Neuroscience

Nitric oxide selectively suppresses $I_{H}$ currents mediated by HCN1-containing channels

Cornelia Kopp-Scheinpflug$^1$, Beatrice M. Pigott$^2$ and Ian D. Forsythe$^2$

$^1$Division of Neurobiology, Department Biology II, Ludwig-Maximilians-University Munich, Großhadernerstr. 2, 82152 Planegg-Martinsried, Germany
$^2$Department of Cell Physiology and Pharmacology, University of Leicester, Leicester LE1 9HN, UK

Key points

- The superior olivary complex (SOC) exhibits a spectrum of HCN1 and HCN2 subunit expression, which generate $I_{H}$ currents with fast and slow kinetics, respectively.
- Neuronal nitric oxide synthase (nNOS) was broadly distributed across the SOC.
- NO hyperpolarizes the half-activation voltage of HCN1-mediated currents and caused a slowing of the $I_{H}$ current kinetics in the respective nuclei (medial and lateral superior olives and superior paraolivary nucleus). This signalling was independent of cGMP.
- NO also caused a depolarizing shift in the half-activation voltage of HCN2-mediated $I_{H}$ currents, increasing activation at resting potentials; this was cGMP-dependent.
- Thus, NO signalling suppressed fast HCN1-mediated currents and potentiated slow HCN2-mediated currents, modulating the overall kinetics and magnitude of the endogenous $I_{H}$.

Abstract

Hyperpolarization-activated non-specific cation-permeable channels (HCN) mediate $I_{H}$ currents, which are modulated by cGMP and cAMP and by nitric oxide (NO) signalling. Channel properties depend upon subunit composition (HCN1–4 and accessory subunits) as demonstrated in expression systems, but physiological relevance requires investigation in native neurons with intact intracellular signalling. Here we use the superior olivary complex (SOC), which exhibits a distinctive pattern of HCN1 and HCN2 expression, to investigate NO modulation of the respective $I_{H}$ currents, and compare properties in wild-type and HCN1 knockout mice. The medial nucleus of the trapezoid body (MNTB) expresses HCN2 subunits exclusively, and sends inhibitory projections to the medial and lateral superior olives (MSO, LSO) and the superior paraolivary nucleus (SPN). In contrast to the MNTB, these target nuclei possess an $I_{H}$ with fast kinetics, and they express HCN1 subunits. NO is generated in the SOC following synaptic activity and here we show that NO selectively suppresses HCN1, while enhancing $I_{H}$ mediated by HCN2 subunits. NO hyperpolarizes the half-activation of HCN1-mediated currents and slows the kinetics of native $I_{H}$ currents in the MSO, LSO and SPN. This modulation was independent of cGMP and absent in transgenic mice lacking HCN1. Independently, NO signalling depolarizes the half-activation of HCN2-mediated $I_{H}$ currents in a cGMP-dependent manner. Thus, NO selectively suppresses fast HCN1-mediated $I_{H}$ and facilitates a slow HCN2-mediated $I_{H}$, so generating a spectrum of modulation, dependent on the local expression of HCN1 and/or HCN2.
Introduction

Hyperpolarization-activated cyclic nucleotide-modulated channel (HCN) subunits are encoded by the HCN1–4 gene family and form the molecular substrate of $I_{h}$ currents that regulate resting membrane potential (RMP) and excitability in the brain and heart (Santoro et al. 1998; Robinson & Siegelbaum, 2003; Wahl-Schott & Biel, 2009). Each HCN channel is formed by a tetrameric complex of subunits, with both homo- and heteromeric channels being suggested (Biel et al. 2009). All four subunits are expressed in the brain, but studies show that they differ in activation kinetics (HCN1: fast, HCN2: intermediate, HCN3 and 4: slow), voltage-dependent activation (half-activation voltages for HCN1: −70 mV, HCN2: −95 mV, HCN3: −85 mV, HCN4: −100 mV) and the depolarizing shift in activation voltage mediated by cyclic nucleotides (HCN2 and 4: most sensitive, HCN1 and 3: less sensitive) (Robinson & Siegelbaum, 2003; Biel et al. 2009; Wahl-Schott & Biel, 2009; Shah, 2014). These findings are mainly based on research using cell expression systems, but there is increasing evidence that site-specific accessory subunits, local kinase and/or phosphatase activity also contribute to shaping the characteristics of $I_{h}$ currents in native neurons (Santoro et al. 2009; Wahl-Schott & Biel, 2009). It is therefore essential to study HCN kinetics and modulation in native neurons with intact intracellular signalling.

Throughout the nervous system, synaptic activity can generate nitric oxide (NO). This messenger molecule is involved in the regulation of synaptic transmission and neuronal function (Garthwaite, 2008; Steinert et al. 2008, 2011; Hardingham et al. 2013) through binding of NO to its receptor, guanylyl cyclase (sGC), and subsequent production of cGMP. NO–cGMP signalling could regulate native HCN-mediated currents directly through changes in cGMP concentrations, or indirectly through effects on cGMP-dependent protein kinases or phosphodiesterases. Cyclic nucleotides (cAMP, cGMP) and NO modulate HCN channel activity in many brain areas (Pape & Mager, 1992; Ingram & Williams, 1996; Garthwaite et al. 2006; Wilson & Garthwaite, 2010; Neitz et al. 2011, 2014).

The medial nucleus of the trapezoid body (MNTB) expresses exclusively HCN2 and sends inhibitory projections to the HCN1-expressing medial and lateral superior olives (MSO, LSO), and to the superior paralvivary nucleus (SPN), which expresses both HCN1 and HCN2 subunits (Koch et al. 2004). Here we examine HCN subunit-specific roles in neuronal signalling by exploiting the distinctive patterns of HCN1 and HCN2 expression across the superior olivary complex (SOC). The large amplitude of $I_{h}$ currents (surpassing those of CA1 neurons by 10-fold) increases the reliability of observing modulatory effects, and the use of HCN1-knockout (KO) mice allowed differentiation between NO modulation of HCN1 and HCN2 subunits.

The results show that NO suppresses HCN1-mediated $I_{h}$ currents by an sGC-independent negative shift in the voltage dependence of activation. Simultaneously NO facilitates HCN2-dominated $I_{h}$ currents by a GC-dependent positive shift in activation. For neurons that express both HCN1 and HCN2, the net effect is to slow $I_{h}$ kinetics and increase the $I_{h}$ contribution to resting conductances, thereby depolarizing the RMP and reducing the peak conductance activated by hyperpolarization.

Methods

Brain slice preparation

Experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and as revised by European Directive 2010/63/EU. The HCN1-KO mice (obtained from Edinburgh University) were backcrossed onto a CBA/Ca background. CBA/Ca and HCN1-KO mice (postnatal days (P)13–21) of either sex were killed by decapitation and brainstem slices containing the SOC were prepared as described previously (Johnston et al. 2008). Briefly, transverse slices were cut in an ice-cold (4 °C) low sodium artificial cerebrospinal fluid (aCSF), incubated at 37 °C for 1 h in normal aCSF, and then stored at room temperature (~20 °C) in a slice-maintenance chamber until transferred to the slice rig at 37 °C for recording. The normal aCSF comprised (in mM): NaCl 125, KCl 2.5, NaHCO$_3$ 26, glucose 10, NaH$_2$PO$_4$ 1.25, sodium pyruvate 2, myo-inositol 3, CaCl$_2$ 2, MgCl$_2$ 1 and ascorbic acid 0.5; pH was 7.4 when bubbled with 95% O$_2$/5% CO$_2$. In the low sodium aCSF, NaCl was replaced by 250 mM sucrose.
and CaCl$_2$ and MgCl$_2$ concentrations were 0.1 and 4 mM, respectively. All chemicals were obtained from Sigma (St Louis, MO, USA), unless otherwise noted.

**Electrophysiology**

A slice was transferred to a Peltier-controlled recording chamber (37 ± 1°C) on the stage of a Nikon FN600 microscope (Nikon, Tokyo, Japan). Individual cells were visualized using differential interference contrast (DIC) optics and 63× (0.9 NA) water immersion objectives. Whole-cell patch-clamp recordings were made using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA) and pClamp 10 software (Molecular Devices), sampling at 50 kHz and filtering at 10 kHz. Patch pipettes were pulled from filamented borosilicate glass (GC150F-7.5, Harvard Apparatus, Edenbridge, UK) using a two-stage vertical puller (PC-10 Narishige, Tokyo, Japan) and filled with a solution containing (in mM): potassium gluconate 97.5, KCl 32.5, Hepes 40, EGTA.

**Figure 1. Expression of HCN1 and HCN2 within the mouse SOC**

A, intense immunofluorescence for HCN1 (green) was predominant in cell bodies and fibres in the MSO and LSO, sparse in the SPN and absent in the MNTB, where immunofluorescence was of background level. Micrographs taken at high magnification show immunopositive cell soma in the LSO (A$_i$), MSO (A$_ii$) and SPN (A$_iii$) but MNTB principal neurons showed little or no specific labelling (A$_iv$; HCN1 = green; different sections to A). B, HCN2 immunofluorescence (green) was most intense in cell bodies and fibres in the MNTB and SPN, weaker in the LSO and comparable to background in the MSO. B$_{i-iv}$, high magnification images show the presence of immunopositive cell soma in the LSO (B$_i$), SPN (B$_ii$) and MNTB (B$_iv$) but not MSO (B$_iii$; green = HCN2; different sections to B). Asterisks (*) in B$_iv$ mark principal cell bodies. C, control sections for the specificity of the secondary antibody (primary antibodies omitted) showed little non-specific staining (green). Dashed line surrounds MNTB. A, B and C are composites of two photographs. Tissue sections shown in A–A$_{iv}$ were adjacent to those shown in B–B$_{iv}$. Cell nuclei were counterstained with DAPI (blue). Scale bar in A = 150 μm for A, B and C and 25 μm for A$_{i-iv}$ and B$_{i-iv}$.
5, MgCl$_2$ 1, Na$_2$phosphocreatine 5 (pH adjusted to 7.2 with KOH). Final resistances were 3–4 MΩ. Whole-cell access resistances were <10 MΩ and series resistances were routinely compensated for by 70%. All voltages were corrected for an 11 mV liquid junction potential.

The amplitudes of the $I_{H}$ currents were measured during a hyperpolarizing voltage command, measured as the difference between the instantaneous current and the sustained currents. Activation kinetics of $I_{H}$ were voltage-dependent, and fitted by a double exponential function, dominated by the fast component. Activation in the HCN1-KO was much slower and was well fit by a single exponential function with similar values to the slow time constant of the wild-type data. Voltage-dependent half-activation was determined by fitting a Boltzmann function to the plot of the conductance ratio ($G/G_{max}$) vs. the applied voltage.

**Immunohistochemistry**

Methods for immunohistochemistry were adapted from Kopp-Scheinpflug et al. (2011). Briefly, male or female adult mice (P30–60), wild-type or neuronal nitric oxide synthase KO mice (nNOS-KO, lacking exon 6) (Gyurko et al. 2002) bred into a CBA/Ca background were decapitated and their brainstems rapidly frozen in Lamb OCT embedding matrix (ThermoFisher Scientific, Waltham, MA, USA). Transverse, 12 μm-thick tissue sections were prepared on Menzel-Gläser Polysine slides using a cryostat (Microm HM 560, ThermoFisher Scientific). For HCN1 and HCN2 labelling, sections were fixed in 4% paraformaldehyde (PFA) diluted in PBS (25 min at 4 °C). Sections to be stained for nNOS were exposed to 99.8% methanol (10 min, −20 °C) instead of PFA as this procedure was found to yield superior antibody specificity in separate experiments (unpublished data). Sections were then washed for 5 min, three times in PBS containing 0.1% Triton X-100 (PBS-T) and blocked in PBS-T plus 1% BSA and 10% normal goat serum (1 h at room temperature; serum concentrations were tripled for nNOS staining; Vector Labs, Burlingame, CA, USA). Washes with PBS-T were repeated and the antibodies for nNOS (1:1000; SC-648, Santa Cruz, Dallas, TX, USA), HCN1 (1:500; APC-056, Alomone, Jerusalem, Israel) or HCN2 (1:1000; APC-030, Alomone) were diluted in blocking solution and applied for 18–20 h at 4 °C in a humid environment. Control sections for the selectivity of the secondary antibodies received blocking solution minus antisera. Sections were then washed for 10 min, three times in PBS-T and incubated with Alexa Fluor 488 goat anti-rabbit IgG (1:1000 in blocking solution; Life Technologies, Carlsbad, CA, USA) for 1 h at room temperature in the dark. Washes were repeated and sections were counterstained (DAPI, Vector Labs), mounted and photographed using a Leica DM2500 fluorescence microscope (Leica, Wetzlar, Germany). Photographs are representative of data from at least two sections from each of three animals. Experimental and control sections were prepared, stained and photographed in parallel and while the experimenter was blinded to genotype. Control and experimental photographs were adjusted equally for brightness and contrast using Adobe Photoshop (Adobe, Mountain View, CA, USA).

**Statistics**

Statistical analyses were performed with SigmaPlot/SigmaStat (SPSS Science, Chicago, IL, USA). Individual data points represent the data collected from single neurons and bar graphs show mean values ± standard error of the mean (SEM). Values of $n$ indicate the number of neurons, which were from at least three different animals. Statistical comparisons between multiple different data sets were made using one-way repeated measures ANOVA. For data following non-normal distributions, significance was assessed using the Kruskal–Wallis ANOVA on ranks test. Differences

---

**Figure 2. nNOS immunostaining predominates in the MNTB and SPN**

A, immunofluorescence for nNOS (green) in tissue from a wild-type (WT) mouse was apparent in cell soma, fibres (arrows) and neuropil throughout the MNTB, SPN, MSO and LSO. B, identical staining procedures applied to tissue from a nNOS knockout (KO) mouse showed little or no staining (green), thereby validating the specificity of the immunofluorescence for nNOS in the WT. Inset: secondary antibody control (no primary antibody was used). Image shows MNTB in a knockout section and is representative of the entire SOC. A and B are merged composites of two images to show the full lateral extent of the SOC. Nuclei were counterstained with DAPI (blue). Scale bar = 150 μm and applies to both panels and inset.
between just two data sets in a classical before/after treatment were assessed by Student’s *t* test (normal distribution) or Mann–Whitney rank sum test. Normality was evaluated by the Shapiro–Wilk test. Differences were considered statistically significant when \( P < 0.05 \) (****: \( P \leq 0.001 \); ****: \( P \leq 0.01 \); *: \( P \leq 0.05 \)).

**Results**

**Mouse SOC neurons express HCN1, HCN2 and nNOS**

The SOC includes the MNTB and its target nuclei, the MSO, LSO and SPN. Immunohistochemistry for HCN1 and HCN2 in the mouse SOC revealed a distinct distribution for each subunit that was consistent with the kinetics of \( I_H \) measured in each nucleus. HCN1 immunofluorescence (green in Fig. 1A) was intense in cell bodies and fibres and was predominant in those nuclei with the fastest \( I_H \) (MSO and LSO), moderate in SPN and absent where \( I_H \) exhibited slow kinetics (MNTB). The specificity of this immunofluorescence for HCN1 was verified previously using HCN1-KO tissue (Kopp-Scheinpflug et al. 2011). In contrast to the HCN1 antibody, the HCN2 antibody, which generates comparable labelling in wild-type and HCN1-KO tissue (Kopp-Scheinpflug et al. 2011), strongly stained MNTB neuron soma (Fig. 1B). The LSO and SPN were also immunopositive for HCN2, but unlike in the MNTB, HCN2 labelling in LSO and SPN was concentrated in the neuropil (Fig. 1Bi and Bi). No expression of HCN3 or HCN4 was observed in SOC neurons, while some minor staining of HCN4 was observed in axonal tracts (data not shown). Overall this is consistent with immunohistochemical localization in the rat (Koch et al. 2004; Notomi & Shigemoto, 2004) and the focus of this paper on somatic currents mediated by HCN1 and HCN2.

In neurons, NO is generated by nNOS, and consistent with previous findings in hamster (Reuss et al. 2000), gerbil and mouse (Yassin et al. 2014), immunofluorescence for nNOS was observed in neuronal soma throughout the MNTB and SPN, as well as in fibres and neuropil throughout MNTB, SPN, MSO and LSO (Fig. 2A). The specificity of the antibody staining for nNOS was validated here by the absence of immunostaining in tissue from nNOS-KO mice (Fig. 2B).

![Figure 3. NO reduces membrane potential sag in MSO, LSO and SPN neurons, but not MNTB neurons](image-url)
NO modulates ionic conductances in SOC neurons without changing the overall firing pattern

MNTB neurons send inhibitory projections to the LSO, the MSO and the SPN. In the target neurons this inhibition hyperpolarizes the membrane and activates $I_{h}$. MNTB neurons are also known to generate NO upon synaptic activity (Steinert et al. 2008). To investigate the effect of NO on $I_{h}$ in each nucleus, whole-cell patch-clamp recordings were obtained from neurons of the mouse SOC before and during bath application of an NO donor sodium nitroprusside (SNP; 100 μM). In response to 200 ms depolarizing current injections (Fig. 3A, black traces) all SOC neurons responded with one or more action potentials according to their established firing properties (Brew & Forsythe, 1995; Barnes-Davies et al. 2004; Scott et al. 2005; Felix et al. 2011; Kopp-Scheinflug et al. 2011). The response to sustained outward current injections (200 ms duration) shows a rapid initial hyperpolarization, followed by the classic ‘sag’ in membrane potential that is indicative of the presence of $I_{h}$ (Fig. 3A, blue traces). In contrast to the large sag observed in MSO and LSO neurons, in SPN neurons the sag was more moderate and of slower time course, while MNTB neurons exhibited the slowest and smallest amplitude sag. The ratio between the peak hyperpolarization and the membrane potential after 100 ms ($R_1$, Fig. 3A top left) and 200 ms ($R_2$, Fig. 3A top left) has been used to quantify the sag and evaluate the contribution of HCN1- vs. HCN2-mediated currents to $I_{h}$ (Nolan et al. 2007). A large difference between $R_1$ and $R_2$ indicates a more slowly activating $I_{h}$ conductance, such as those dominated by HCN2 subunits. In contrast, when the membrane potential quickly relaxes back to more depolarized values, it reflects $I_{h}$ with more rapid kinetics as expected for the HCN1-dominated currents of the MSO, LSO and SPN. In agreement with our immunohistochemical evidence (Fig. 1), a between-group comparison of the HCN1 contribution based on $\Delta R_3 - R_1$ between the four nuclei revealed that MNTB neurons were similar across all four nuclei (MSO: 0.021 ± 0.002, n = 9; LSO: 0.013 ± 0.002; n = 10) and SPN (0.019 ± 0.003; n = 24) are all significantly different (smaller) from MNTB (0.033 ± 0.002; n = 15; P ≤ 0.001), but not significantly different from each other (P = 0.105; Fig. 3B)

An initial estimation of the degree to which HCN channels were affected by NO was achieved by monitoring current-clamp changes in the voltage sag before (Fig. 3C, grey traces) and during NO exposure (Fig. 3C, blue traces). Activation of nitrergic signalling reduced the magnitude and slowed the time-course of the sag in MSO, LSO and SPN neurons (Fig. 3C) but not in MNTB neurons. Following NO exposure the R ratios were similar across all four nuclei (MSO: 0.021 ± 0.004, n = 5; LSO: 0.019 ± 0.004, n = 5; SPN: 0.025 ± 0.005, n = 9; MNTB: 0.028 ± 0.004, n = 4; P = 0.705; Fig. 3D), implying NO-mediated suppression of a fast HCN1-mediated component in the MSO, LSO and SPN.

---

**Figure 4. NO slows the activation time constant and hyperpolarizes the half-activation voltage of HCN1-dominated currents**

A, control: fast $I_{h}$ currents in an MSO cell evoked by step commands from a holding potential of −61 to −131 mV (voltage protocol plotted below). Following application of an NO donor (100 μM SNP), $I_{h}$ currents evoked over the same voltage range were slower to activate (grey traces). Inset: superimposed traces from control (black trace) and following NO exposure (grey trace) show the slowed $I_{h}$ activation kinetics. B, the voltage-dependent activation curve for the cell shown in A was plotted as $G / G_{\text{max}}$ and fitted to a Boltzmann equation. C, mean activation curves were plotted from individual cells and fitted to a Boltzmann equation for WT controls (black squares), HCN1-knockout (HCN1KO, white squares) and WT following NO exposure (grey circles). D, voltage-dependent half-activation was shifted towards more hyperpolarizing values in HCN1KO mice and following NO exposure. E, in the HCN1KO the HCN conductance was significantly reduced, while NO exposure shifted the activation voltage without changing the maximum conductance. F, the absence (HCN1KO) or reduction of HCN1 contribution (NO condition) led to slower activation time constants of MSO currents. Average data are shown as mean ± SEM; values of $n$ are indicated by individual data points and by the number in each bar. **P ≤ 0.001. Significance was assessed by one-way ANOVA or Kruskal–Wallis one-way ANOVA on ranks.
NO hyperpolarizes \(I_H\) half-activation and slows its kinetics to values observed in HCN1-KO mice

To test the hypothesis that NO selectively suppresses HCN1-mediated currents, we focused on MSO neurons, which show a strong HCN1 contribution (Fig. 3), and compared these data with \(I_H\) measurements from HCN1-KO mice. Under voltage-clamp, control MSO \(I_H\) currents activated quickly upon hyperpolarization to voltages only slightly negative to the RMP (Fig. 4A, B). While maximum \(I_H\) conductance was significantly reduced in MSO neurons of HCN1-KO mice, application of the NO donor SNP caused no significant change in conductance (\(G_{max}\) control: 21.6 ± 1.1 nS, \(n = 40\); \(G_{max}\) HCN1-KO: 7.65 ± 1.4 nS, \(n = 9\); \(G_{max}\) NO: 17.6 ± 2.1 nS, \(n = 13\); \(P < 0.001\); Fig. 4E). We controlled for rundown over time; recordings made over a 25 min time course showed no significant change in half-activation voltage (Fig. 5).

The fast activation time constant of \(I_H\) under control conditions is indicative of a strong contribution of HCN1 subunits in the MSO, corroborating our immunohistochemical results (Fig. 1). The suppression of this fast \(I_H\) by NO exposure suggests a mechanism specific for HCN1 and this hypothesis was tested by comparing MSO \(I_H\) \(V_{1/2}\) and \(\tau\) between wild-type and HCN1-KO mice (Fig. 4C–F). The positive half-activation voltage of control (WT) MSO \(I_H\) currents (\(V_{1/2}: -83.5 \pm 1.1\) mV, \(n = 40\)) was shifted in the hyperpolarizing direction by deletion of HCN1 subunits in HCN1-KO mice (\(V_{1/2}: -111.3 \pm 3.3\) mV, \(n = 9\); \(P < 0.001\), Fig. 4C, D) or NO signalling in WT MSO neurons (\(V_{1/2}: -107.8 \pm 3.9\) mV, \(n = 13\), Fig. 4C, D). The inward current that remained following NO application was tested for its sensitivity to the \(I_H\) blocker ZD7288. A concentration of 20 \(\mu M\) ZD7288 blocked 85.5 ± 5.1% (\(n = 3\)) of the remaining current, confirming that although this current had slower kinetics, it was an \(I_H\) current. Together these results are consistent with the hypothesis that NO signalling suppresses activation of an HCN1-mediated \(I_H\) current by inducing a leftward shift of the activation curve. The absence (HCN1-KO) or reduction of HCN1 contribution (NO exposure) led to slower activation time constants compared to wild-type MSO currents (\(\tau_{\text{wild-type}}\): 19.1 ± 0.8 ms, \(n = 40\); \(\tau_{\text{HCN1-KO}}\): 168.3 ± 14.4 ms, \(n = 9\); \(\tau_{\text{NO}}\): 36.9 ± 4.4 ms, \(n = 13\); \(P \leq 0.001\); Fig. 4F).

NO depolarizes activation of HCN2-mediated currents in a cGMP-dependent manner

MNTB neurons are characterized by exclusive HCN2 expression (Fig. 1), consistent with the slow sag in

**Figure 5.** No significant changes in amplitude or half-activation voltage of HCN1 currents over 20 min

A, consecutively recorded fast \(I_H\) currents in an MSO cell evoked by step commands from a holding potential of −61 to −131 mV. Lower inset shows the instantaneous current (\(i_{\text{inst}}\)) and the onset of the \(I_H\) current. B, mean data showing the amplitudes of the instantaneous current (\(i_{\text{inst}}\)), the sustained current (\(i_{\text{sust}}\)) and the difference current (\(i_{H} = i_{\text{sust}} - i_{\text{inst}}\)). C, activation curves were plotted for the neuron shown in A and fitted to a Boltzmann equation (see Methods). D, mean data for voltage-dependent half-activation for control recordings and recordings from the same cell 20 min later. The range between the dotted lines in D represents the mean ± SD of the control \(V_{1/2}\) \(I_H\) in 40 MSO neurons.

© 2015 The Authors. The Journal of Physiology published by John Wiley & Sons Ltd on behalf of The Physiological Society
membrane potential during hyperpolarization (Fig. 3). Voltage-clamp recordings of MNTB neurons revealed further characteristics of HCN2-like $I_H$ currents, including slow activation kinetics of $50.8 \pm 2.8$ ms ($n = 25$) and hyperpolarized half-activation voltages of $-97.68 \pm 0.68$ mV ($n = 25$; Fig. 6B, C). The absence of HCN1 from MNTB neurons was reinforced by a comparison of $I_H$ currents between wild-type

![Image of Figure 6](image-url)

**Figure 6.** NO depolarizes the half-activation voltage of slow HCN2 channels in a cGMP-dependent manner

A, slow $I_H$ currents in an MNTB neuron evoked by step commands. NO exposure (grey traces) did not change activation kinetics. B, voltage-dependent activation was depolarized during NO exposure in MNTB neurons. C, mean activation curves were plotted from individual cells and fitted to a Boltzmann equation for WT controls (black squares), HCN1-knockout (HCN1$^{KO}$; white squares) and WT following NO exposure (100μM SNP; grey circles). D, mean voltage-dependent activation was more depolarized following NO exposure, but no difference was found between half-activation voltages of WT and HCN1$^{KO}$ mice. E and F, conductance and activation kinetics were not altered by HCN1$^{KO}$ or NO exposure. G, mean activation curves for control (black squares), increased intracellular cGMP (1 mM; white circles) and increased cAMP (1 mM; grey circles). H, increased intracellular cGMP or cAMP mimicked NO-mediated depolarization of half-activation voltages for HCN2 currents. Dotted lines indicate the mean ± SD of $V_{1/2}$ during NO application. Average data shown as mean ± SEM; $n$, numbers are indicated by individual data points and by the number in each bar. **$P \leq 0.01$, ***$P \leq 0.001$. Significance was assessed by one-way ANOVA or Kruskal–Wallis one-way ANOVA on ranks.
and HCN1-KO MNTB neurons, which revealed no significant differences in activation voltage ($V_{1/2}$wild-type: −96.2 ± 1.2 mV, $n = 25$; $V_{1/2}$HCN1-KO: −97.3 ± 4.6, $n = 6$; $P = 0.991$), activation kinetics ($\tau$wild-type: 50.8 ± 2.8 ms, $n = 25$; $\tau$HCN1-KO: 60.6 ± 7.2 ms, $n = 6$; $P = 0.240$) or overall conductance ($G_{\text{wild-type}}$: 4.7 ± 0.3 nS, $n = 25$; $G_{\text{HCN1-KO}}$: 4.8 ± 0.5 nS, $n = 6$; $P = 0.856$; Fig. 6C–F).

In contrast to the suppression of HCN1-mediated $I_H$ currents in the MSO, NO caused a depolarizing shift in the activation voltage in the MNTB ($V_{1/2}$NO: −89.3 ± 0.9 mV, $n = 6$; $P \leq 0.05$; Fig. 6B, C, E) and no change in the activation kinetics ($\tau_{\text{NO}}$: 61.3 ± 3.1 ms, $n = 6$; $P = 0.097$; Fig. 6A, F). This depolarizing shift was mimicked by adding 1 mM cGMP or cAMP to the intracellular patch solution ($V_{1/2}$cGMP: −88.0 ± 3.5 mV, $n = 6$; $P = 1.000$; $V_{1/2}$cAMP: −82.6 ± 3.1 mV, $n = 7$; $P = 0.275$; Fig. 6G, H), suggesting involvement of the classical NO/cGMP pathway.

**Discussion**

We have exploited the differences in endogenous expression between nuclei of the SOC to examine the subunit-specific signalling modulation of native $I_H$ currents by NO. HCN1 and HCN2 subunits are expressed widely in the CNS (Shah, 2014; He et al. 2014). HCN1 is highly expressed in specific neuron types in cortex, cerebellum, hippocampus and brainstem (Moosmang et al. 1999; Koch et al. 2004) and HCN1-dominated currents have fast kinetics, are active at RMPs and often found in fast-spiking interneurons. In contrast, HCN2-mediated $I_H$ currents activate more slowly and at more negative voltages (Santoro et al. 2000; Aponte et al. 2006; Wahl-Schott & Biel, 2009) so contributing less to resting conductances.
Here we argue that the presence of HCN1 subunits provides an opportunity for activity-dependent suppression of $I_{H}$ currents via NO signalling. In contrast, the slower HCN2-dominated $I_{H}$ currents are enhanced by NO. Downstream of NO, HCN1-mediated currents are suppressed in a cGMP-independent manner while HCN2-mediated currents are enhanced via the NO-receptive sGC/cGMP pathway.

**Modulation of HCN1**

A major function of HCN1 channels is to increase membrane conductance and thereby accelerate or control the membrane time constant in a localized region of a neuron (Pape & McCormick, 1989; Maccaferri & McBain, 1996; Gasparini & DiFrancesco, 1997; Kopp-Scheinpflug et al. 2011). HCN1 channels are abundantly expressed...
in hippocampal and cortical pyramidal neuron dendrites where they serve to dampen excitability around threshold and modulate the propagation and integration of synaptic potentials, especially those originating from remote sites (Magee, 1998). In cerebellar Purkinje neurons conditional deletion of HCN1 decreased $I_{\text{H}}$ currents and slowed the recovery of the membrane potential following inhibitory postsynaptic potential (IPSP) trains, without changing excitatory postsynaptic potential (EPSP) kinetics (Rinaldi et al. 2013). This is consistent with a role for HCN1 in regulating integration at hyperpolarized potentials, be that mediated by IPSPs or by hyperpolarizing after-potentials (AHP). If HCN1 is suppressed (e.g. by NO), the balance between IPSP and EPSP integration would favour inhibition, which switches from phasic to tonic, and the recovery of the membrane potential after an action potential would be prolonged. This could contribute to mechanisms of anaesthetics such as propofol, isoflurane and halothane, which have been reported to suppress HCN1-containing channels (Chen et al. 2005a,b, 2009).

The modulation of HCN1 by NO observed here was independent of the classic NO/cGMP pathway. cGMP-independent mechanisms of IH modulation have been observed previously in hypoglossal motoneurons (Wenker et al. 2012). Mechanisms for regulation of HCN1 that are cGMP-independent could involve channel auxiliary subunits, such as Trip8b, which regulates the trafficking of HCN1 channels (Santoro et al. 2009) and acts to hyperpolarize the channel’s half-activation voltage. Trip8b reduces the depolarizing effect of cAMP on $I_{\text{H}}$ activation, consistent with the modest effects of cAMP seen here in the MSO, LSO and SPN neurons (Santoro et al. 2009; Hu et al. 2013). Trip8b possesses several potential serine/threonine phosphorylation sites (Santoro et al. 2009) where protein kinase C (PKC) has the potential to phosphorylate and could interact with NO signalling via S-nitrosylation (Gopalakrishna et al. 1993), which would be independent of cGMP.

**Modulation of HCN2**

$I_{\text{H}}$ currents with slow kinetics, such as in MNTB or in neurons of the deep cerebellar nucleus, activate at rather negative voltages and are modulated by cAMP, indicative of HCN2 (Banks et al. 1993; Chen et al. 2005b). HCN2 channels are also sensitive to cGMP, raising the potential for their modulation by an NO/sGC-coupled mechanism (Wilson & Garthwaite, 2010). ATP is typically included in patch solutions, which as suggested by Wilson and Garthwaite (2010), could inhibit NO-activated sGC activity (Roy et al. 2008). We did not add ATP to the intracellular patch pipette solution and we could observe NO/cGMP-mediated modulation of the HCN2-dominated $I_{\text{H}}$ in MNTB neurons. Consistent with this result, cGMP-dependent depolarization of $I_{\text{H}}$ activation was reversed when the NO–cGMP pathway was disrupted in sGC (NO receptor) knockout mice, resulting in the increased intracellular cGMP (1 mM; grey bars), in the presence of ODQ alone (1 μM; black bars) and with NO (100 μM SNP; red bars) for cells in the MSO (C) and in the SPN (D). The range between the dotted lines in C and D represents the mean ± SD of the control $V_{1/2}$ in the respective nuclei.
in more negative activation voltages (Neitz et al. 2011, 2014).

**Functional consequences of $I_{H}$ modulation**

NO is generated in neurons by nNOS upon glutamatergic synaptic transmission (Garthwaite, 2008; Steinert et al. 2008, 2011; Hardingham et al. 2013). We have shown that NO signalling can activate or suppress $I_{H}$ currents by cGMP-dependent or cGMP-independent mechanisms contingent on the channel subunit composition: HCN1-mediated currents are suppressed by NO, while HCN2-mediated currents are enhanced by NO.

nNOS is associated with postsynaptic densities (Brenman et al. 1996) and is widely distributed in the brain, as are HCN1 and HCN2 subunits (Notomi & Shigemoto, 2004). The localization of HCN1 and HCN2 to somatic, dendritic or even axonal and synaptic compartments is highly controlled (Cuttle et al. 2001; Huang et al. 2012; Shah, 2014; Huang & Trussell, 2014) and the ability of $I_{H}$ to increase or decrease neuronal excitability depends strongly on this subcellular location. At dendritic locations, where nNOS resides, local $I_{H}$ currents regulate the membrane time constant and thereby control temporal summation, while somatic $I_{H}$ depolarizes the resting membrane potential and determines threshold for action potential firing (Magee, 1999; Poolos, 2010; Shah, 2014). Dendritic $I_{H}$ stabilizes the integrative properties of the neurons to ensure an input/output function that is independent from the cells’ previous firing activity (Nolan et al. 2003). However, activity-dependent firing is important to establish different forms of plasticity. $I_{H}$ tunes this equilibrium; activation renders the neuron less dependent on firing history while suppression enables plasticity. This means that, for example, the loss of dendritic $I_{H}$ currents in hippocampal CA1 neurons or cerebellar Purkinje neurons causes enhanced stability of place fields (Giocomo et al. 2011; Hussaini et al. 2011). Suppression of $I_{H}$ currents, as occurs during prolonged synaptic stimulation, will produce a net hyperpolarization that can reduce dendritic filtering, remove location-dependent

---

**Figure 9. Differential expression of HCN subunits between the SOC nuclei underlies differential actions of NO in regulating the RMP and action potential (AP) threshold**

A, bar graph of mean (± SEM) and individual data points for RMP measured under current clamp in the SPN, MSO, LSO and MNTB in control (filled symbols) and following NO application (open symbols). Only the MNTB showed no significant change. B, current threshold for triggering an AP was measured as the positive current required to be injected to fire the first AP and was significantly reduced in MSO neurons following NO application. C, the number of APs evoked per trial at the current threshold was not altered following NO application. D, in the SPN, rebound AP firing (inset) triggered on repolarization, following a hyperpolarizing current injection, was reduced following NO application as indicated by the box. ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05. Significance was assessed by Student’s t test.

© 2015 The Authors. The Journal of Physiology published by John Wiley & Sons Ltd on behalf of The Physiological Society
variability in temporal integration and thereby enhance synchronization of firing across neuronal populations (Magee, 1999, 2000). Facilitation of spatial learning and memory via suppression of HCN1 (Nolan et al. 2004) has not been linked to a physiological signalling cascade, but the results reported here suggest that NO-mediated suppression of HCN1 could fulfil this role.

**Differential role of HCN1 versus HCN2 in the auditory nuclei**

In auditory brainstem neurons $I_{H}$ currents are many fold larger in amplitude than elsewhere in the brain (Golding et al. 1999; Koch & Grothe, 2003; Leao et al. 2006; Felix et al. 2011; Kopp-Scheinpfug et al. 2011) and are often paired with low-voltage activated potassium currents ($I_{KL}$), which mediate an opposing outward current (Cao & Oertel, 2011; Khurana et al. 2011, 2012; Golding & Oertel, 2012). These two conductances are active at resting membrane potentials, serving to lower input resistances and regulate threshold and firing patterns. Their action also generates very fast membrane time constants, which limit temporal summation and favours coincidence detection.

In the brainstem of WT CBA/Ca mice neurons possess $I_{H}$ currents with distinct kinetics (fast and slow). HCN1-KO mice lack a fast kinetic component and the remaining $I_{H}$ has slow kinetics similar to $I_{H}$ currents mediated by HCN2 subunits. NO has distinct modulatory actions on these two $I_{H}$ kinetic components: the first is independent of modulation by cGMP, shifting the fast HCN1 activation to the left (so it is less effectively activated). The second is a cGMP-dependent rightward shift in the activation kinetics of the slow HCN2-dominated $I_{H}$. Together these observations are consistent with the hypothesis that native HCN1 channels are homomeric. The $I_{H}$ current remaining in the HCN1-KO mice is consistent with the kinetics of an HCN2 current (perhaps including HCN4 subunits) and suggests that the dual expression and independent differential modulation of HCN1- versus HCN2-containing channels then allows switching between two distinct kinetic forms of the $I_{H}$ current (fast and slow) rather than formation of heteromeric assemblies with intermediate properties.

If unopposed by $I_{KL}$, $I_{H}$ will depolarize the membrane potential and deactivate. In contrast, suppression of $I_{H}$ currents will produce a net hyperpolarization pushing the membrane potential further from action potential threshold (Liu et al. 2014). Indeed blockade of $I_{H}$ in auditory neurons causes a reduction in excitability in vivo (Shaikh & Finlayson, 2003). In the mammalian auditory system moderate firing rates are able to drive nNOS-positive neurons in the MNTB sufficiently to generate NO, suggesting that NO signalling is active during normal physiology with increasing importance at more intense stimuli (Steinert et al. 2008, 2011). In the MNTB, low acoustic background conditions (corresponding to low NO levels) will only activate $I_{HL}$ and $I_{KL}$ during or following an action potential. As the background activity increases, temporal summation will be limited to ensure typical single action potential responses and entrainment to high stimulus frequencies. Increasing synaptic activity will initiate NO signalling in HCN2-dominated MNTB neurons leading to enhanced $I_{H}$ currents at resting membrane voltages (see Fig. 6). The $I_{H}$-induced depolarization will recruit $I_{KL}$ at rest so that together the two conductances will decrease the duration of synaptic potentials and guarantee that only large, fast EPSPs will evoke action potentials. NO signalling also shifts the set of potassium conductances available to MNTB neurons from domination by Kv3- to Kv2-mediated (Steinert et al. 2011). Kv2.2 currents activate at lower voltages and together with the increased $I_{H}$ ensure faithful entrainment to high stimulus frequencies, especially during periods of high acoustic input (Tong et al. 2013).

In the mouse MSO, HCN1-dominated $I_{H}$ is large, fast- activating and active at rest during low activity conditions when there is presumably little NO signalling. This situation is comparable with that in the ventral, high-frequency region of the MSO in gerbils (Baumann et al. 2013). The slower time course $I_{H}$ observed in the dorsal part of the gerbil MSO (Baumann et al. 2013) seems at least in part to be carried by HCN4 subunits (Khurana et al. 2012) but HCN4 was not detected in cell bodies of the mouse MSO (our unpublished observations). Increasing sound stimulation will trigger NO signalling in the MSO, LSO and SPN and suppress HCN1-mediated $I_{H}$, and this would decrease temporal precision (Khurana et al. 2012). On the other hand, an activity-dependent loss of HCN1 would enhance plasticity, as suggested in the hippocampus and entorhinal cortex (Nolan et al. 2004; Giocomo et al. 2011; Hussaini et al. 2011). Overall, increased neuronal firing activity augments the contribution of NO signalling, which suppresses HCN1-mediated currents and enhances HCN2-mediated currents. With HCN1 current activation shifted to the far left, a depolarizing (rightward) shift of HCN2 currents would cause greater activation at resting membrane potentials and shift overall $I_{H}$ kinetics from fast to slow. In contrast, reduced acoustic input would cause a rapid decline of NO signalling (Steinert et al. 2011), corroborating findings that long-term acoustic deprivation (and hence minimal NO signalling) caused an enhancement of HCN1-dominated currents and suppressed HCN2 currents (Hassfurth et al. 2009).

We conclude that NO modulation in the auditory brainstem suppresses the contribution from HCN1-dominated...
channels and enhances that from HCN2-containing channels. This favours activation of \( I_{H1} \) currents with slow kinetics and a more negative activation voltage. The differential expression of HCN1 (MSO, SPN, LSO) and HCN2 (MNTB, SPN) between the nuclei of the SOC allows for a spectrum of \( I_{H1} \) kinetics (fast to slow) that on activation of NO signalling become dominated by slow kinetics; this phenomenon may underlie activity-dependent control of \( I_{H1} \) signalling in many areas of the brain.

**References**

Aponte Y, Lien CC, Reisinger E & Jonas P (2006). Hyperpolarization-activated cation channels in fast-spiking interneurons of rat hippocampus. *J Physiol* **574**, 229–243.

Banks MI, Pearce RA & Smith PH (1993). Hyperpolarization-activated cation current (\( I_{H1} \)) in neurons of the medial nucleus of the trapezoid body: voltage-clamp analysis and enhancement by norepinephrine and cAMP suggest a modulatory mechanism in the auditory brainstem. *J Neurophysiol* **70**, 1420–1432.

Barnes-Davies M, Barker MC, Osmani F & Forsythe ID (2004). Kv1 currents mediate a gradient of principal neuron excitability across the tonotopic axis in the rat lateral superior olive. *Eur J Neurosci* **19**, 325–333.

Baumann VJ, Lehnert S, Leibold C & Koch U (2013). Tonotopic organization of the hyperpolarization-activated current (\( I_{H1} \)) in the mammalian medial superior olive. *Front Neural Circuits* **7**, 117.

Biel M, Wahl-Schott C, Michalakis S & Zong X (2009). Hyperpolarization-activated cation channels: from genes to function. *Physiol Rev* **89**, 847–885.

Brenman JE, Chao DS, Gee SH, McGee AW, Craven SE, Santillano DR, Wu Z, Huang F, Xia H, Peters MF, Frohener SC & Bredt DS (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and \( \alpha 1 \)-syntrophin mediated by PDZ domains. *Cell* **84**, 757–767.

Brew HM & Forsythe ID (1995). Two voltage-dependent K\(^+\) conductances with complementary functions in postsynaptic integration at a central auditory synapse. *J Neurosci* **15**, 8011–8022.

Cao XJ & Oertel D (2011). The magnitudes of hyperpolarization-activated and low-voltage-activated potassium currents co-vari in neurons of the ventral cochlear nucleus. *J Neurophysiol* **106**, 630–640.

Chen X, Shu S & Bayliss DA (2005a). Suppression of \( I_{H1} \) contributes to propofol-induced inhibition of mouse cortical pyramidal neurons. *J Neurophysiol* **94**, 3872–3883.

Chen X, Shu S, Kennedy DP, Willcox SC & Bayliss DA (2009). Subunit-specific effects of isoflurane on neuronal \( I_{H1} \) in HCN1 knockout mice. *J Neurophysiol* **101**, 129–140.

Chen X, Siros JE, Lei Q, Talley EM, Lynch C, 3rd & Bayliss DA (2005b). HCN subunit-specific and cAMP-modulated effects of anesthetics on neuronal pacemaker currents. *J Neurosci* **25**, 5803–5814.

Cuttle MF, Rusznak Z, Wong AY, Owens S & Forsythe ID (2001). Modulation of a presynaptic hyperpolarization-activated cationic current (\( I_{H1} \)) at an excitatory synaptic terminal in the rat auditory brainstem. *J Physiol* **534**, 733–744.

Felix RA, 2nd, Fridberger A, Leijon S, Berrebi AS & Magnusson AK (2011). Sound rhythms are encoded by postinhibitory rebound spiking in the superior paraolivary nucleus. *J Neurosci* **31**, 12566–12578.

Garthwaite G, Bartus K, Malcolm D, Goodwin D, Kollb-Sielecka M, Doolendiya C & Garthwaite J (2006). Signaling from blood vessels to CNS axons through nitric oxide. *J Neurosci* **26**, 7730–7740.

Garthwaite J (2008). Concepts of neural nitric oxide-mediated transmission. *Eur J Neurosci* **27**, 2783–2802.

Gasparini S & DiFrancesco D (1997). Action of the hyperpolarization-activated current (\( I_{H1} \)) blocker ZD 7288 in hippocampal CA1 neurons. *Pflugers Arch* **435**, 99–106.

Giocomo LM, Hussaini SA, Zheng F, Kandel ER, Moser MB & Moser EI (2011). Grid cells use HCN1 channels for spatial scaling. *Cell* **147**, 1159–1170.

Golding NL, Ferragamo MF & Oertel D (1999). Role of intrinsic conductances underlying responses to transients in octopus cells of the cochlear nucleus. *J Neurosci* **19**, 2897–2905.

Golding NL & Oertel D (2012). Synaptic integration in dendrites: exceptional need for speed. *J Physiol* **590**, 5563–5569.

Gopalakrishna R, Chen ZH & Gundimeda U (1993). Nitric oxide and nitric oxide-generating agents induce a reversible inactivation of protein kinase C activity and phorbol ester binding. *J Biol Chem* **268**, 27180–27185.

Gyurko R, Leupen S & Huang PL (2002). Deletion of exon 6 of the neuronal nitric oxide synthase gene in mice results in hypogonadism and infertility. *Endocrinology* **143**, 2767–2774.

Hardingham N, Dachtler J & Fox K (2013). The role of nitric oxide in pre-synaptic plasticity and homeostasis. *Front Cell Neurosci* **7**, 190.

Hassfurth B, Magnusson AK, Grothe B & Koch U (2009). Sensory deprivation regulates the development of the hyperpolarization-activated current in auditory brainstem neurons. *Eur J Neurosci* **30**, 1227–1238.

He C, Chen F, Li B & Hu Z (2014). Neurophysiology of HCN channels: from cellular functions to multiple regulations. *Prog Neurobiol* **112**, 1–23.

Hu L, Santoro B, Saponaro A, Liu H, Moroni A & Siegelbaum S (2013). Binding of the auxiliary subunit TRIP8b to HCN channels shifts the mode of action of cAMP. *J Gen Physiol* **142**, 599–612.

Huang H & Trussell LO (2014). Presynaptic HCN channels regulate vesicular glutamate transport. *Neuron* **84**, 340–346.

Huang Z, Lujan R, Martinez-Hernandez J, Lewis AS, Chetkovich DM & Shah MM (2012). TRIP8b-independent trafficking and plasticity of adult cortical presynaptic HCN1 channels. *J Neurosci* **32**, 14835–14848.

Hussaini SA, Kempadoo KA, Thauault SJ, Siegelbaum SA & Kandel ER (2011). Increased size and stability of CA1 and CA3 place fields in HCN1 knockout mice. *Neuron* **72**, 643–653.
Ingram SL & Williams JT (1996). Modulation of the hyperpolarization-activated current (I_{h}) by cyclic nucleotides in guinea-pig primary afferent neurons. J Physiol 492, 97–106.

Johnston J, Griffin SJ, Baker C, Skrzypiec A, Chernova T & Forsythe ID (2008). Initial segment Kv2.2 channels mediate a slow delayed rectifier and maintain high frequency action potential firing in medial nucleus of the trapezoid body neurons. J Physiol 586, 3493–3509.

Khurana S, Liu Z, Lewis AS, Rosa K, Chetkovich D & Golding NL (2012). An essential role for modulation of hyperpolarization-activated current in the development of binaural temporal precision. J Neurosci 32, 2814–2823.

Khurana S, Remme MW, Rinzel J & Golding NL (2011). Dynamic interaction of I_{h} and I_K,CA2 during trains of synaptic potentials in principal neurons of the medial superior olive. J Neurosci 31, 8936–8947.

Koch U, Braun M, Kapfer C & Grothe B (2004). Distribution of HCN1 and HCN2 in rat auditory brainstem nuclei. Eur J Neurosci 20, 79–91.

Koch U & Grothe B (2003). Hyperpolarization-activated current (I_{h}) in the inferior colliculus: distribution and contribution to temporal processing. J Neurophysiol 90, 3679–3687.

Kopp-Scheinpflug C, Tozer AJ, Robinson SW, Tempel BL, Maccaferri G & McBain CJ (1996). The hyperpolarization-activated cation current of principal neurons of the medial superior olive. J Neurosci 16, 1151-1158.

Koelle J, Wong A, Nettleton J & Mertens J (2001). Immunohistochemical localization of I_{h} channel subunits, HCN1–4, in the rat brain. J Comp Neurol 437, 241–276.

Pape HC & Mager R (1992). Nitric oxide controls oscillatory activity in thalamocortical neurons. Neuron 9, 441–448.

Pape HC & McCormick DA (1989). Noradrenaline and serotonin selectively modulate thalamic burst firing by enhancing a hyperpolarization-activated cation current. Nature 340, 715–718.

Poolos NP (2010). Genetic loss of HCN1 channels is exciting, but is it epileptic? Epilepsy Curr 10, 49–51.

Reuss S, Schaeffer DF, Laages MH & Riemann R (2000). Evidence for increased nitric oxide production in the auditory brainstem of the aged dwarf hamster (Phodopus sungorus): an NADPH-diaphorase histochemical study. Mech Ageing Dev 112, 125–134.

Rinaldi A, Deferali C, Mialot A, Garden DL, Beraneck M & Nolan MF (2013). HCN1 channels in cerebellar Purkinje cells promote late stages of learning and constrain synaptic inhibition. J Physiol 591, 5691–5709.

Robinson RB & Siegelbaum SA (2003). Hyperpolarization-activated cation currents: from molecules to physiological function. Ann Rev Physiol 65, 453–480.

Roy B, Halvey EJ & Garthwaite J (2008). An enzyme-linked receptor mechanism for nitric oxide-activated guanylyl cyclase. J Biol Chem 283, 18841–18851.

Santoro B, Chen S, Luthi A, Pavlidis P, Shumyatsky GP, Tibbs GR & Siegelbaum SA (2000). Molecular and functional heterogeneity of hyperpolarization-activated pacemaker channels in the mouse CNS. J Neurosci 20, 5264–5275.

Santoro B, Liu JT, Yao H, Bartsch D, Kandel ER, Siegelbaum SA & Tibbs GR (1998). Identification of a gene encoding a hyperpolarization-activated pacemaker channel of brain. Cell 93, 717–729.

Santoro B, Piskorowski RA, Pian P, Hu L, Liu H & Siegelbaum SA (2009). TRIP8b splice variants form a family of auxiliary subunits that regulate gating and trafficking of HCN channels in the brain. Neuron 62, 802–813.

Scott LL, Mathews PJ & Golding NL (2005). Post-hearing developmental refinement of temporal processing in principal neurons of the medial superior olive. J Neurosci 25, 7887–7895.

Shah MM (2014). Cortical HCN channels: function, trafficking and plasticity. J Physiol 592, 2711–2719.
Shaikh AG & Finlayson PG (2003). Hyperpolarization-activated ($I_{h}$) conductances affect brainstem auditory neuron excitability. *Hearing Res* **183**, 126–136.

Steinert JR, Kopp-Scheinflug C, Baker C, Challiss RA, Mistry R, Haustein MD, Griffin SJ, Tong H, Graham BP & Forsythe ID (2008). Nitric oxide is a volume transmitter regulating postsynaptic excitability at a glutamatergic synapse. *Neuron* **60**, 642–656.

Steinert JR, Robinson SW, Tong H, Haustein MD, Kopp-Scheinflug C & Forsythe ID (2011). Nitric oxide is an activity-dependent regulator of target neuron intrinsic excitability. *Neuron* **71**, 291–305.

Tong H, Kopp-Scheinflug C, Pilati N, Robinson SW, Sinclair JL, Steinert JR, Barnes-Davies M, Allfree R, Grubb BD, Young SM, Jr & Forsythe ID (2013). Protection from noise-induced hearing loss by Kv2.2 potassium currents in the central medial olivocochlear system. *J Neurosci* **33**, 9113–9121.

Wahl-Schott C & Biel M (2009). HCN channels: structure, cellular regulation and physiological function. *Cell Mol Life Sci* **66**, 470–494.

Wenker IC, Benoit JP, Chen X, Liu H, Horner RL & Mulkey DK (2012). Nitric oxide activates hypoglossal motoneurons by cGMP-dependent inhibition of TASK channels and cGMP-independent activation of HCN channels. *J Neurophysiol* **107**, 1489–1499.

Wilson GW & Garthwaite J (2010). Hyperpolarization-activated ion channels as targets for nitric oxide signalling in deep cerebellar nuclei. *Eur J Neurosci* **31**, 1935–1945.

Yassin L, Radtke-Schuller S, Asraf H, Grothe B, Hershfinkel M, Forsythe ID & Kopp-Scheinflug C (2014). Nitric oxide signalling modulates synaptic inhibition in the superior paraolivary nucleus (SPN) via cGMP-dependent suppression of KCC2. *Front Neural Circuits* **8**, 65.

---

**Additional Information**

**Competing interests**

None of the authors has any conflicts of interest, beyond the acknowledgment that I.D.F. is a Senior Editor for *The Journal of Physiology*.

**Author contributions**

C.K.S. conducted electrophysiological experiments and analysed data. B.M.P. performed immunohistochemistry. C.K.S. and I.D.F. jointly conceived the project and wrote the manuscript.

**Funding**

This work was funded by the Medical Research Council (MRC, MR/K005170/1) and Deutsche Forschungsgemeinschaft (DFG, KO2207/3-1).

**Acknowledgements**

We are grateful to Matt Nolan (Edinburgh University, UK) for providing the HCN1-KO mice and to Olga Alexandrowa (Ludwig-Maximilians-University Munich, Germany) for sharing her expertise with cell reconstruction.