causing an increase in excitability (Hoffman and Johnston, 1998). The decreased threshold for AP generation induced by LTP-IE in TMNs, suggests that calcium influx and subsequent PKA activation could act by altering voltage-dependent conductances that begin to activate around the AP threshold. Which conductances are affected, and whether this occurs through modulation of existing channels or insertion or removal of new channels, remains to be determined.

TMN excitability is dynamically modulated by required protein kinases and phosphatases. The general role of this modulation may be to adjust their excitability to ensure

appropriate contraction and relaxation of jaw driving muscles to different physiological challenges affecting mastication, swallowing and suckling. Rhythmical oral-motor activities, such as sucking and chewing, are thought to be under the control of a network of brainstem neurons typically referred to as a central pattern generator (CPG) (Lund et al., 1998). In the case of the trigeminal system, the findings that TMNs exhibit nonlinear intrinsic membrane characteristics imparted by the presence of NMDA receptors and that these synaptic connections are functional during oral-motor behaviors (Katakura and Chandler, 1990) suggest that the final rhythmical motoneuronal output is determined by a complex interaction between rhythmically occurring trigeminal premotoneuron oscillations and intrinsic motoneuronal membrane oscillations (Kim and Chandler, 1995). Few findings are available regarding the development of the circuitry underlying oral-motor rhythmical activity and, in particular, the properties of TMNs during the transition from rhythmical sucking behavior to adult-like mastication (Turman et al., 1999). The present findings are of critical importance to a complete understanding of the cellular factors controlling oral-motor behavior. Indeed, it has been reported that the process of induction depends exclusively on the activation of NMDA receptors in other neurons of the trigeminal system (Hamba et al., 2000; Youn, 2008). Although rhythmical oral-motor behaviors are produced by brainstem CPGs that project to TMNs, the intrinsic properties of the TMNs shape the final output pattern (Chandler and Goldberg, 1982; Enomoto et al., 2002; Kogo et al., 1996; Lund, 1991; Nakamura and Katakura, 1995; Tanaka et al., 1999). These properties can amplify motoneuronal output. Clearly, ion currents affect multiple aspects of membrane excitability to shape neuronal activity. Moreover, modification of these currents in certain pathophysiological conditions has now been established (Ashcroft, 2000). It remains to be seen whether any oral-motor disorders resulting in abnormal jaw movements and pain states are associated with dysfunction of ion currents and activity in brainstem neurons. Although plasticity of intrinsic

electrical properties is not the only form of homeostatic plasticity present in trigeminal neural circuits, future investigations of the types of channel conductances affected by plasticity and whether this change occurs through modulation of channels will be useful in elucidating the mechanisms controlling the trigeminal system.

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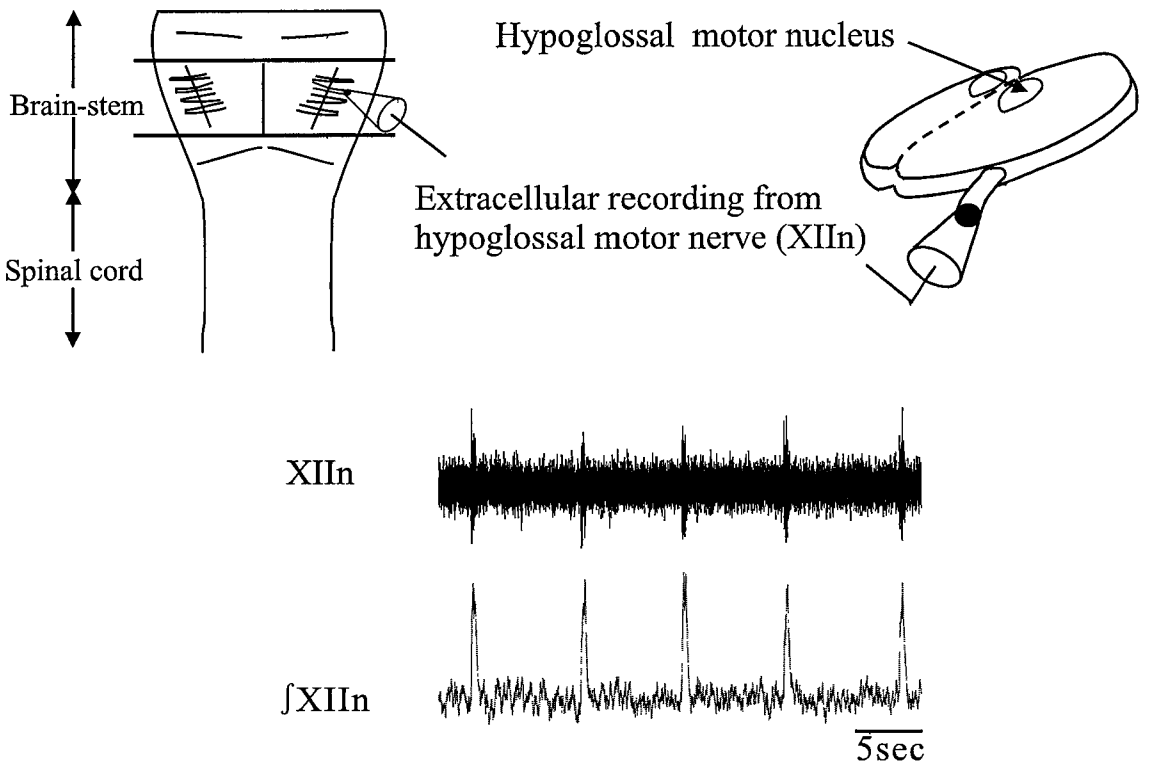
A


Brain-stem
Spinal cord
Hypoglossal motor nucleus
Extracellular recording from
hypoglossal motor nerve (XIIn)
XIIn
∫XIIn
5sec

B

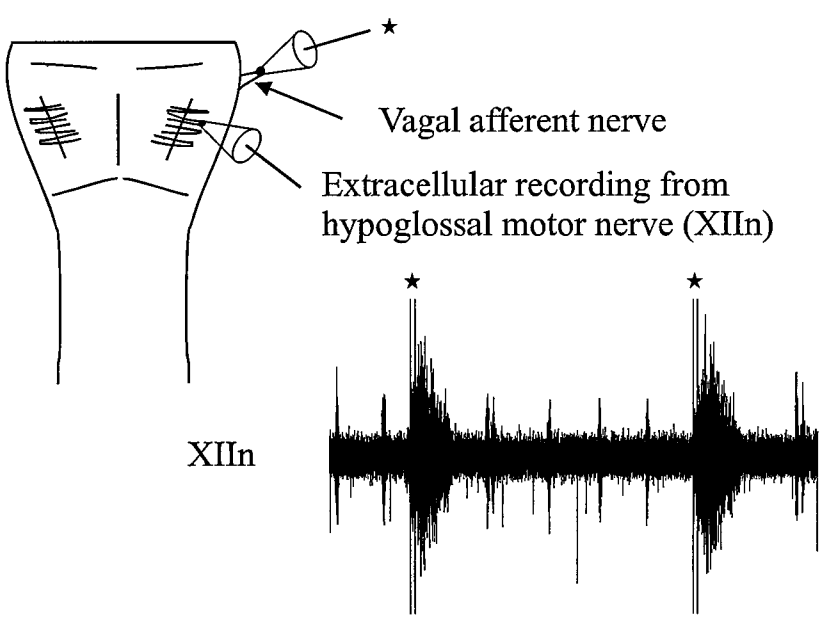

Vagal afferent nerve
Extracellular recording from
hypoglossal motor nerve (XIIn)
XIIn

Fig. 1. Preparation and extracellular recording. (*A*) Brainstem preparation transected from the inter-collicular level to the fourth cervical ventral nerve (C4) level (left). Scheme of a sagittal view of the brain slice which was cut at the pontomedullary and medulla-spinal cord junction, including the hypoglossal motor nucleus and hypoglossal motor nerve (right). Respiratory activities (bottom) that were spontaneously generated were recorded from the hypoglossal motor nerve by extracellular recording (n = 20). Traces are raw signals of extracellular recordings from a hypoglossal (XIIn) motoneuron population and integrated signals of hypoglossal discharges ([integral] XIIn). (*B*) In response to electrical stimulation (asterisks) of the vagal afferent nerve, burst discharges, which defined as swallowing activity, were induced (n = 5).

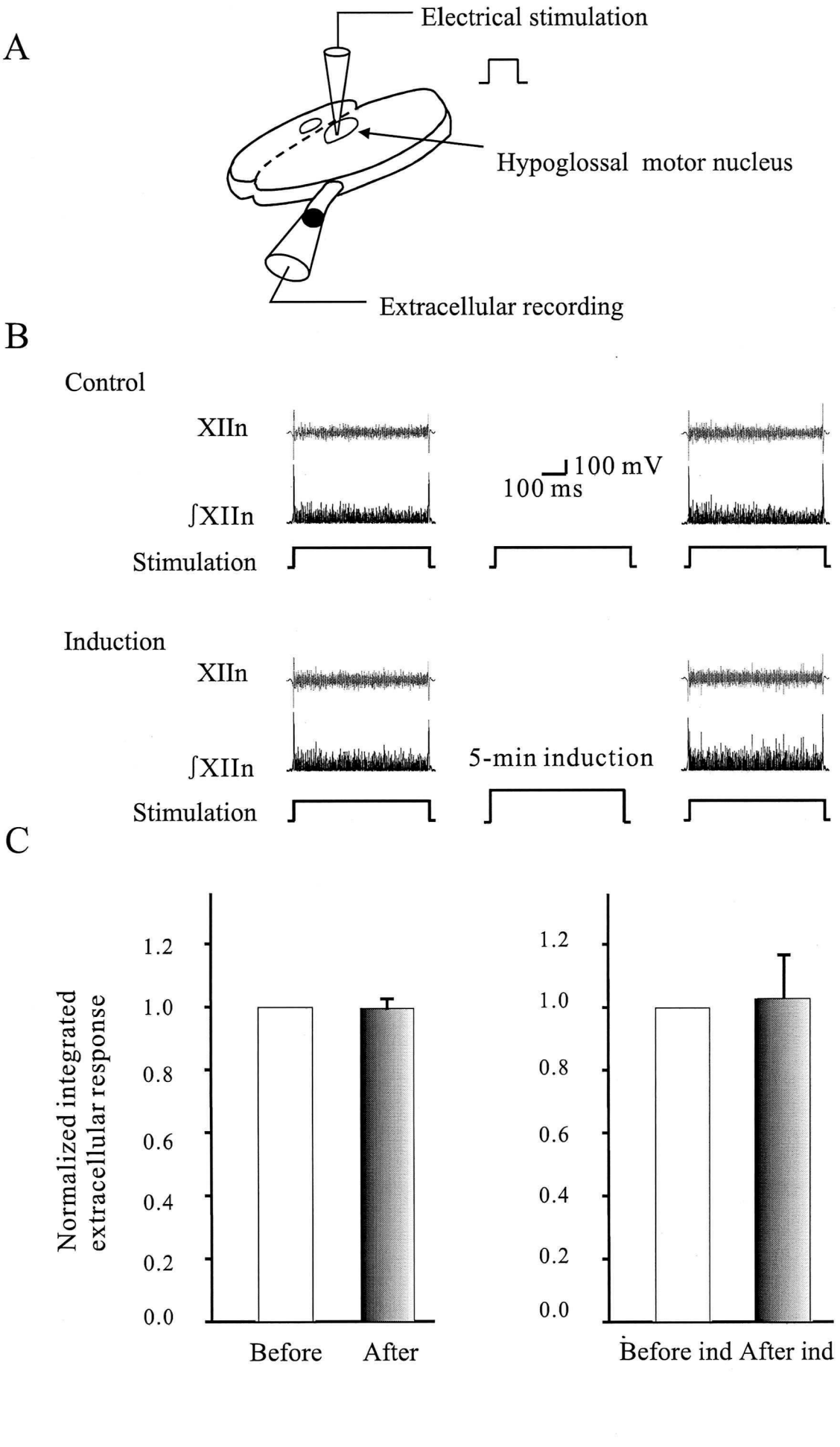

A
Electrical stimulation
Hypoglossal motor nucleus
Extracellular recording
B
Control
XIIn
∫XIIn
Stimulation
100 mV
100 ms
Induction
XIIn
∫XIIn
Stimulation
5-min induction
C
Normalized integrated extracellular response
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Before
After
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Before ind
After ind

Fig.2. Extracellular recording from hypoglossal motoneuron (HMN) population. (*A*) Focal stimulation (400-500 μV) was delivered via a concentric bipolar electrode. The exposed tip of the stimulator was positioned on the motor nucleus. (*B*) Example of a control recording and induction. Constant-amplitude current pulses were delivered every 30 seconds to measure intrinsic excitability. Extracellular discharge from the HMN population remained stable throughout the recording ($n = 20$) (top). The HMNs were stimulated with an electrical stimulus 1.5 to 2 times the size of the test pulse (500 ms) (every 5 s for 5 min) injected for stimulation of induction. Following the induction stimulus, there was no change in the excitability of HMNs ($n = 20$) (bottom). (*C*) Integrated area of the resultant HMN population response before and after induction. Extracellular responses were rectified and integrated area was calculated. There was no significant change between the amplitude of extracellular response before induction and after that.

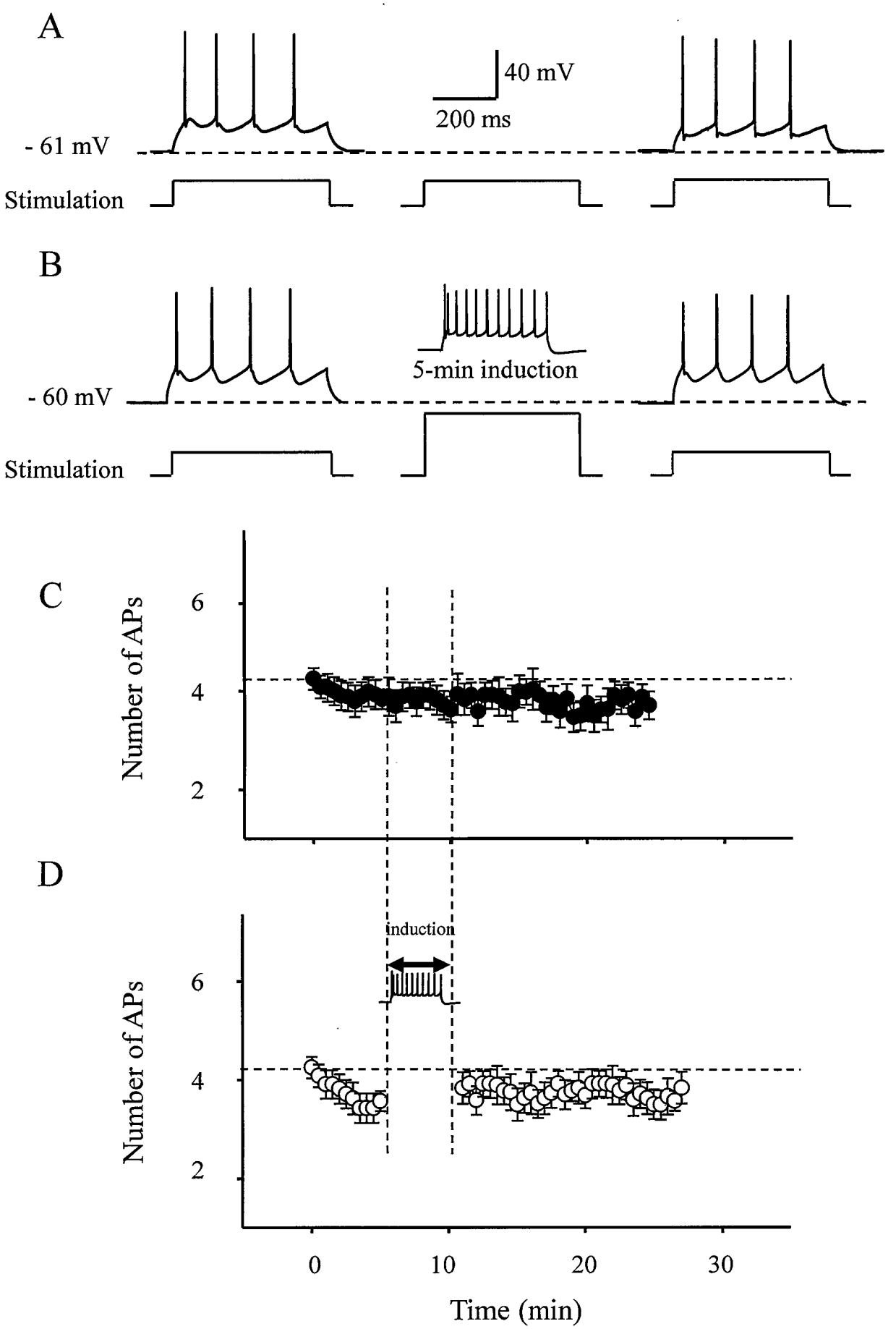

A
40 mV
200 ms
- 61 mV
Stimulation
B
5-min induction
- 60 mV
Stimulation
C
Number of APs
6
4
2
D
induction
Number of APs
6
4
2
0
10
20
30
Time (min)

Fig.3. Whole-cell patch-clamp recording from a single hypoglossal motoneuron. (*A, C*) 500-ms DC test pulse was injected every 30 s to evoke 4 to 5 APs. Trace shows the voltage response to the test pulse. In control recordings, the response to the test pulse remained constant for the duration of recording (n = 20). (*B, D*) Inducing neurons to fire at higher frequencies for 5 min (20-30 Hz for 500 ms every 5 s) could not increase the frequency of Aps elicited by the test pulse (n = 20).

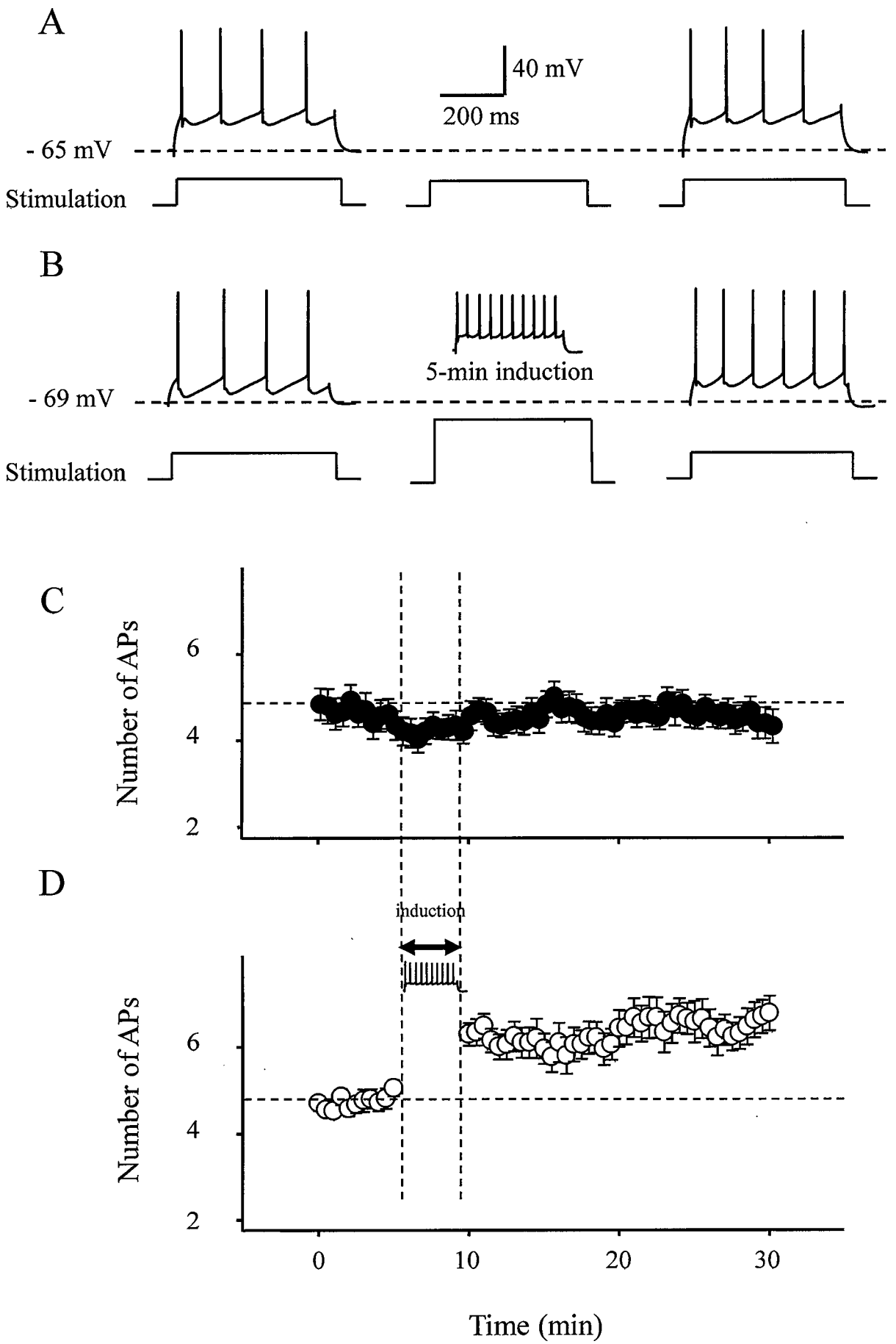

A
40 mV
200 ms
- 65 mV
Stimulation
B
5-min induction
- 69 mV
Stimulation
C
Number of APs
6
4
2
D
induction
Number of APs
6
4
2
0
10
20
30
Time (min)

Fig.4. Whole-cell patch-clamp recording from a single trigeminal motoneuron. (*A, C*) 500-ms DC test pulse was injected every 30 s to evoke 4 to 5 APs. Trace shows the voltage response to the test pulse. In control recordings, the response to the test pulse remained constant for the duration of recording (n = 23). (*B, D*) Inducing neurons to fire at higher frequencies for 5 min (20-30 Hz for 500 ms every 5 s) increased the frequency of Aps elicited by the test pulse (n = 21).

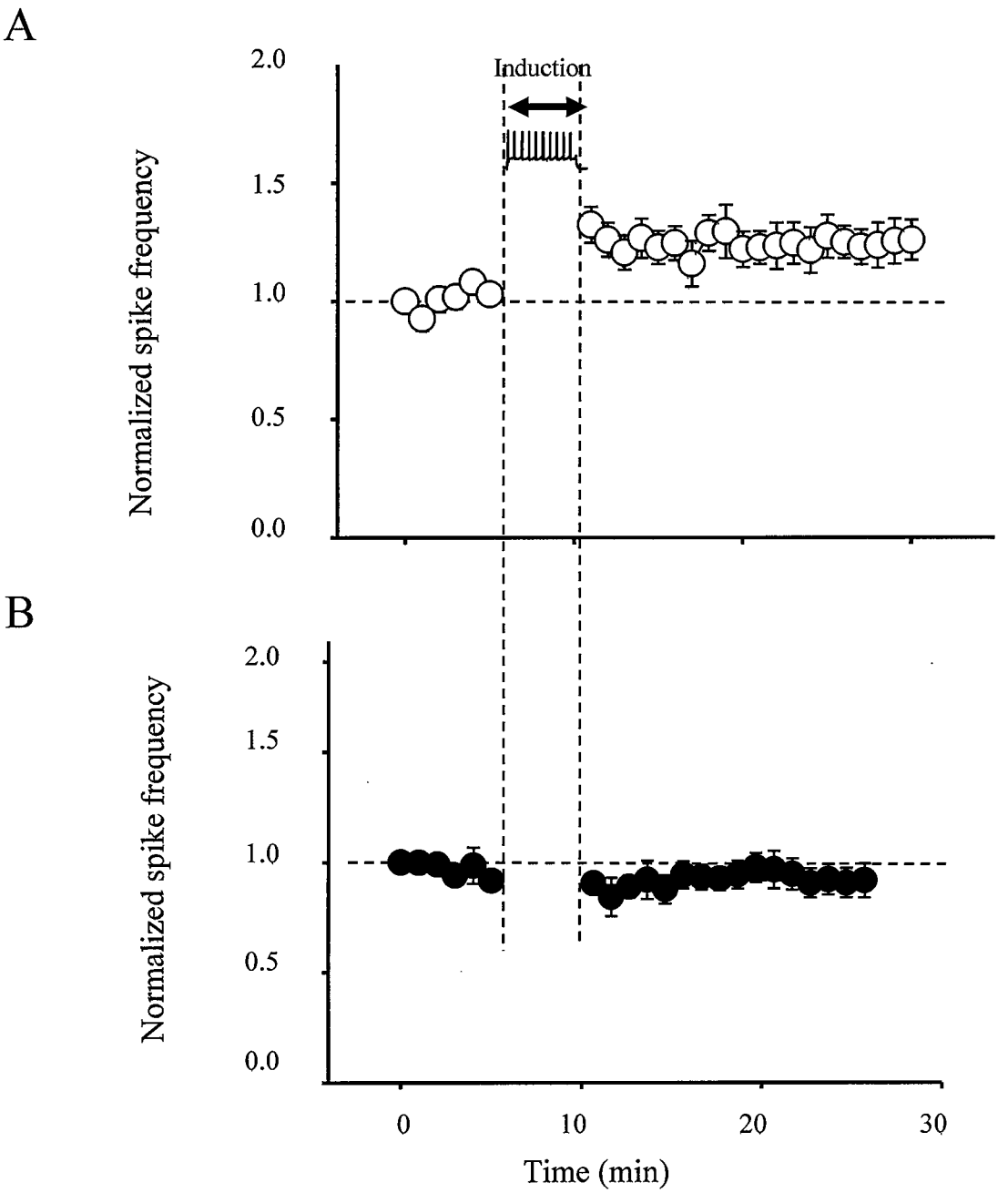


Fig. 5. Average time courses of induced recordings. (*A*) Spike rate was averaged for all TMNs given the induction stimulus. Induction produced a significant increase in spike rate evoked by the test pulse (n = 21). (*B*) Averaged spike rate in HMNs given the induction stimulus, exhibited no significant change (n = 20).

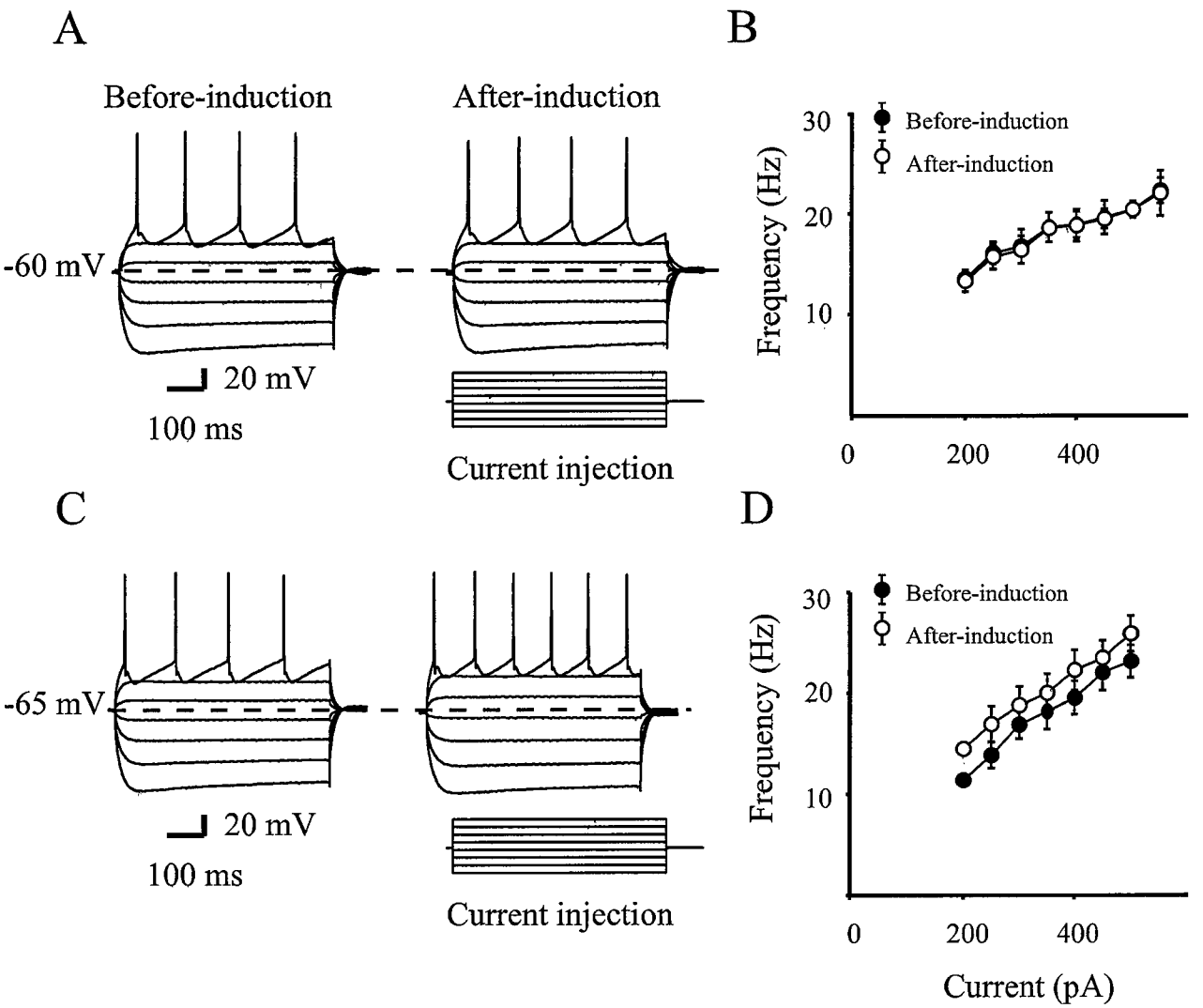
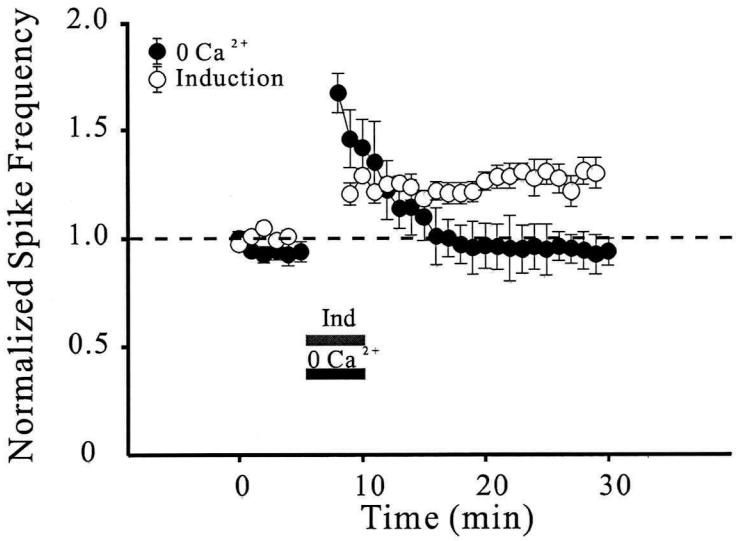
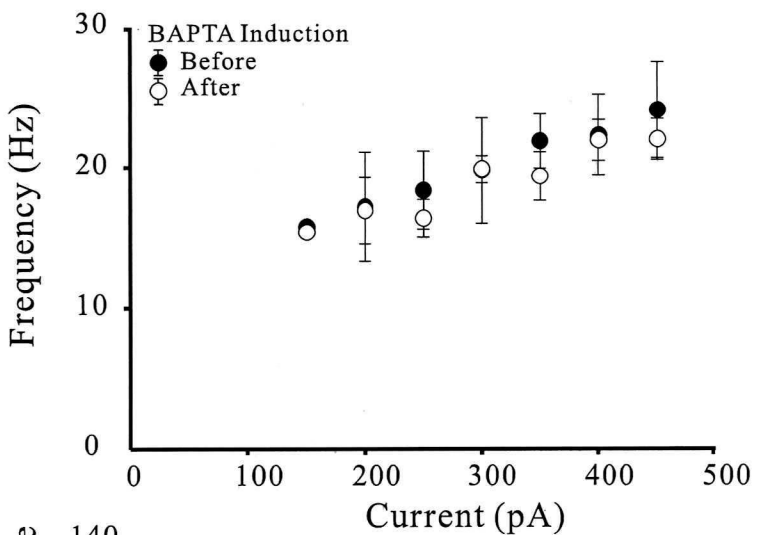


Fig. 6. Firing properties of hypoglossal and trigeminal motoneurons (HMNs and TMNs). (*A, C*) 500-ms current steps of increasing intensity were delivered every 5 s for HMNs and TMNs. Traces are examples of the firing patterns for both motoneurons evoked at three levels of current stimulation before (left) and after induction (right). (*B, D*) Average firing rates before and after induction are plotted against current intensity and revealed *F-I* curve. In HMNs group (*B*) post-induction frequency was similar to that pre-induction ($n = 20$) while in TMNs group (*D*) induction caused a leftward shift in the average *F-I* curve ($n = 15$).

A


Normalized Spike Frequency
2.0
1.5
1.0
0.5
0
0 Ca 2+
Induction
Ind
0 Ca 2+
0
10
20
30
Time (min)

B


Frequency (Hz)
30
20
10
0
BAPTA Induction
Before
After
0
100
200
300
400
500
Current (pA)

C

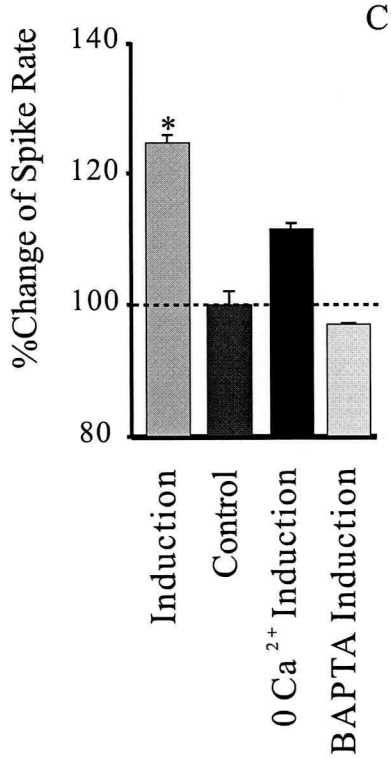

%Change of Spike Rate
140
120
100
80
*
Induction
Control
0 Ca 2+ Induction
BAPTA Induction

Fig. 7. LTP-IE is Ca^{2+} dependent. (*A*) Bath application (black bar) of nominally 0 Ca^{2+} during the induction period prevented LTP-IE. Nominally 0mM Ca^{2+} artificial cerebrospinal fluid (ACSF) produced a temporary increase in excitability that reversed as regular ACSF was returned to the bath (n = 8). (*B*) Buffering intracellular Ca^{2+} with bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA, 10 mM intracellular) also prevented LTP-IE. When BAPTA was included in the pipette, there was no change in the *F-I* curve after induction (n = 7). (*C*) A change in spike rate was not distinguishable from control when induction was run with nominal 0 Ca^{2+} or BAPTA (* $p < 0.05$).

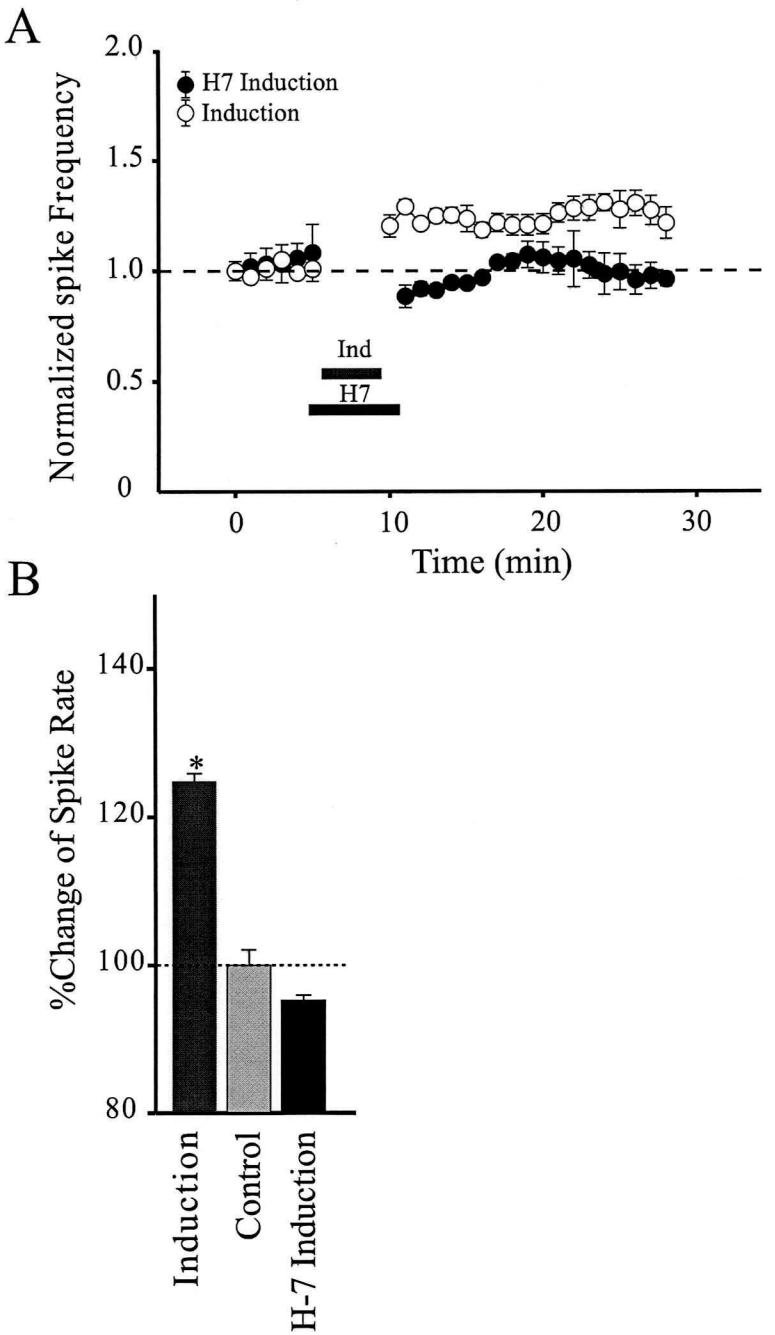


Fig. 8. LTP-IE depends on protein kinase activation. *(A)* H-7, a broad spectrum protein kinase inhibitor, completely blocked the increase in excitability after induction ($n = 8$). H-7 was bath applied (black bar) 5 min before the induction stimulus and terminated 10 min later, always after the end of the induction stimulus (gray bar). *(B)* H-7 also prevented the change in spike rate (* $p < 0.05$).

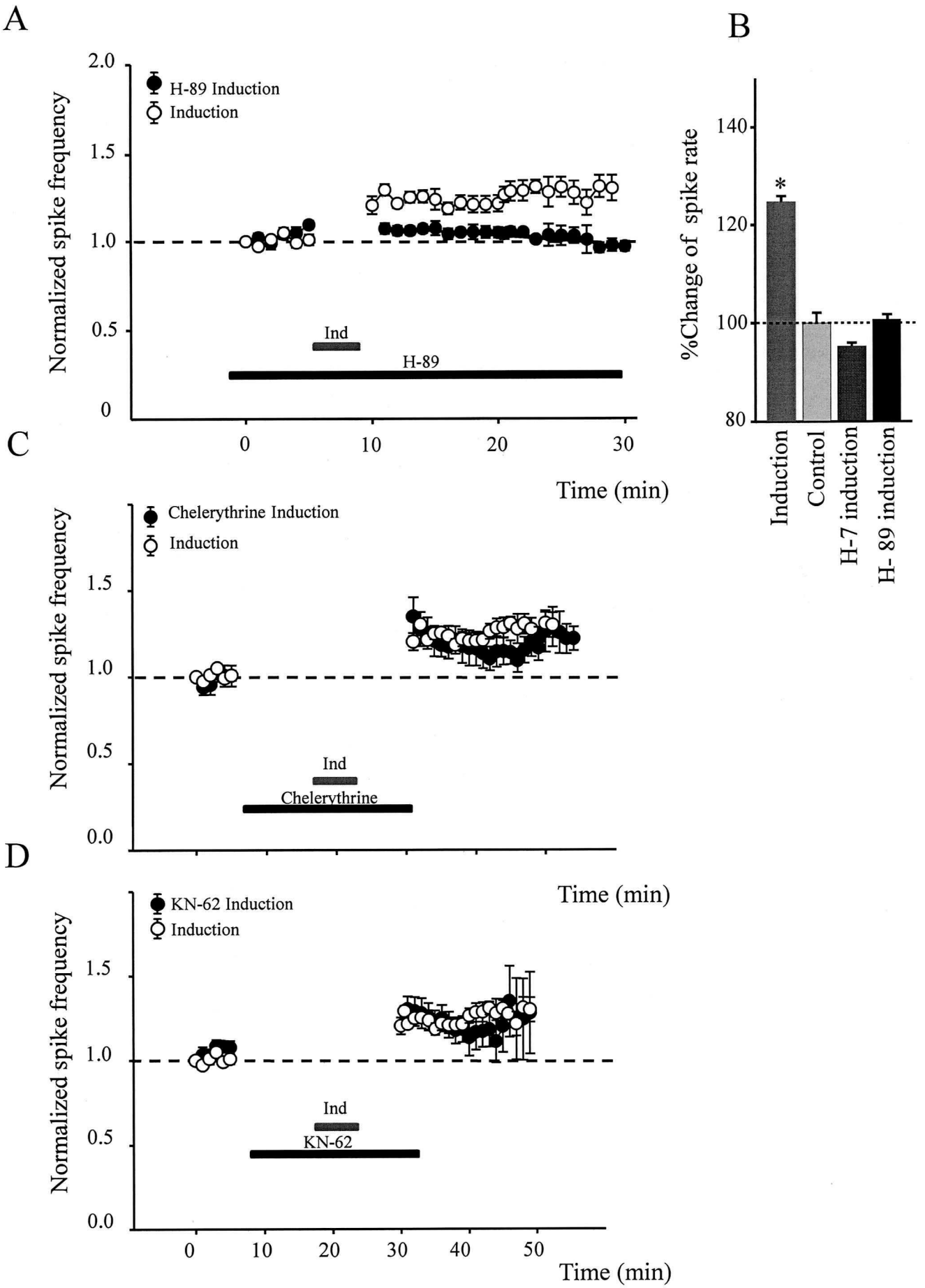

A
B
C
D
H-89 Induction
Induction
Normalized spike frequency
Ind
H-89
Time (min)
%Change of spike rate
Induction
Control
H-7 induction
H- 89 induction
Chelerythrine Induction
Chelerythrine
KN-62 Induction
KN-62

Fig. 9. LTP-IE depends on protein kinase activation. (*A*) Intracellular application (black bar) of PKA inhibitor, H-89 (100 μM), prevented the increase in excitability produced by the induction protocol (n = 8). (*B*) H-89 also prevented the change in spike rate. (*C*) and (*D*), LTP-IE was still produced when PKC and CaMKII activities were inhibited using 30 min bath application (black bar) of chelerythrine (10 μM, n = 6) and KN62 (10 μM, n = 10) (* $p <$ 0.05).

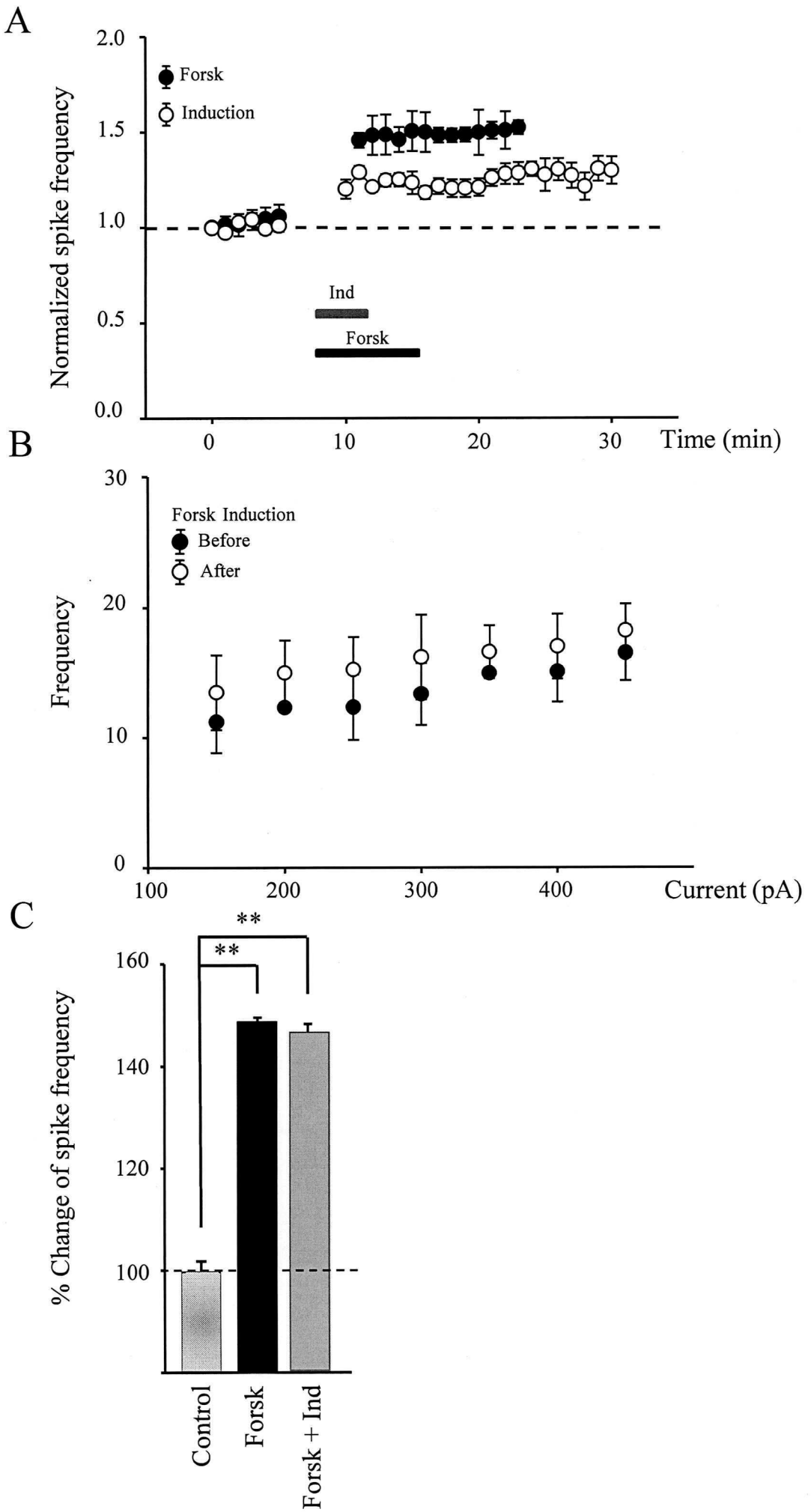

A
2.0
1.5
1.0
0.5
0.0
Forsk
Induction
Ind
Forsk
Normalized spike frequency
0
10
20
30
Time (min)
B
30
20
10
0
Forsk Induction
Before
After
Frequency
100
200
300
400
Current (pA)
C
**
**
160
140
120
100
% Change of spike frequency
Control
Forsk
Forsk + Ind

Fig. 10. PKA activation mimicked and occluded LTP-IE. (*A*) 10-min bath application of forskolin (Forsk) (black bar) produces a long-lasting increase in excitability that closely resembled LTP-IE ($n = 10$, $p < 0.05$). (*B*) Bath application of Forsk produced a leftward shift in the *F-I* curve. (*C*) To determine if the increased excitability induced by Forsk occluded firing-induced LTP-IE, an induction stimulus was run (Forsk + Ind) following bath application of Forsk. This did not cause any further change in excitability ($n = 10$,** $p < 0.005$).

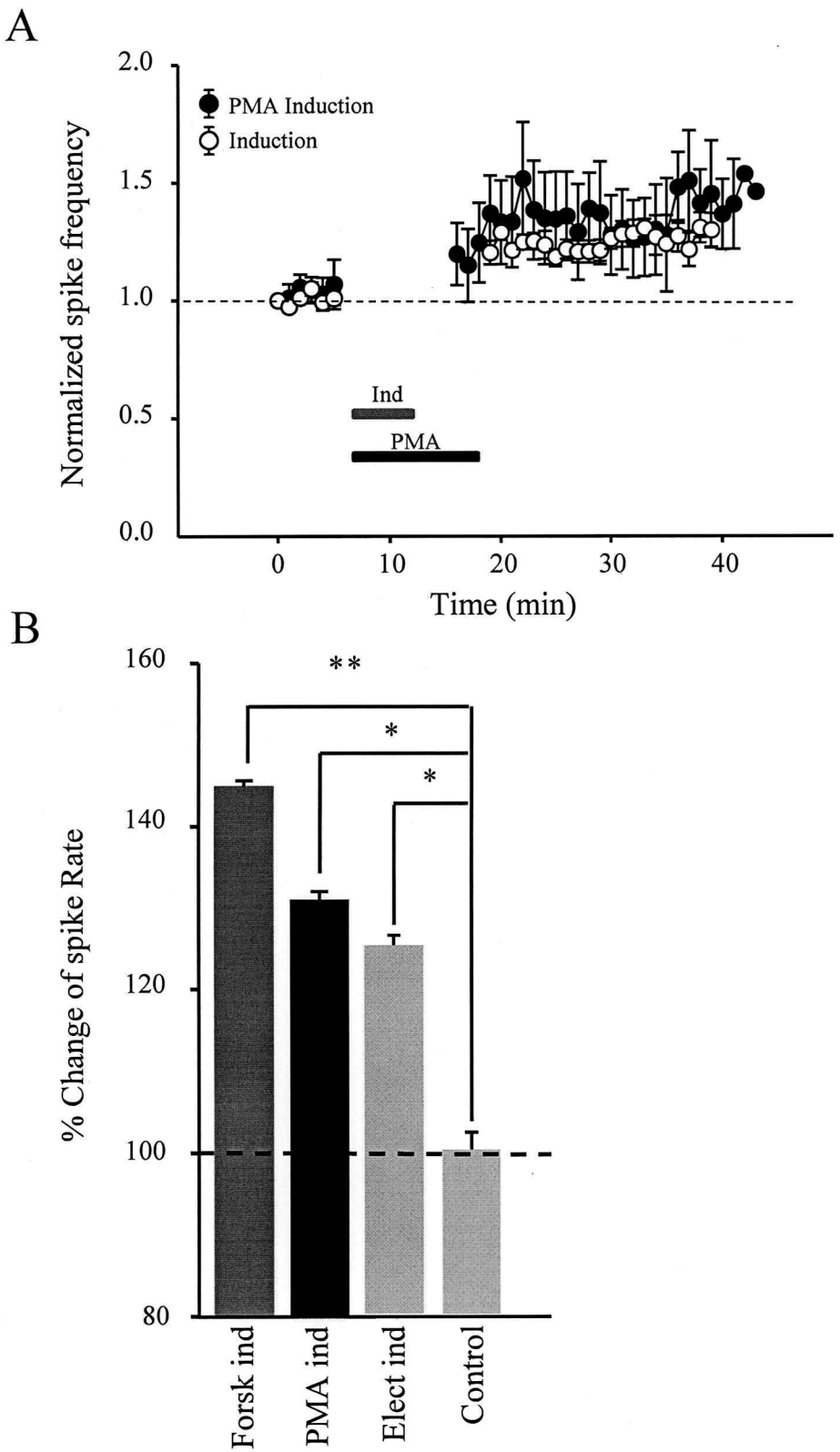


Fig. 11. PKC activation and LTP-IE. (*A*) 10-min bath application of phorbol 12-myristate 13-acetate (PMA) (black bar) increased the spike rate ($n = 6$, $p < 0.05$). (*B*) Change in spike rate was significantly different between Forsk and PMA (* $p < 0.05$, ** $p < 0.005$).