Inhibition of Resting Potassium Conductances by Long-Term Activation of the NO/cGMP/Protein Kinase G Pathway: A New Mechanism Regulating Neuronal Excitability

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Glutamate-induced excitotoxicity, the most common pathological mechanism leading to neuronal death, may occur even with normal levels of glutamate if it coincides with a persistent enhancement of neuronal excitability. Neurons expressing nitric oxide (NO) synthase (NOS-I), which is upregulated in many human chronic neurodegenerative diseases, are highly susceptible to neurodegeneration. We hypothesized that chronic production of NO in damaged neurons may increase their intrinsic excitability via modulation of resting or “leak” K^+ currents. Peripheral XIIth nerve injury in adult rats induced *de novo* NOS-I expression and an increased incidence of low-threshold motor units, the latter being prevented by chronic inhibition of the neuronal NO/cGMP pathway. Accordingly, sustained synthesis of NO maintained an enhanced basal activity in injured motoneurons that was slowly reverted (over the course of 2–3 h) by NOS-I inhibitors. In slice preparations, persistent, but not acute, activation of the NO/cGMP pathway evoked a robust augment in motoneuron excitability independent of synaptic activity. Furthermore, chronic activation of the NO/cGMP pathway fully suppressed TWIK-related acid-sensitive K^+ (TASK) currents through a protein kinase G (PKG)-dependent mechanism. Finally, we found evidence for the involvement of this long-term mechanism in regulating membrane excitability of motoneurons, because their pH-sensitive currents were drastically reduced by nerve injury. This NO/cGMP/PKG-mediated modulation of TASK conductances might represent a new pathological mechanism that leads to hyperexcitability and sensitizes neurons to excitotoxic damage. It could explain why *de novo* expression of NOS-I and/or its overexpression makes them susceptible to neurodegeneration under pathological conditions.

Key words: hypoglossal motoneurons; nerve injury; neuronal excitability; NOS-I upregulation; TASK channels; neurodegenerative diseases

Introduction

Disturbance of intracellular Ca^{2+} homeostasis is a central event in glutamate-induced excitotoxicity, the major mechanism of neuronal death caused by brain injury, ischemia, or neurodegenerative diseases (Lipton, 2004; Zhang et al., 2006). Excitotoxic damage may occur even with normal levels of glutamate if the normal block of NMDA receptors by Mg^{2+} is relieved, a condition that would potentiate Ca^{2+} influx through these receptors (Lipton, 2004). Consequently, sustained changes in excitability and membrane potential (V_m) induced by neuronal injury (González-Forero et al., 2004a,b) might exacerbate excitotoxic processes. Understanding of the mechanisms that make neurons

susceptible to excitotoxic death is of great basic and clinical relevance. Upregulation and/or *de novo* expression of the neuronal isoform of nitric oxide (NO) synthase (NOS-I), which synthesizes the highly reactive gas NO, is a common hallmark of several human chronic neurodegenerative conditions, such as Parkinson's and Alzheimer's diseases and amyotrophic lateral sclerosis (ALS) (Moreno-López and González-Forero, 2006). Increased levels of NO have also been detected in animal models of stroke and neurodegeneration, which are associated with excitotoxic cell death (Lipton, 2004; Zhang et al., 2006). Furthermore, NOS-I-expressing neurons are highly susceptible to neurodegeneration (Thorns et al., 1998). NO physiologically regulates neuronal excitability by modulating diverse ionic channels through soluble guanylyl cyclase (sGC)/protein kinase G (PKG) activation (Ahern et al., 2002). However, it is unknown whether persistent activity of the NO/cGMP pathway is causally related to the enhanced neuronal excitability after injury.

Neuronal excitability is a dynamic rather than fixed variable, which allows adjustment of postsynaptic sensitivity to afferent activity. Modulation of resting K^+ currents, which are fundamental in determining resting V_m and input resistance (R_N), has a profound impact on neuronal excitability. The KCNK family of

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two-pore-domain K^+ channels plays a major role in control of both variables in mammalian cells (Bayliss et al., 2003). Among them, the pH-sensitive subunits TWIK-related acid-sensitive K^+-1 (TASK-1) and TASK-3 are widely coexpressed throughout the brain, with particularly high expression levels in motoneurons (Talley et al., 2001). TASK currents can be modulated by multiple neurotransmitter systems, including those associated with awakening and alertness states. This could serve to couple neuronal responsiveness to afferent drive and behavioral status (McCormick and Bal, 1997; Talley et al., 2000; Bayliss et al., 2003). Interestingly, K^+ channel openers have potential for the treatment of several brain disorders characterized by neuronal hyperexcitability, such as spasticity or epileptic seizures (Wua and Dworetzky, 2005). Therefore, modulation of TASK channels might potentially represent a new therapeutic strategy, and these channels might become important drug targets.

We show that the autocrine activation of the neuronal NO/cGMP pathway induced by XIIth nerve injury enhances excitability of the motoneuron pool. In addition, whole-cell recordings from motoneurons demonstrated that chronic, but not acute, stimulation of the NO/cGMP pathway leads to a pronounced increase in intrinsic excitability, via a PKG-mediated inhibition of TASK-like pH-sensitive “leak” K^+ currents. Accordingly, we describe a new mechanism for regulating neuronal excitability that could be the basis for synaptic potentiation and neuron sensitization to excitotoxic death during neurological disorders.

Materials and Methods

Animals, obtained from an authorized supplier (Animal Supply Services, University of Cádiz, Cádiz, Spain), were cared for and handled in accordance with the guidelines of the European Union Council (86/609/UE) and the Spanish regulations (BOE 67/8509-12 and BOE 1201/2005) on the use of laboratory animals. Statistical tests applied to each data set are indicated in the figure legends. Data are presented as mean $\pm$ SEM.

In vivo approaches

Extracellular unitary recordings of hypoglossal motoneurons. Adult male Wistar rats (250–400 g) were anesthetized with chloral hydrate (0.5 g/kg, i.p.), and the right hypoglossal (XIIth) nerve was thoroughly crushed with microdissecting tweezers for 30 s just proximal to the nerve bifurcation, as described previously (González-Forero et al., 2004b). Animals were allowed to survive 7 d after surgery and then prepared for extracellular recordings (González-Forero et al., 2004b). Briefly, rats were anesthetized (as above) and additionally injected intramuscularly with atropine (0.2 mg/kg) and dexamethasone sodium phosphate (0.8 mg/kg). Teflon-isolated silver bipolar electrodes were fixed around the right XIIth nerves. Trachea, bladder, and femoral artery and vein were cannulated. Subsequently, animals were vagotomized, decerebrated, paralyzed with gallamine triethiodide (20 mg/kg, i.v., initially; 4 mg/kg, i.v., as needed), and mechanically ventilated. Expired CO_2 and O_2 were monitored continuously (Eliza duo; Gambro Engström, Bromma, Sweden). During the experiment, the end-tidal CO_2 (ET_{CO_2}) was changed (~ 3 to $\sim 7.5\%$) by adjusting tidal volume and/or respiratory rate. Expired O_2 (14–19%) was always higher than values below which hypoxia-induced alterations have been reported (Hwang et al., 1983). Femoral arterial blood pressure (95 ± 15 mmHg) and rectal temperature ($37 \pm 1^\circ C$) were continuously monitored and kept stable. Glass micropipettes (1–3 M Ω), filled with 2 M NaCl, were placed under visual guidance and advanced through the brainstem into the hypoglossal nucleus (HN). The correct position of the micropipette was confirmed by recording the characteristic inspiratory pattern and the presence of the antidromic field potential elicited by electrical stimulation of the ipsilateral XIIth nerve. Hypoglossal motoneurons (HMNs) were identified by their antidromic activation from the XIIth nerve and by the collision test (González-Forero et al., 2004b; Sunico et al., 2005). The electrical signals were amplified and filtered at a bandwidth of 10 Hz to 10 kHz for display and digitalization

purposes. Responses of HMNs were recorded in response to changes in ET_{CO_2} from hypocapnic ($\sim 3\%$) to hypercapnic ($\sim 7.5\%$) conditions. Only inspiratory HMNs discharging at basal conditions ($ET_{CO_2} = 4.8$ – 5.2%) were considered in this study. Unitary discharge activity, percentages of expired CO_2 and O_2 , and arterial pressure recordings were amplified, filtered, digitized, and stored in a computer using a PowerLab/8SP analog-to-digital interface (ADInstruments, Castle Hill, Australia) for off-line analysis. The mean unitary firing rate (mFR) (spikes per second) in each burst was measured over the range of ET_{CO_2} tested. Because mFR changed proportionally to alterations in ET_{CO_2} (see Fig. 1B), both parameters were positively correlated using linear regression analysis (see Fig. 1C). It was thus possible to obtain an equation characterizing the behavior of an HMN in response to changes in ET_{CO_2} : $mFR = S_{mFR} \times ET_{CO_2} + I_{mFR}$, where S is the slope (i.e., the neuronal gain or sensitivity of mFR relative to ET_{CO_2} variations), and I is the ordinate intercept (i.e., the theoretical mFR value when $ET_{CO_2} = 0\%$). Theoretical recruitment threshold (Th) was calculated as the abscissa intercept from the $mFR-ET_{CO_2}$ regression line (see Fig. 1C).

Administration of NOS and sGC inhibitors. To study the involvement of the NO/cGMP pathway in motoneuron alterations induced by peripheral nerve lesions, injured rats were daily administered (i.p.) with the broad-spectrum NOS inhibitor N^G -nitro-L-arginine methyl ester (L-NAME; 90 mg $\cdot$ kg $^{-1}$ $\cdot$ d $^{-1}$; Sigma, St. Louis, MO), the inactive stereoisomer D-NAME (90 mg $\cdot$ kg $^{-1}$ $\cdot$ d $^{-1}$; Sigma), the relatively specific NOS-I inhibitor 7-nitroindazole (7-NI; 30 mg $\cdot$ kg $^{-1}$ $\cdot$ d $^{-1}$; Sigma), or the specific sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 2 mg $\cdot$ kg $^{-1}$ $\cdot$ d $^{-1}$; Sigma), beginning on the day of nerve crush. Treatment with 7-NI did not affect arterial blood pressure, whereas L-NAME administration caused a transient elevation of this parameter that returned to control values 6 h after injection (Moreno-López et al., 2004). The levels of cerebral NOS-I and endothelial isoform of NOS (NOS-III) remained as in control animals after chronic treatment with L-NAME (Moreno-López et al., 2004). Perfusion or recording sessions were conducted at least 18 h after the last injection of drugs. To rule out the possibility that systemic NOS inhibition had its own effects on the functional characteristics of HMNs, we studied their firing properties in a separate group of noninjured rats treated with L-NAME (90 mg $\cdot$ kg $^{-1}$ $\cdot$ d $^{-1}$) for 7 d.

Electroneurographic recordings of XIIth nerves. In a series of experiments conducted on untreated adult rats at 7 d after injury, basal activity of XIIth nerves was monitored continuously for a minimum period of 8 h, from 1 h before to 7 h after drug administration. Under ketamine: xylidine anesthesia (35:1 mg/kg, i.m.), both nerves were dissected from experimental animals, and Teflon-isolated silver bipolar electrodes were placed 3–4 mm proximal to their bifurcation. Electrodes were electrically isolated from neighboring tissue with Vaseline jelly and Parafilm. During the recording sessions, supplemental doses of ketamine:xylidine (5:0.2 mg $\cdot$ kg $^{-1}$ $\cdot$ hr $^{-1}$, i.m.) were given as necessary to maintain a stable level of anesthesia. Spontaneous activity from both nerves was recorded in monopolar mode, AC coupled, amplified, and filtered (10 Hz to 10 kHz). The electroneurographic signals were integrated ($\tau = 20$ ms), and the area of each burst was determined using a parabola automatically fitted to the integrated burst activity provided by the Chart software (ADInstruments). The ratio between the activities of the crushed relative to the intact nerve was analyzed at 1 h intervals throughout each experiment and plotted against time. Mean values were calculated from bursts occurring over a period of 5 min at each time sampled.

In vitro whole-cell recordings from HMNs in brainstem slices

Whole-cell patch-clamp experiments were performed on brainstem slices from 6- to 9-d-old animals either intact or lesioned unilaterally at postnatal day 3 (P3). Rat pups were anesthetized by hypothermia (placing on ice for 10–15 min) and decapitated, and their brainstems were quickly removed. Dissection was in ice-cold ($<4^\circ C$) sucrose artificial CSF (S-aCSF) bubbled with 95% O_2 and 5% CO_2 . S-aCSF composition was as follows (in mM): 26 NaHCO $_3$, 10 glucose, 3 KCl, 1.25 NaH $_2$ PO $_4$, 2 MgCl $_2$, and 218 sucrose. Transverse slices (300–400 μ m thickness) were cut around the obex using a vibratome (Series 1000; Technical Products International, St. Louis, MO). Slices were transferred to normal oxygen-

ated aCSF (in which sucrose was substituted by 130 mM NaCl and 2 mM CaCl_2), incubated for 1 h at 36°C, and then allowed to stabilize at room temperature ($\sim 22^\circ\text{C}$) for at least 30 min.

To study the influence of the NO/cGMP/PKG pathway on the membrane excitability of HMNs, different combinations of activators and/or blockers were added to the bath solution. Either drugs were applied acutely (10 min during recordings) or slices were preincubated with drugs for a minimum of 4 h before the recordings and also during the recordings ("chronic" treatments). Untreated control slices and those exposed to acute treatments were also maintained for the same 4 h period in standard aCSF before recordings. To clearly isolate the influence of the NO/cGMP/PKG pathway on intrinsic excitability and avoid possible modulatory effects from afferent synaptic inputs, a series of experiments involving chronic treatments were performed under conditions of minimal levels of synaptic activity. In this protocol, both preincubation and recording were made in a modified aCSF containing low Ca^{2+} (0.5 mM CaCl_2), high Mg^{2+} (6 mM MgCl_2), and a mixture of synaptic blockers: bicuculline methiodide (10 μM ; Sigma), strychnine hydrochloride (0.25 μM ; Sigma), AP-5 [D(-)-2-amino-5-phosphonopentanoic acid; 50 μM ; Tocris Cookson, Ballwin, MO], CNQX (6-cyano-7-nitroquinoxaline-2,3-dione; 10 μM ; Tocris Cookson), D-tubocurarine chloride (30 μM ; Sigma). Because chronic and acute exposure to NO donors resulted in a constant depolarization of HMNs (see Results), we tested the effects of a persistent depolarization on the membrane parameters in a separate group of experiments. In this case, slices were preincubated and recorded with high- K^+ aCSF (5 mM KCl and 1.25 mM KH_2PO_4).

After preincubations, the slices were individually transferred into the recording chamber and perfused continuously (at a rate of 2 ml/min) with different solutions at 31°C . Whole-cell recordings were obtained from somata of HMNs visually identified based on their location and characteristic size and shape (Talley et al., 2000) using a Nikon (Tokyo, Japan) Eclipse CFI60 microscope equipped with infrared (IR) differential interference contrast, a 40 $\times$ water-immersion objective, and an IR camera system (TILL Photonics, Pleasanton, CA). Patch pipettes were pulled from 1.5 mm outer diameter borosilicate glass using a PP-830 puller (Narishige, Tokyo, Japan). Patch electrodes (1.5–3 M Ω resistance) contained the following (in mM): 17.5 KCl, 122.5 K-gluconate, 9 NaCl, 1 MgCl_2 , 10 HEPES, 0.2 EGTA, 3 Mg-ATP , and 0.3 GTP-Tris with pH buffered to 7.2. Current- and voltage-clamp recordings were obtained and low-pass Bessel filtered at 10 kHz with a MultiClamp 700B amplifier. Data were digitized at 20 kHz with a Digidata 1332A analog-to-digital converter and acquired using pCLAMP 9.2 software (Molecular Devices, Foster City, CA). Only recordings with access resistance between 5 and 20 M Ω were considered acceptable for analysis. The access resistance was checked throughout the experiments, and recording was abandoned if it changed $>15\%$. Series resistance was routinely compensated 65–75%. The pipette offset potential was zeroed before the cells were patched. Leak or liquid junction potentials were not corrected. In the voltage-clamp mode, all tests were performed in presence of tetrodotoxin (TTX; 1 μM ; Alomone Labs,

Figure 1. Chronic activation of the NO/cGMP pathway modifies the recruitment distribution of hypoglossal motoneurons after nerve injury in adult rats. **A**, Photomicrographs obtained from coronal brainstem sections showing the induction of a new enzymatic source of NO and the presence of its target within the HN. **a1**, **a2**, NADPH-d visualized histochemistry in sections obtained in control (**a1**) and at 7 d after lesion (**a2**). **a3**, **a4**, Confocal photomicrographs of a section obtained from a control animal in which HMNs are double labeled with both the retrograde marker FG (**a3**) injected in the tongue and sGC antisera (**a4**). Scale bars, 250 μm . **B**, Instantaneous firing rate [spikes (sp) per second] histograms for representative HMNs recorded at two different levels of ET_{CO_2} concentrations in a control animal and 7 d after ipsilateral XIIth nerve crushing in animals receiving chronic treatment with D-NAME or L-NAME. **C**, Regression lines of mFR and ET_{CO_2} obtained from HMNs ($n = 5$ per condition), in control and 7 d after XIIth nerve injury from receiving the specified treatments. The abscissa intercept in the regression line indicates the theoretical recruitment Th for each motor unit. **D**, Distribution histograms of Th obtained in HMNs from control and lesioned animals treated with D-NAME, L-NAME, or ODQ. All distributions except those from crushed nontreated (data not shown) and D-NAME-treated groups were fitted by Gaussian peak equations ($r > 0.9$; $p < 0.0001$). Arrows point to the mean of each group. **E**, Cumulative probability histograms of Th obtained in control and at 7 d after lesion receiving the indicated treatments. Histograms were fitted by exponential functions ($r > 0.95$; $p < 0.0001$); control, $y = -0.03 + 0.48\exp(0.18x)$; crushed, $y = 0.19 + 0.54\exp(0.1x)$; crushed D-NAME, $y = -0.24 + 0.97\exp(0.07x)$; crushed L-NAME, $y = -0.03 + 0.22\exp(0.34x)$; crushed ODQ, $y = -0.2 + 0.53\exp(0.19x)$. * $p < 0.05$; Kolmogorov–Smirnov test with respect to the control group.

Jerusalem, Israel). Neurons were initially held near the resting potential (-65 mV), and then voltage-clamp protocols consisting either of depolarizing ramps or command steps were applied. In some experiments, pH sensitivity of HMNs was evaluated by perfusing sequentially the same cell with aCSF with varying pH levels (pH 6.2, 7.2, and 8.2). Hydrogen ion concentration was adjusted adding either HCl or NaOH to aCSF. In some experiments, responsiveness of HMNs to thyrotropin-releasing hormone (TRH) applications (10 μM ; superfused for 15 s; Sigma) was tested.

Treatments with drugs that modulate the NO/cGMP/PKG pathway. Two NONOates, NO-generating compounds and a cell-permeable cGMP analog, were used to discriminate between acute and chronic effects of NO on membrane properties. The short-half-life NO donor

Figure 2. Persistent activation of the NO/cGMP pathway induces a severe increase in the excitability of hypoglossal motoneurons. **A**, Confocal photomicrographs through the HN obtained from a neonatal rat (P7) immunostained for sGC (**a1**). **a2**, **a3**, An area shown using high magnification demonstrates that all of the HMNs identified by selective retrograde tracing with FG (**a2**) also express sGC (**a3**). Scale bars: **a1**, 250 μ m; **a2**, **a3**, 50 μ m. **B–E**, Changes in the V_m (**B**), current threshold for firing (I_{Th} ; **C**), mean R_N (**D**), and mean $f-I$ slope (**E**) of HMNs ($n = 20–38$) induced by either acute or chronic incubations with the listed drugs under conditions of normal spontaneous or pharmacologically reduced synaptic activity. **C**, Inset, Examples of voltage responses to short (5 ms) depolarizing current pulses obtained in different HMNs recorded under conditions of normal spontaneous synaptic activity in control or after chronic preincubations with the indicated drugs. The horizontal dotted line marks -60 mV. **D**, Inset, Voltage responses to a series of depolarizing and hyperpolarizing current pulses (1 s duration, 0.02 nA increments) from different HMNs recorded in control slices or after incubations with the specified drugs under conditions of normal spontaneous synaptic activity. **B–E**, Horizontal gray bars represent means $\pm$ SEM of the control group. Striped bars indicate data obtained under conditions of reduced synaptic activity. * $p < 0.05$; one-way ANOVA; *post hoc* Tukey's test relative to the control groups with spontaneous and reduced synaptic activity, respectively.

2-(*N,N*-diethylamino)-diazolot-2-oxide (DEA/NO; 10 or 20 μ M; Sigma) or cell-permeable cGMP analog 8-Br-cGMP (1 mM; Sigma) was used for acute applications and added to the bath after controls have been accomplished and superfused for 10 min before measurements were taken again. For chronic treatments (>4 h exposure), the long-half-life NO donor (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]-diazene-1-ium-1,2-diolate-NO (DETA/NO; 1 mM; Sigma), the sGC

inhibitor ODQ, (20 μ M), 8-Br-cGMP (0.1 mM), and the cell-permeable inhibitor of PKG, guanosine, 3',5'-cyclic monophosphorothioate, 8-4-chlorophenylthio-, Rp-isomer (Rp-8-pCPT-cGMPS, 10 μ M; EMD Biosciences, La Jolla, CA), were added either alone or in different combinations both to the preincubation and recording solutions.

Data analysis. In current-clamp mode, the resting V_m , the current threshold for firing (I_{Th}), R_N , and the slope of the relationship between average firing frequency and injected current ($f-I$ curves) were measured. I_{Th} was determined as the lowest depolarizing current pulse (5 ms) required to elicit an action potential in 50% of cases. R_N was calculated from the current–voltage ($I-V$) plots obtained by injecting a series of depolarizing and hyperpolarizing current pulses intracellularly (1 s; -0.2 to 0.2 nA). The resulting data points were then fitted with a least-squares regression line, and R_N was estimated as the slope of the lines. Finally, $f-I$ curves were constructed applying intracellular injections of depolarizing current pulses (1 s; steps of 0.02 nA) and plotting the mFR within each burst against current pulse amplitude.

In voltage-clamp recordings, the holding current ($I_{holding}$) required to keep V_m at -65 mV was measured. The protocols to obtain $I-V$ relationships consisted either of increasing voltage ramps (2 s duration) from -120 to -40 mV or voltage steps applied in 5 mV increments between -50 and -120 mV from a baseline holding potential of -65 mV. The slope conductance (G_{slope}) was calculated as the slope of the $I-V$ linear fits generated by the ramp protocol within the voltage range of -100 to -40 mV. Similarly, input conductance (G_N) was determined as the slope of the $I-V$ linear fits obtained from current responses to voltage steps. In this case, the instantaneous component was measured in a time window between the settling of the transient capacitive current and the onset of the time-dependent current (I_h), ~ 10 ms after the onset of the step.

Histological procedures

Animals were anesthetized with ketamine and xylidine–dihydrothiazine (as above), injected intraventricularly with heparin, and perfused transcardially first with PBS, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, at 4°C. The brains were removed, postfixed for 2 h in the same fixative solution, and cryoprotected by overnight immersion in 30% sucrose in PB at 4°C. Serial coronal sections (30 μ m thick) from brainstem were obtained using a cryostat and stored at -20°C in a cryoprotectant solution (glycerol:PBS, pH 7.4, 1:1 in volume).

Nitric neurons were identified by NADPH diaphorase (NADPH-d) histochemistry. NADPH-d activity was made visible by incubation of the tissue in a mixture containing 1 mM β -NADPH, 1 mM nitroblue tetrazolium, and 0.1% Triton X-100 in 0.1 M Tris buffer, pH 8.0, for 30 min at 37°C. After extensive washing, the tissue was dehydrated, mounted with DePeX (Serva, Heidelberg, Germany), and analyzed under light microscopy. In a separate group, 50 μ l of a solution containing 1% of the retrograde tracer aminostilbamidine methanesul-

phate was used for retrograde tracing. The animals were perfused with PBS, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, at 4°C. The brains were removed, postfixed for 2 h in the same fixative solution, and cryoprotected by overnight immersion in 30% sucrose in PB at 4°C. Serial coronal sections (30 μ m thick) from brainstem were obtained using a cryostat and stored at -20°C in a cryoprotectant solution (glycerol:PBS, pH 7.4, 1:1 in volume).

Figure 3. TASK channels as feasible targets for the chronic action of NO. **A**, Time series illustrating changes in I_{holding} for a representative HMN voltage clamped at -65 mV in response to variations in extracellular pH. **B**, Voltage responses of a P8 HMN to the same depolarizing current pulse (1 s, 0.5 nA) obtained during superfusion with either alkaline (pH 8.2) or acidic (pH 6.2) aCSF. In both cases, the baseline membrane potential was kept constant at -60 mV (dotted line) by DC-current injection through the patch pipette. **C**, Whole-cell current responses evoked by the application of TRH (10 μ M for 15 s) in representative HMNs from control and DEA/NO-treated slices. Membrane potential was held at -65 mV.

fonate [FluoroGold (FG); Invitrogen, Eugene, OR] in PBS was injected into the tip of the tongue to identify HMNs. These animals were perfused 5–7 d after the tracer injection. For the analysis of sGC immunostaining, free-floating sections were rinsed in PBS and immersed in 2.5% (w/v) bovine serum albumin, 0.25% (w/v) sodium azide, and 0.1% (v/v) Triton X-100 in PBS for 30 min, followed by incubation at 4°C with the primary antiserum solution containing a polyclonal antibody raised in goat against sGC β 1 (1:100; 2 d; Santa Cruz Biotechnology, Santa Cruz, CA). Subsequently, the tissue was rinsed in PBS and incubated for 2 h at room temperature with an anti-goat IgG conjugated with Cy3 (cyanine 3; 1:400; Jackson ImmunoResearch, West Grove, PA). Finally, sections were washed with PBS and mounted on slides with a solution containing propyl gallate (0.1 mM in PBS:glycerol, 1:9). Omission of the primary antibody resulted in no detectable staining. Immunoreactivity was analyzed using a Leica (Nussloch, Germany) confocal microscope.

Results

Injured HMNs have the biochemical machinery for autocrine NO signaling

To explain how NO can regulate the functional status of HMNs, it is essential to identify its potential sources and targets within the HN. Although HMNs are normally deficient in NOS-I, its expression can be induced by axon injury (Sunico et al., 2005). To illustrate this change, slices obtained from adult rats were processed for NADPH-d histochemistry to label NOS-expressing structures. Under control conditions, only some scattered nitrergic neurons were observed at various locations throughout the HN (Fig. 1a1). However, 7 d after XIIth nerve crush, the number of positive cell bodies on side ipsilateral to the injured nerve increased dramatically relative to the intact side (375.5 ± 7.6 vs 21.1 ± 4.7 ; $n = 3$ animals) (Fig. 1a2). We have previously demonstrated that the upregulated isoform was NOS-I (Sunico et al., 2005). The hypothesis of an autocrine modulation would be supported if the major target of NO, the sGC, is also present in HMNs. To test this, HMNs were identified by retrograde tracing with FG (Fig. 1a3) and immunostained for sGC (Fig. 1a4). We found that all FG-positive neurons also expressed sGC, whereas

only a small fraction ($<10\%$) of sGC-positive cell bodies lacked FG labeling. In conclusion, after injury to the XIIth nerve, HMNs express the molecular substrates necessary for autocrine action of the NO/cGMP pathway.

Autocrine action of the NO/cGMP pathway modifies the recruitment scheme of the hypoglossal motor pool *in vivo*

In vivo recordings showed that mFR of HMNs was positive and linearly related to changes in ET_{CO_2} (Fig. 1B,C). Recruitment Th for each recorded hypoglossal motor unit can be defined as the theoretical ET_{CO_2} concentration at which the motoneuron begins to discharge (that is, the abscissa intercept in the mFR– ET_{CO_2} regression line) (Fig. 1C, Th). In the control group, the mean Th was $-1.2 \pm 0.8\%$, and its distribution was well fitted with a Gaussian function (Fig. 1D). This recruitment scheme was preserved in both the sham-operated and L-NAME-treated intact groups (data not shown). However, 7 d after nerve crush, distribution of Th was distorted, and the mean Th was reduced ($-13.8 \pm 5.2\%$). Cumulative sum histograms showed that in the insulted group, the proportion of motor units that would have been active at 0% of ET_{CO_2} (75.6%) was much higher than in the control pool (41.5%) (Fig. 1E). These alterations were indeed accompanied by an expansion of the recruitment range and a reduction in the recruitment gain (i.e., the recruitment rate throughout the whole ET_{CO_2} range) (Fig. 1E, compare exponential growth rates). Similar changes in Th distribution and mean Th ($-13.0 \pm 4.8\%$) were observed in the injured group that received chronic administration of the inactive stereoisomer D-NAME (Fig. 1C–E). On the contrary, chronic inhibition of either NO synthesis with L-NAME or 7-NI (data not shown) or sGC activity with ODQ preserved the Gaussian distribution of Th values and its mean ($1.8 \pm 0.5\%$, $-0.8 \pm 1.5\%$, and $0.9 \pm 0.4\%$, respectively), although they were slightly shifted toward higher Ths (Fig. 1C–E). Together, these results suggest that decreases in recruitment Th and disorganization of the recruitment pattern in the injured motor pool are mediated by the action of NO synthesized by the induced NOS-I acting via sGC. We addressed the hypothesis that these effects are the consequence of the changes in the intrinsic membrane properties of HMNs in the following experiments.

Acute versus chronic signaling through the NO/cGMP pathway differentially regulates the excitability of HMNs

Because *in vitro* experiments were performed on rat pups, we confirmed that the NO-sensitive enzymatic machinery is already present at that age. Brainstem sections containing FG-labeled HMNs were further processed for sGC immunohistochemistry (Fig. 2A). As in adults, all FG-identified motoneurons were also immunoreactive for sGC (Fig. 2a2,a3).

We first tested HMN sensitivity to a short-half-life NO-generating compound, DEA/NO, applied acutely. Superfusion of slices with DEA/NO (10 μ M), but not with the vehicle, for 10 min caused a significant and reproducible depolarization of the V_m in all HMNs tested (3.8 ± 0.74 mV) (Fig. 2B) that was associated with a reduction in the I_{Th} (0.76 ± 0.05 and 0.62 ± 0.02 nA for control and DEA/NO, respectively) (Fig. 2C). This acute exposure had, however, no effects on R_N (Fig. 2D) or $f-I$ slope (Fig. 2E). A twofold increase in DEA/NO concentration did not result in a proportional increase in its effects (Fig. 2B–E), indicating that a saturating effect was already achieved with the lower concentration. Effects of DEA/NO were blocked by ODQ (Fig. 2B,C), indicating an sGC-mediated action.

Adult HMNs are characterized by an uninterrupted rhythmic

Figure 4. Sustained signaling through the NO/cGMP/PKG pathway inhibits TASK-like pH-sensitive K^+ conductances. **A**, Examples of current responses to voltage-step commands (range, -115 to -40 mV; 5 mV increments) recorded under acidification (pH 6.2) and alkalization (pH 8.2) from representative HMNs after chronic incubations with the listed drugs. PKG-i, Rp-8-pCPT-cGMPs. Instantaneous currents were measured at the end of the capacitive transient (triangles). Insets, Current-clamp recordings of the voltage responses to a series of depolarizing and hyperpolarizing current pulses (0.5 s duration, 0.04 nA increments) from the same HMNs. Asterisks indicate the time point used to measure the peak voltage response. Dotted lines indicate either zero current or -60 mV in voltage-clamp and current-clamp recordings, respectively. **B**, Currents evoked by ramping the membrane from -100 to -45 mV (40 mV/s) plotted against holding potential (V_h) at both pH levels for the same HMNs as illustrated in **A**. Linear regression analysis ($r > 0.9$) was used to calculate G_{slope} from these I - V relationships. Insets, I - V relationships of the pH-sensitive current obtained by graphical subtraction of ramp currents recorded at pH 8.2 and pH 6.2 for each neuron. **C**, Averaged data ($n = 6-8$) of instantaneous I - V relationships in the control and chronically treated groups in response to extracellular acidification and alkalization. G_N values were calculated as the slopes of the linear regression lines fitted to I - V plots.

discharge mediated by glutamatergic inputs. Because NOS-I is upregulated in injured motoneurons, it is highly probable that this repetitive activation leads to a sustained production of NO. In an attempt to mimic this condition, slices were preincubated with a long-half-life NONOate, DETA/NO, for at least 4 h before the beginning of recordings. As with acute DEA/NO applications, this chronic treatment also induced V_m depolarization (5.6 ± 0.74 mV) (Fig. 2B) and a marked decrease in I_{Th} (0.39 ± 0.03 nA) (Fig. 2C). In addition, the prolonged exposure to DETA/NO caused significant increases in both R_N ($22.8 \pm 6.7\%$) (Fig. 2D) and f - I slope ($27.4 \pm 5.5\%$) (Fig. 2E). These effects were absent in slices coincubated with DETA/NO and ODQ, indicating that they were also mediated via sGC (Fig. 2D,E). To test whether changes in R_N and f - I slope result from sustained depolarization, rather than from NO itself, slices were chronically incubated in aCSF containing high K^+ . This led to reductions in I_{Th} (0.29 ± 0.03 nA) and membrane depolarization (5.3 ± 0.56 mV), which were similar to those after chronic incubation with DETA/NO. However, this treatment caused no changes in R_N and f - I slope (Fig. 2B–E). Finally, to test whether the effects of NO on HMNs are direct rather than result from modification of the synaptic inputs to these neurons, some slices were preincubated and recorded in a modified aCSF with low Ca^{2+} , high Mg^{2+} , and a

mixture of neurotransmitter receptor blockers (see Materials and Methods). Under these conditions of reduced synaptic activity, neurons also exhibited reduced R_N and higher I_{Th} and f - I slopes (Fig. 2B–E). In this environment of reduced synaptic activity, chronic effects of DETA/NO were also similar to those found under normal conditions of spontaneous synaptic activity (Fig. 2B–E). Likewise, all of the modulatory effects of NO were essentially mimicked by the permeant cGMP-analog 8-Br-cGMP (Fig. 2B–E) and, therefore, most probably cGMP mediated.

Persistent activation of the NO/cGMP/PKG cascade inhibits TASK-like pH-sensitive K^+ conductances

We next investigated the possibility that the sustained exposure to NO exerted its effects via inhibition of TASK pH-sensitive resting K^+ conductances. Because these channels are highly expressed in motor nuclei (Talley et al., 2000; Berg et al., 2004), we tested the effects of pH on HMNs of P6–P9 rats. Voltage-clamp recordings from HMNs held at -65 mV in the continuing presence of TTX showed that acidification of the aCSF resulted in an inward shift in I_{holding} , whereas alkalization induced a change in the outward direction (Fig. 3A). In current-clamp mode, perfusion with an acidic aCSF increased the excitability of HMNs. Figure 3B illustrates a response of one HMN to a positive current pulse under alkaline (pH 8.2) and acidic (pH 6.2) conditions and demonstrates a greatly increased excitability at acidic pH. Thus, *in vitro* recordings demonstrate functional characteristics consistent

with expression of TASK-like pH-sensitive K^+ conductances on neonatal HMNs. Given the similarity between the effects of acidification and chronic influence of NO on motoneuron excitability, we postulated that both manipulations may target the same ion channels. Closure of TASK channels is also controlled by a number of transmitters and neuromodulators such as TRH (Talley et al., 2000, 2003; Chen et al., 2006). Under voltage clamp (holding potential, -65 mV), TRH induced an inward shift of the I_{holding} in all HMNs tested from control slices (-0.348 ± 0.67 nA; $n = 5$). Interestingly, chronic treatment of slices with DETA/NO almost fully inhibited the TRH-induced response ($-75.2 \pm 6.7\%$; $n = 5$) (Fig. 3C), which suggested a contribution of TASK channels as possible effectors for the chronic action of NO on motoneuron excitability.

To investigate in more detail the role of TASK channels in mediating such effects, we next compared the pH sensitivity of HMNs recorded from control and chronically treated slices. I - V relationships were constructed using both voltage-step and voltage-ramp protocols (Fig. 4) (see Materials and Methods). In control HMNs, current responses to voltage steps in alkalized solution (pH 8.2) were much larger than in acidified (pH 6.2) solution, indicating a larger G_N at the higher pH value (Fig. 4A). Similarly, the slope conductance G_{slope} , obtained from the slope

Figure 5. Effects of modulators of the NO/cGMP/PKG pathway on the pH sensitivity of hypoglossal motoneurons. **A**, Summary of changes in V_m , I_{holding} , R_N , and G_N induced by acidification from a basal alkaline pH for each listed treatment group. $\#p < 0.05$; one-way ANOVA; *post hoc* Tukey's test with respect to the control group. **B**, Averaged I – V relationships of the pH-sensitive current component obtained after subtracting mean instantaneous leak currents at pH 6.2 from those at pH 8.2 in Figure 4C. In all cases, these data were well fitted ($r > 0.9$) with the Goldman–Hodgkin–Katz equation (solid lines) and currents reversed between -90 and -100 mV, which was close to the reversal potential predicted theoretically by the Nernst equation for K^+ .

of the I – V curves generated by the ramp protocol, was attenuated at pH 6.2 and increased at pH 8.2 (Fig. 4B). Therefore, changing the external pH from 8.2 to 6.2 reversibly induced increases in R_N and significant reductions in G_N and G_{slope} (Fig. 4). Modulation of TASK-like pH-sensitive K^+ currents was almost entirely suppressed after preincubations with DETA/NO or 8-Br-cGMP (Fig. 4). Accordingly, the pH-sensitive component of the whole-cell current calculated by subtracting ramp traces at pH 6.2 from those at pH 8.2 was significantly reduced compared with controls (Fig. 4B, insets). This inhibitory effect was prevented, however, when DETA/NO and Rp-8-pCPT-cGMPs, a potent cell-permeable inhibitor of PKG, were coadded to the incubation medium (Fig. 4A,B). Averaged I – V plots generated by the voltage-step protocol at both pH values are illustrated in Figure 4C for each treatment. Mean G_N ratios at pH 6.2/pH 8.2 were 16.7/26.7, 14.4/15.5, 19.2/19.7, and 23.3/31.2 nS for the control, DETA/NO-, 8-Br-cGMP-, and DETA/NO plus Rp-8-pCPT-cGMPs-treated groups, respectively, which indicates that the “high-conductance” status at pH 8.2 was more profoundly affected by the treatments used to stimulate the NO/cGMP pathway. A summary of the HMN responsiveness to pH (reflected as percentage change for several membrane parameters in response to pH changes) exhibited by HMNs under different drug treatments is depicted in Figure 5A. The pH-sensitive current in each group was obtained by subtracting the currents recorded at pH 8.2 from those obtained at pH 6.2 using the voltage-step protocol (Fig. 5B). In all four cases, the subtracted currents reversed close to the reversal potential calculated for K^+ ions (approximately

-90 mV) and were well fitted by the Goldman–Hodgkin–Katz constant-field equation ($r > 0.98$) (Fig. 5B), thus displaying properties of an outward rectifier K^+ conductance. These currents were almost completely inhibited after persistent activation of the NO/cGMP pathway, whereas the inhibition of PKG completely abolished this blocking effect (Fig. 5B). Together, these results indicated that the persistent action of the NO/cGMP/PKG pathway increases HMN excitability through a specific inhibitory action on TASK-like pH-sensitive leak K^+ conductances.

Acute exposure to NO or cGMP increases a net inward current without changing the overall input conductance

Because chronic activation of the NO/cGMP cascade caused more profound changes in membrane excitability parameters than short-term exposure (Fig. 2B–D), we investigated whether acute NO/cGMP-induced depolarizations were mediated through a different subset of conductances. Application of DEA/NO or 8-Br-cGMP for 10 min also evoked pronounced inward shifts in the I_{holding} relative to control preexposure values (difference, -133.1 ± 25.9 pA and -136.6 ± 14.2 pA, respectively), which reversed entirely after 10 min washout. Likewise, the mean I_{holding} of the HMN pool treated chronically with DETA/NO shifted inward (difference, -91.0 ± 18.6 pA) relative to that measured in the control group. Thus, an inward drift in the I_{holding} is induced by either chronic DETA/NO or acute DEA/NO application, which accounts for V_m depolarization in both cases. Nevertheless, the absence of changes in R_N during acute DEA/NO or 8-Br-cGMP treatments and the presence of this effect after chronic exposure to DETA/NO or 8-Br-cGMP suggests that NO via cGMP could be acting on different subsets of ionic channels depending on the time of exposure. Acute DEA/NO application had no effect on the slope of the I – V curves even though it depolarized HMNs and caused a robust shift in the I_{holding} (Fig. 6A,B). Such “short-term” depolarizing action was preserved even in the presence of TTX and a mixture of synaptic blockers (difference, 3.6 ± 0.5 mV) without significantly affecting R_N ($4.7 \pm 6.8\%$ with respect to the control), which excludes the possibility that any change in R_N could be obscured by a parallel modification in synaptic inputs. Interestingly, acute infusion of DEA/NO resulted in V_m depolarization that was larger than that induced by acidification to pH 6.2 (Fig. 6C), a pH value known to block almost completely TASK-like acid-sensitive conductances (Berg et al., 2004). Furthermore, when DEA/NO was applied after bath acidification, it induced an additional depolarization (Fig. 6C). In summary, whereas cells chronically treated with DETA/NO ($n = 28$) showed changes in V_m , I_{holding} , R_N , and G_N with respect to the control pool ($n = 27$), short-term exposure to either DEA/NO or 8-Br-cGMP modified only V_m and I_{holding} (Fig. 6D). These experiments suggest that the NO/cGMP pathway acts via two different mechanisms regulating HMN excitability depending on the longevity of its action (Fig. 7D). Short-term action of the NO/cGMP cascade could involve either a TASK-independent conductance or a combination of effects on different subtypes of resting conductances without a net impact on the overall R_N . On the contrary, chronic effects involve the reduction of resting TASK-like K^+ conductances via cGMP synthesis and PKG activation, which reduces membrane conductance, depolarizes HMNs, and increases their excitability.

Inhibition of TASK conductances underlies increases in excitability of injured HMNs

In light of the previously described results, we proceeded to ask whether XIIth nerve injury upregulated intrinsic excitability of

Figure 6. Short-term exposure to NO depolarizes hypoglossal motoneurons without changing the overall resting conductance. **A**, Current traces obtained from a motoneuron in response to voltage steps in control aCSF, at the end of a 10 min incubation with DEA/NO and again after 10 min of washout. Insets, Voltage responses to current injections recorded from the same HMN in the course of application of DEA/NO. Dotted lines, asterisks, and triangles represent the same as in Figure 4A. **B**, Averaged I - V relationships ($n = 9$) of instantaneous current responses in control aCSF, in the presence of DEA/NO and after 10 min washout. Inset, Mean G_N from HMNs before and during DEA/NO infusion and after washout. **C**, Current-clamp recordings from an HMN showing the effects of DEA/NO on V_m in control aCSF (top trace) or after steady-state change in V_m caused by extracellular acidification. **D**, Summary of changes (expressed as absolute percentage variation) in V_m , R_N , I_{holding} , and G_N induced by chronic incubations with DETA/NO and acute treatments with DEA/NO or 8-Br-cGMP ($n = 7$). $*p < 0.05$; one-way ANOVA; *post hoc* Tukey's test relative to the control group. $^{\#}p < 0.05$; one-way ANOVA for repeated measurements; *post hoc* Tukey's test relative to the preexposure control condition.

HMNs and, if so, whether this change was directly linked to the inhibition of TASK-like conductances. To that end, we recorded HMNs in slices obtained from 6- to 8-d-old rat pups after XIIth nerve crush at P3 and analyzed their sensitivity to pH variations (Fig. 7A,B). Whole-cell recordings in the presence of TTX showed that injured motoneurons had very high R_N ($108.5 \pm 10.7 \text{ M}\Omega$). This represented an increase of 153.3% over the averaged R_N calculated for the control group. In parallel with this observation, we found a considerable reduction in the responsiveness of lesioned HMNs to pH changes (Fig. 7A,B). Therefore, we can conclude that nerve crush induces a reduction in TASK-like pH-sensitive K^+ conductances in motoneurons comparable with that induced by chronic incubations with NO donors in intact motoneurons.

Given that changes in excitability induced by a sustained synthesis of NO seem to represent a long-term mechanism, we could expect that its effects should be slow in onset and reversal. Electroneurographic recordings in adult lesioned animals at 7 d after injury were used to follow the time course of the ratio between the injured and intact whole-nerve activities just after administering D-NAME, L-NAME, or 7-NI. As previously reported by us, nerve activity on the lesioned side was significantly reduced relative to the intact side (Fig. 7C,D, top traces), an effect that correlated well with the loss of afferent inputs on motoneurons (González-Forero et al., 2004b). Systemic administration of D-NAME did not alter this ratio over the course of a 7 h experiment (Fig. 7C,E, black circles). On the contrary, the NOS inhibitor L-NAME unbalanced even more this ratio, inducing a progressive decline that reached statistical significance at 2 h after injection, peaked at 3 h, and then was maintained for the next 4 h period (Fig. 7D,E, open circles). Similar dynamics was observed using 7-NI (data not shown). Thus, at 5 h after drug administration, nerve activity on the lesioned side was significantly reduced relative to the control

side in L-NAME- ($-26.0 \pm 8.0\%$) and 7-NI-treated animals ($-19.3 \pm 3.6\%$), whereas it remained unchanged in the D-NAME-treated group ($-0.2 \pm 9.6\%$). These results strongly support the notion that NO operates in adult injured motoneurons and is required to maintain an enhanced basal activity.

Discussion

This study provides the first account of a mechanism that could be an important component in a complex physiopathological response to nerve injury and possibly some neurodegenerative disorders. We provide strong evidence that in HMNs, persistent, but not acute, activation of NO/cGMP pathway leads to a pronounced enhancement of neuronal excitability through a PKG-dependent inhibition of TASK-like K^+ currents. In neurons lacking NOS-I but innervated by nitrergic afferents, this regulatory mechanism could serve to tightly couple postsynaptic excitability to synaptic use. Likewise, it could also provide a mean to increase excitability of membrane areas adjacent to repeatedly stimulated glutamatergic postsynaptic densities on NOS-I-expressing neurons, in which Ca^{2+} influx and NO production are spatially and functionally coupled (Garthwaite et al., 1988; Watanabe et al., 2003). This same link between postsynaptic activation, NO production, and increase of excitability might render nitrergic neurons highly susceptible to excitotoxic damage and death in the course of neurodegenerative and traumatic brain diseases (Thorns et al., 1998; Moreno-López and González-Forero, 2006).

Typically, axotomized motoneurons exhibit features of an enhanced excitability, (González-Forero et al., 2004a) which are accompanied by *de novo* expression of NOS-I (Yu, 1997; Sunico et al., 2005; Moreno-López and González-Forero, 2006). Recruitment order in a motor pool is primarily determined by intrinsic membrane properties, although synaptic input organization and the patterns of afferent activity could eventually modify Th range or recruitment gain (Gustafsson and Pinter, 1985; Heckman and Binder, 1993; Cope and Sokoloff, 1999). Because axonal injury induces predominantly the loss of excitatory synaptic terminals on adult HMNs (Sumner, 1975) and also a pronounced increase in R_N of both neonatal and adult HMNs (Moreno-López and González-Forero, 2006), we can reasonably assume that changes in the recruitment pattern arise from this enhanced intrinsic excitability and not from the altered synaptic drive, which according to its own sign would be expected to result in an opposed effect. The increased incidence of low-Th units in the lesioned pool was prevented by chronic inhibition of NOS-I or sGC *in vivo*. These data, along with the lack of expression of the inducible isoform of NOS (NOS-II) after injury (Sunico et al., 2005), and the absence of effects of L-NAME in intact animals, indicate that the changes in recruitment distribution are mediated by the *de novo* production of NO in damaged motoneurons acting via an autocrine signaling loop that enhances cGMP synthesis and intrinsic excitability.

This idea receives support from previous studies demonstrat-

ing that bath application of NO increases intrinsic neuronal excitability *in vitro* (Bains and Ferguson, 1997; Shaw et al., 1999; Yang and Hatton, 1999; Abudara et al., 2002; Pose et al., 2003). Although experimental approaches using exogenous NO application do not exactly mimic the transient and discrete synthesis of NO by NOS-I-expressing neurons in response to synaptic stimulation, they confirm that some membrane properties determining intrinsic excitability are modulated by NO. The production of NO is coupled to synaptic activity via NMDA receptors and Ca^{2+} /calmodulin-dependent NOS-I activation (Garthwaite et al., 1988). NO released as a result of this action might modulate intrinsic excitability through PKG activation and/or S-nitrosylation of multiple channel proteins (Ahern et al., 2002). In experiments designed to reproduce such short-term NO actions, several studies revealed reversible changes in excitability consisting of V_m depolarization and decreases in I_{Th} , without parallel alterations in R_N (Bains and Ferguson, 1997; Shaw et al., 1999; Yang and Hatton, 1999; Abudara et al., 2002; Pose et al., 2003; Wang et al., 2006). However, even in the lesioned state, HMNs are characterized by an uninterrupted inspiratory activity mediated by glutamatergic inputs (Wang et al., 2002; González-Forero et al., 2004b). It could be expected that, under these conditions, NOS-I will be persistently activated to produce NO, leading perhaps to even more profound alterations in neuronal excitability. In agreement with this hypothesis, chronic incubations with a NONOate induced an additional increase in R_N and f - I slope in a synaptic-independent way. Similar conclusions could be reached from previous works in cerebellar and striatal neurons, in which NOS or sGC inhibitors caused hyperpolarization and decreases in R_N (Wall, 2003; West and Grace, 2004). It is unlikely that additional effects of the chronic treatment on neuron excitability could be a consequence of the differences in NO concentrations released by different NONOates in this study, because a four-fold increase in DETA/NO concentration, relative to the one used here, had no effects or even reduced R_N in sensory and motor neurons during acute exposures (Abudara et al., 2002; Pose et al., 2003).

It is logical to expect that ionic channels exerting a powerful control over membrane excitability are likely to be the downstream targets for the NO/cGMP signaling. Resting voltage-independent leak K^+ conductances are determinants for setting intrinsic excitability. Motoneurons ex-

Figure 7. Peripheral nerve injury downregulates TASK channel function and increases the basal activity of injured motoneurons. **A**, Top, Time series illustrating changes in I_{holding} in response to variations in extracellular pH for a P7 HMN held at -65 mV and recorded 4 d after ipsilateral XIth nerve crushing. Bottom, Current-clamp recordings of the voltage responses to a series of depolarizing and hyperpolarizing current pulses (0.5 s duration, 0.04 nA increments) from the same HMN at the indicated extracellular pH values. **B**, Summary of changes in I_{holding} and R_N induced by acidification from a basal alkaline pH in intact (control) and crushed P6–P9 HMNs ($n = 5$). Control data (illustrated here for comparison) are the same as those shown in Figure 5A. $^{\#}p < 0.05$; one-way ANOVA; *post hoc* Tukey's test with respect to the control group. **C**, **D**, Integrated bilateral recordings from XIth nerves in adult animals at 7 d after lesion obtained 1 min before (top traces) and 5 h after (bottom traces) administration of the indicated drugs. **E**, Time course of the ratio between the crushed and intact whole-nerve activities recorded from adult animals at 7 d after lesion after administration (at time 0) of D-NAME or L-NAME. Data were expressed as percentage of the measurements taken 5 min before drug administration (100%; triangle). Inset, The area contained within the integral of each inspiratory burst was used to construct the ratio of activities for the crushed/intact sides. $n = 5$ for D-NAME and $n = 3$ for L-NAME. $^{\#}p < 0.05$; two-way ANOVA; *post hoc* Tukey's test relative to both the preadministration value in the same group and the D-NAME-treated group.

press particularly high levels of TASK pH-sensitive channels, which provide a substantial component of their background K^+ current (Talley et al., 2001; Bayliss et al., 2003; Berg et al., 2004). Inhibition of TASK-like conductances leads to membrane depolarization and increases in R_N and intrinsic excitability (Talley et al., 2000, 2003; Bayliss et al., 2003). In this way, *in vitro* recordings from HMNs subjected to prolonged exposures to DETA/NO confirmed that the main action of NO involves inhibition of TASK-like conductances. These results, along with changes in the recruitment pattern of the lesioned pool, still leave open the possibility that a similar modulatory action by endogenous NO may be exerted in HMNs after axonal injury. Evidence for this hypothesis comes from three findings: (1) pH sensitivity of neonatal HMNs was greatly reduced after nerve injury; (2) chronic administration of NOS-I or sGC inhibitors prevented reduction of recruitment Ths induced by nerve crushing in the lesioned HN; and (3) administration of NOS-I inhibitors to adult animals at 7 d after nerve crushing produces a long-latency decrease (>2 h) in the ratio between the injured and intact whole-nerve activities, indicating the activation of an NO-induced long-term mechanism that maintains an increased basal activity in injured HMNs.

Continuous, but not transient, presence of a NONOate in the extracellular solution shifted inward the I_{holding} and increased R_N of HMNs. The decrease in membrane conductance by prolonged NO exposures was associated with a marked loss of sensitivity to pH variations and inhibition of a background current that displayed features of TASK-like conductances (i.e., reversal close to E_K^+ , outward rectification, and modulation by proton concentrations) (Talley et al., 2000; Berg et al., 2004). This almost complete blockade of TASK-like K^+ currents was mimicked by 8-Br-cGMP and prevented by a PKG inhibitor, indicating the involvement of the NO/cGMP/PKG cascade. On the contrary, acute NO or cGMP incubations evoked similar inward shifts in the I_{holding} but lacked effects on background conductances. NO action was independent of synaptic activity and therefore not contaminated by possible changes in synaptic function. Subsequently, it is reasonable to assume that the acute NO/cGMP effects could reflect an action on either a TASK-independent conductance or a combination of background conductances that compensate each other to avoid a net variation in the R_N . In line with this idea, depolarization induced by acute NO incubations occurred even in an acidified extracellular solution, which almost completely inhibits TASK-mediated conductances (Berg et al., 2004). This leads us to the conclusion that the main action of acute NO on I_{holding} and V_m must be associated with effects other than modulation of TASK-like conductances.

Neurotransmitter modulation of TASK channels (Talley et al., 2000) occurs via direct association of the activated $G_{\alpha q}$ subunit with the channel (Chen et al., 2006). Such direct interaction would account for the fast neuromodulatory actions on this conductance but disagree with the slower regulation by NO. It is likely that NO/cGMP/PKG-mediated modulation of TASK-like conductances occurs through mechanisms other than simple PKG-mediated channel phosphorylation, perhaps involving additional signaling pathways, regulation of gene or protein expression, and/or trafficking of the channels (Sauzeau et al., 2003; Renigunta et al., 2006).

NOS-I upregulation and elevated levels of NO have been causally associated with neurodegeneration in the course of a broad range of neurological disorders (Lipton, 2004; Moreno-López and González-Forero, 2006). For instance, in animal models of ALS, NO causes hyperexcitability and sensitizes motoneurons to death (Raoul et al., 2002; Kuo et al., 2004, 2005), whereas its

inhibition prevents experimentally induced parkinsonism (Hantraye et al., 1996; Itzhak and Ali, 1996; Moreno-López and González-Forero, 2006). Likewise, NOS-I-expressing neurons in the entorhinal cortex and hippocampus are highly vulnerable to neurodegeneration in the progression of the Alzheimer's disease (Thorns et al., 1998). Therefore, although a relationship between NOS-I expression and susceptibility to neuronal death seems evident, the cellular mechanisms underlying such deleterious processes remain elusive. Interestingly, differences in the expression level and pattern of TASK channels has been also related to a variable susceptibility to ischemic damage and apoptotic cell death (Patel and Lazdunski, 2004; Taverna et al., 2005). Our results provide a way to integrate these facts and propose a hypothesis whereby TASK channel inhibition via persistent autocrine activation of the NO/cGMP/PKG cascade could sensitize NOS-expressing neurons to excitotoxic damage in brain neurodegenerative processes via a sustained increase in their excitability.

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