Synaptic activity-independent persistent plasticity in endogenously active mammalian motoneurons

Christopher M. Bocchiaro* and Jack L. Feldman†

Systems Neurobiology Laboratory, Departments of Neurobiology and Physiological Science, University of California, Los Angeles, CA 90095-1763

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Potentiation and depression of glutamate receptor function in hippocampal, cerebellar, and cortical neurons are examples of persistent changes in synaptic function that underlie important behavioral adaptations such as learning and memory. Persistent changes in synaptic function relevant for motor behaviors have not been demonstrated in mammalian motoneurons. We demonstrate that adaptive changes in (±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrobromide (AMPA) receptor function at endogenously active synapses occur in motoneurons in neonatal rodents. We found a form of serotonin (5-HT)-dependent synaptic plasticity in hypoglossal (XII) motoneurons, which control tongue muscles affecting upper airway function, that is metamodulated by metabotropic glutamate receptors. Episodic, but not continuous, activation of postsynaptic 5-HT type 2 (5-HT_2) receptors on hypoglossal (XII) motoneurons leads to long-lasting increases in their AMPA receptor-mediated respiratory drive currents and associated XII nerve motor output. Antagonism of group-I metabotropic glutamate receptors blocks induction of the 5-HT-induced increase in excitability. We propose that this activity-independent postsynaptic 5-HT-mediated plasticity represents the cellular mechanism underlying long-term facilitation, i.e., persistent increases in respiratory motor output and ventilation seen in humans and rodents in response to episodic hypoxia. Loss of activity in XII motoneurons is common during sleep causing snoring and, in serious cases, airway obstruction that interrupts breathing, a condition known as obstructive sleep apnea. These results may provide the basis for rationale development of therapeutics for obstructive sleep apnea in humans.

excitability | respiration | breathing | learning | apnea

Motoneurons produce behavior by integrating and transforming patterns of premotoneuronal activity into commands for skeletal muscle contraction. The principal fast excitatory neurotransmitter affecting motoneurons is glutamate (1), acting mainly through (±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrobromide (AMPA)-type receptors (2). Changes in AMPA receptor function contribute to neuronal plasticity, such as occurs in long-term potentiation and depression in the hippocampus, cerebellum, cortex, and dorsal spinal cord (3–5); this is partly mediated through intracellular second messengers affecting the functional characteristics of AMPA receptors (3, 6, 7). Whether similar long-term plasticity at AMPA synapses can be induced in mammalian motoneurons, which are often viewed as passive relays (1), is not known.

Long-lasting changes in neuronal excitability are an important component of adaptive behavior. Such changes in motoneuronal excitability could play a role in the fine tuning and/or coordination of movement and in motor learning. Persistent, compensatory increases in breathing movements, termed long-term facilitation (LTF) (8), can be induced in neonatal[‡] and adult (10–12) rats by episodic but not continuous exposure to hypoxia. In humans who are persistent snorers, episodic hypoxia during sleep induces LTF in upper airway dilator muscles (which are innervated by the XII nerve) (13–15). LTF is associated with increases in phrenic and/or XII nerve (XIIn) inspiratory-modulated discharge. Initiation, but not maintenance, of LTF depends on 5-$HT_{2A/C}$ receptor activation (16). The cellular basis for this process in identified neurons, postulated to be behaviorally relevant and allostatic, has not been established (11).

In a brainstem slice from neonatal rat generating endogenous rhythmic respiratory-related activity (17), episodic, but not continuous, stimulation of 5-hydroxytryptamine (serotonin) type 2 (5-HT_2) receptors on XII motoneurons leads to activity-independent long-lasting increases in their AMPA-mediated inspiratory-related drive currents and a concomitant increase in XIIn output. We propose that this represents at least one component of the cellular mechanism underlying LTF of respiratory motor output in mammals. Furthermore, we demonstrate that group-1 metabotropic glutamate receptors (mGluRs) play a permissive role in this 5-HT-dependent mechanism. This long-lasting form of synaptic plasticity, *in vitro* LTF, in motoneurons may contribute to the regulation of breathing essential during sleep and in response to hypoxia.

Methods

Experiments were performed on a medullary slice preparation (Sprague–Dawley rats, 0–3 days old) that spontaneously generates respiratory rhythm (17). The dissection and slicing were performed in an artificial cerebrospinal fluid bubbled with 95% O_2/5% CO_2 at room temperature. The artificial cerebrospinal fluid contained 128 mM NaCl, 3.0 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgSO_4$, 23.5 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, and 30 mM glucose. During electrophysiological recording, the slice was continuously superfused (≈3 ml/min) with artificial cerebrospinal fluid (temperature maintained at 27 ± 1°C) with increased KCl (9 mM).

Electrophysiological Recording. XII motoneurons were visualized with infrared differential interference contrast microscopy. Electrodes (5.0–7.5 MΩ, 1.0- to 1.5-μm tip diameter) pulled from borosilicate glass on a horizontal puller were filled with solution containing 120.0 mM potassium gluconate, 11.0 mM EGTA, 5.0 mM NaCl, 1.0 mM $CaCl_2$, 10.0 mM Hepes, and 2.0 mM ATP (Mg^{2+} salt), pH adjusted to 7.3. Intracellular signals were amplified with a patch-clamp amplifier (Axopatch 1D, Axon Instruments, Foster City, CA); whole cell capacitance was compensated, as was the series resistance. Signals were low-pass-filtered at 10 kHz. Input resistance stability was checked throughout the experiments. During voltage-clamp recordings, XII motoneurons were held at −70 mV. Respiratory-related whole nerve activity was recorded from the cut ends of the XII nerve with a suction electrode, amplified 10–20 K, band-pass-

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Abbreviations: AMPA, (±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrobromide; LTF, long-term facilitation; mGluR, metabotropic glutamate receptor; 5-HT, 5-hydroxytryptamine (serotonin); L-AP3, L-(+)-2-amino-3-phosphonopropionic acid; α-Me-5-HT, α-methyl-5-hydroxytryptamine maleate.

*Present address: GEL-P, Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037.

†To whom correspondence should be addressed. E-mail: feldman@ucla.edu.

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filtered (3–3,000 Hz), and rectified. Peak amplitudes, from 20 consecutive bursts, were measured from the integrated XIIn signal.

Drugs and Drug Application. The following drugs were used: α-methyl-5-hydroxytryptamine maleate (α-Me-5-HT, Tocris Cookson, St. Louis), 5-HT_2 receptor agonist; Pindolol (Tocris Cookson), 5-$HT_{1A/1B}$ receptor antagonist; (*S*)-α-methyl-4-carboxyphenylglycine (Tocris Cookson), nonselective group 1/group 2 mGluR antagonist; L-(+)-2-amino-3-phosphonopropionic acid (L-AP3, Fluka), competitive group 1 mGluR antagonist; AMPA (Sigma), glutamate receptor agonist.

For experiments involving local application, α-Me-5-HT [10 μM; this concentration best mimicked the responses to bath application of 1 μM α-Me-5-HT (18)] was dissolved in artificial cerebrospinal fluid and pressure ejected from a glass pipette (tip diameter, 1.5–3.0 μm; pressure, 10 psi; duration as indicated in *Results*) placed under visual guidance above the XII nucleus.

For experiments involving application of exogenous AMPA, action potential-dependent synaptic transmission was prevented by bath application of tetrodotoxin (1 μM). AMPA (10 μM) was dissolved in artificial cerebrospinal fluid and pressure ejected from a glass pipette (tip diameter, 1.5–3.0 μm; pressure, 10 psi; duration, 50–150 ms; rate, one ejection per min), placed under visual guidance above the neuron being recorded. With this concentration and injection protocol, no evidence of desensitization of AMPA responses was seen.

XII motoneurons were anatomically and physiologically identified. Large (>15-μm diameter), multipolar neurons were visualized in clusters in the XII nucleus located ventral to the fourth ventricle and near the midline. XII motoneurons exhibited rhythmic activity in phase with XII nerve (XIIn) respiratory-modulated motor output.

Data Acquisition and Analysis. Signals were sampled digitally at 20 kHz and stored for analysis by using CLAMPFIT 8.0 (Axon Instruments) and ORIGIN 7.0 (OriginLab). Peak whole-cell amplitudes were averaged from six consecutive respiratory bursts. Averaged peak currents were normalized to the current amplitude immediately following whole cell break-in (control). Recordings of endogenous drive currents were triggered off the integrated XII motor output. The overall charge transfer of the recorded signals was computed by taking the integral of the recorded signal.

Statistical values are reported as means ± SD. Differences between means were calculated by Student's paired *t* test, with $P < 0.05$ level of significance.

Results

In a brainstem slice from neonatal rodent generating respiratory-related rhythm (17), persistent increases in XII motoneuronal activity were generated by episodic bath application (three 3-min agonist applications spaced 5 min apart) of α-methyl-5-HT (α-Me-5-HT; 1 μM), a 5-HT_2 receptor agonist (Fig. 1*A*; see also Fig. 3*D*). Potentiation of motor output and intracellular current was elevated at the end of the third application of α-methyl-5-HT. Compared with activity before application, respiratory-related currents in XII motoneurons at 60 min after application increased by 55 ± 20% (Fig. 1*B*; $n = 5, P < 0.05$), and XII nerve activity increased by 70 ± 22% (Fig. 1 *A* and *B*) ($n = 10$; $P < 0.05$). No change occurred in neuronal input resistance after agonist application (19).

No significant long-term change in XIIn activity was induced by α-Me-5-HT when applied as a continuous 9-min application (Fig. 1*C*) (97 ± 11% at 60 min after application; $n = 7, P > 0.05$). Acute responses during α-Me-5-HT application consisted of increased tonic baseline activity with (Figs. 1*A* and 3*A*) or without (Fig. 1*C*) increased motor output amplitude (20, 21) and were unrelated to persistent increases in motor output. To rule

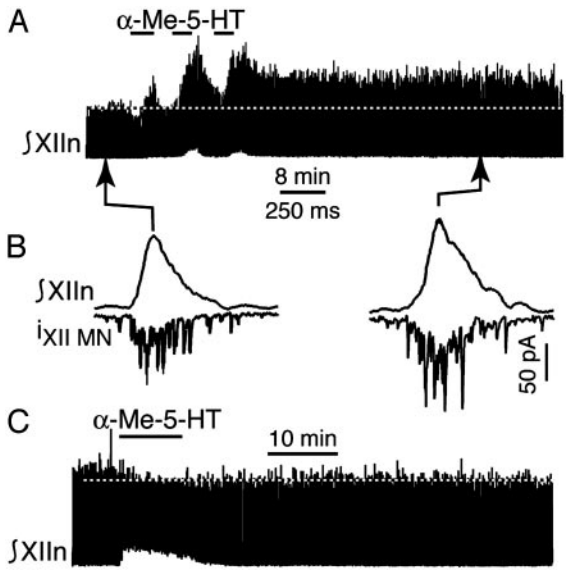

Fig. 1. Episodic but not continuous bath application of α-Me-5-HT induces persistent increases in XII nerve (XIIn) and XII motoneuronal respiratory-modulated drive currents ($i_{XII\ MN}$). (*A*) Effect of three applications of α-Me-5-HT (3 min on, 5 min off) on integrated XIIn (∫XIIn) activity. (*B*) As in *A*, at faster timescale, effect on integrated XIIn activity and endogenous respiratory-related drive currents in whole-cell patched XII motoneurons ($i_{XII\ MN}$). (*C*) Effect of one 9-min continuous application of α-Me-5-HT on integrated XIIn activity. Bar(s) above traces represent α-Me-5-HT application.

out any effects of 5-HT_1 receptors, we repeated the three application protocol in the presence of pindolol (5 μM), a 5-HT_1-receptor antagonist. No difference in the persistent changes in XII motoneuronal activity was seen in these preparations, establishing that the 5-HT_1-receptor subtype did not contribute to *in vitro* LTF.

To establish that persistent excitability increases were initiated within or near the XII nucleus, we applied α-Me-5-HT (10 μM) locally to XII motoneurons by means of a micropipette placed directly over the XII nucleus. Focal episodic application ($n = 7$, $P < 0.05$) produced persistent increases in XII motoneuronal respiratory drive currents (150 ± 21% of control) and XII nerve output (160 ± 26% of control), which lasted at least 60 min without decrement, similar to bath application experiments.

To determine the postsynaptic component of this response, XII motoneurons were synaptically isolated (7). After identification of a XII motoneuron receiving inspiratory-related drive currents in normal bathing medium, tetrodotoxin (0.5 μM) was added to the bath to block action potential-mediated synaptic transmission. Pressure ejection of AMPA (10 μM) from a micropipette positioned above the motoneuron produced postsynaptic AMPA-mediated currents. Control whole-cell patch-clamp recordings were stable over the course of 60 min, with no potentiation or desensitization in the AMPA-induced current (Fig. 2*A*). After episodic 5-HT_2 receptor activation (1 μM, bath applied), AMPA currents were potentiated (peak current, 156 ± 21%; charge transfer, 170 ± 26%; $n = 6, P < 0.05$) for at least 60 min without decrement (Fig. 2*B*).

Respiratory-modulated glutamate release onto XII motoneurons is likely to activate ionotropic receptors and mGluRs, the latter of which can modulate neuronal excitability by means of second messengers (22). To establish whether mGluRs play a role in *in vitro* LTF, mGluR antagonists were bath applied 5–10 min before the episodic application of α-Me-5-HT. Preincubation of the slice with the group 1 mGluR antagonist L-AP3 (300–500 μM) had no discernible effect on respiratory-

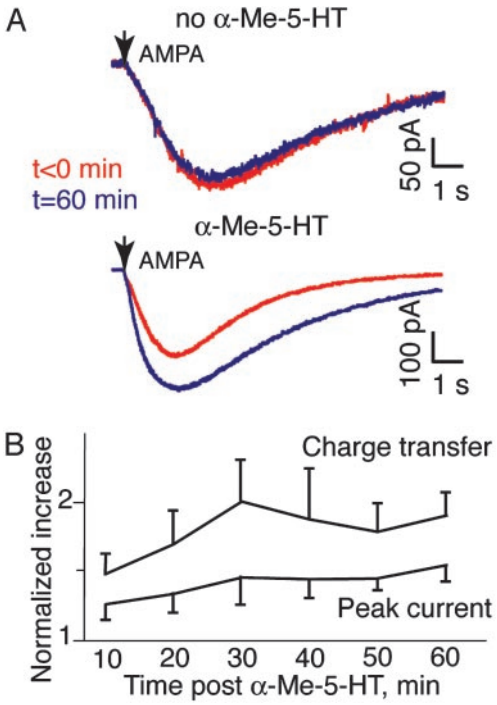

Fig. 2. Episodic α-Me-5-HT application increases currents in synaptically isolated XII motoneurons in response to local puffs of AMPA. (*A Upper*) Whole-cell response to AMPA ejection (arrow) was stable over 60 min in control recordings. (*Lower*) Response increased significantly after episodic α-Me-5-HT application. (*B*) Time course of AMPA-induced currents (peak and charge transfer, i.e., time integral of induced current) after episodic α-Me-5-HT application.

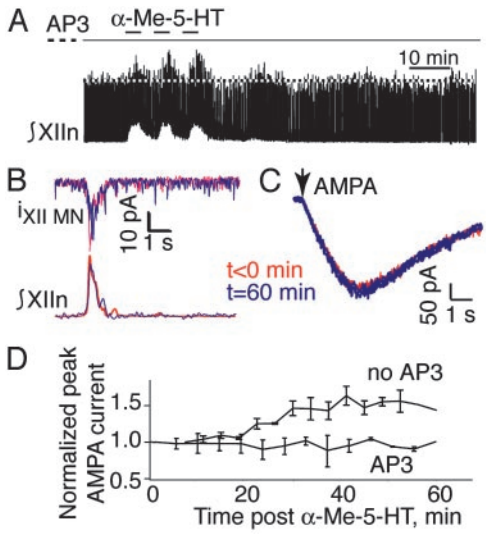

Fig. 3. Antagonism of mGluRs blocks induction of the effects of episodic α-Me-5-HT on AMPA-mediated current. (*A*) After preincubation of the slice with L-AP3, there is no persistent increase in XIIn activity in response to episodic α-Me-5-HT application, although the acute response persists. (*B*) Antagonism of mGluRs blocks the chronic effects of episodic α-Me-5-HT on respiratory-modulated drive current in XII motoneurons. (*C*) L-AP3 also blocks the increase in AMPA-evoked currents in synaptically isolated XII motoneurons. Compare with Fig. 2*A*. (*D*) Time course of changes in AMPA induced current following episodic α-Me-5-HT application with and without AP3.

modulated XII motoneuronal activity or XII motor output. Episodic bath application of α-Me-5-HT could still produce transient increases in activity during application, but there was no induction of persistent changes in XII nerve output (Fig. 3*A*) (98 ± 6%, $n = 7$, $P > 0.05$) and no potentiation of XII motoneuronal respiratory drive currents (Fig. 3*B*) (96 ± 10%, $n = 5$, $P > 0.05$). Similar effects were also seen following preincubation with the group 1/2-mGluR antagonist (*S*)-α-methyl-4-carboxyphenylglycine (250 μM; 103 ± 6%, $n = 3$, $P > 0.05$).

Slices were exposed to L-AP3 (300 μM) and tetrodotoxin, and whole-cell responses to AMPA ejection onto synaptically isolated XII motoneurons were measured before and after episodic α-Me-5-HT bath application. No changes in AMPA-induced currents were seen (Fig. 3 *C* and *D*) (97 ± 7%, $n = 5$, $P > 0.05$). Thus, blocking postsynaptic mGluR activation disrupts 5-HT-dependent AMPA receptor plasticity in XII motoneurons.

Discussion

We demonstrate a form of mammalian motoneuronal synaptic plasticity, *in vitro* LTF, that may subserve a role in motor control and adaptive behavior. Changes in AMPA receptor function are postulated to contribute to neuronal plasticity associated with learning, memory, and injury (3–5). Similar changes in synaptic efficacy have not been demonstrated in mammalian motoneurons, although there can be persistent nonsynaptic changes in excitability, i.e., plateau potentials (1). However, they are in no way related to what we report here, as plateau potentials are not present in XII motoneurons. In addition, plateau potentials are voltage-dependent, which means that they could not contribute to our results because our single-cell experiments were performed in voltage clamp.

Episodic, but not continuous, application of a 5-HT_2 receptor agonist to the XII nucleus leads to a persistent increase in the endogenous respiratory-modulated motor output of the XII nerve. This results from increases in AMPA receptor-mediated respiratory-modulated drive currents in XII motoneurons. Because similar effects were seen on AMPA-induced currents in synaptically isolated XII motoneurons, we conclude that episodic activation of postsynaptic 5-HT_2 receptors on XII motoneurons elicits persistent changes in motoneuronal output by potentiating AMPA receptor function. In the invertebrate *Aplysia californica*, 5-HT also can augment the glutamatergic responses of motoneurons (23–25).

Metamodulation of the persistent change in excitability induced by episodic 5-HT by group 1 mGluRs reveals a form of plasticity in which mGluRs serve a permissive role for the 5-HT-dependent postsynaptic potentiation of AMPA receptors. That this occurs following tetrodotoxin-induced synaptic blockade suggests either that postsynaptic mGluRs are constitutively active in XII motoneurons or that the extracellular concentration of glutamate under these conditions is sufficiently high to activate mGluRs. The mechanism for this interaction remains speculative. Two possibilities are as follows: (*i*) interaction by means of convergent signaling cascades that activate phospholipase C through the heterotrimeric G protein $G_{q/11}$; and (*ii*) direct receptor–receptor interaction, e.g., formation of hetero-oligomers as seen between other pairs of receptors (26). The finding of functional interaction between group 1 mGluRs and 5-HT_2 receptors may not be the unique province of motor control, so that in other systems 5-HT-dependent potentiation of AMPA function may be affected by mGluR activation.

Although not the main focus of this article, the acute 5-HT response can vary between experiments. The long-lasting increases in XII output are not dependent on the initial acute (during application) response to α-Me-5-HT. Fig. 3 demonstrates this point, as there is an acute effect during the application of α-Me-5-HT but no persistent increase in the motor output

(compare with Fig. 1). Our data indicate that the acute response and the long-term response involve different mechanisms.

That our findings depend on episodic rather than continuous 5-HT input is not surprising. 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 (27, 28). In *Aplysia*, short, intermediate, or long-term memory for sensitization can be elicited by different patterns of tail shocks (29).

Reduced activity of XII motoneurons during sleep in humans can lead to collapse of the upper airways, resulting in snoring and obstructive sleep apnea, with consequent episodic hypoxia (14, 30). LTF is postulated to be a regulatory mechanism that increases XII muscle tone in response to this episodic hypoxia, acting to reduce the frequency and severity of airway obstructions (10). Failure of this mechanism may lead to or exacerbate obstructive sleep apnea. We suggest that persistent changes in XII motoneuronal AMPA function in response to episodic 5-HT release induced by acute hypoxia (9) underlies (a significant component of) LTF. Understanding details of this signal transduction mechanism in XII motoneurons may be helpful in understanding and perhaps treating obstructive sleep apnea.

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