Title: Neuromedin U depolarizes rat hypothalamic paraventricular nucleus neurons in vitro by enhancing I\textsubscript{H} channel activity

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Abbreviated title: Qiu et al. \textbullet NMU enhancing I\textsubscript{H} and exciting PVN neurons

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ABSTRACT:

The effect of neuromedin U (NMU) on rat paraventricular nucleus (PVN) neurons was examined using whole-cell patch-clamp recordings. Under current-clamp, 31% of PVN pavocellular neurons (n = 243) were depolarized by 100 nM NMU, but magnocellular neurons were not affected. NMU (10 nM to 1 µM) resulted in increased basal firing rate and depolarization in a dose-dependent manner with an EC$_{50}$ of 70 nM. NMU-induced depolarization was unaffected by co-perfusion with 0.5 µM tetrodotoxin (TTX) + 10 µM CNQX + 10 µM bicuculline. Extracellular application of 70 µM ZD 7288 completely inhibited NMU-induced depolarization. Under voltage-clamp, 1 µM NMU produced negligible inward current but did increase the hyperpolarization-activated current (I$_{H}$) at step potentials less than –80 mV. The effects of NMU on I$_{H}$ were voltage-dependent, and NMU shifted the I$_{H}$ conductance-voltage relationship (V$_{1/2}$) by about 10.8 mV and enhanced I$_{H}$ kinetics without changing the slope constant (k). Extracellular application of 70 µM ZD 7288 or 3 mM Cs$^+$ blocked I$_{H}$ and the effects of NMU in voltage-clamp. These results suggest that NMU selectively depolarizes the subpopulation of PVN pavocellular neurons via enhancement of the hyperpolarization-activated inward current.
INTRODUCTION

Neuromedin U (NMU) is a neuropeptide that is present in the gut and central nervous system (Minamino et al. 1985; Ballesta 1988) and has potent effects on smooth muscle. The two receptors for NMU, NMU-R1 and NMU-R2, are G-protein-coupled receptors (GPCRs) (Howard et al. 2000). NMU-R2 is expressed in the paraventricular nucleus (PVN) of the rat hypothalamus (Raddatz et al. 2000). NMU induces extracellular acidification, arachidonic acid metabolite release and intracellular Ca\(^{2+}\) mobilization in cells expressing NMU-R1 or NMU-R2 (Howard et al. 2000; Shan et al. 2000). Recently, we found that intracerebroventricular (i.c.v.) administration of NMU can provoke an increase in mean arterial blood pressure (MABP), heart rate (HR) and plasma norepinephrine. This suggests that NMU regulates sympathetic nervous system activity and affects cardiovascular function (Chu et al. 2002).

Conductance of K\(^+\) and Na\(^+\) by I\(_{\text{h}}\) channels is activated by membrane hyperpolarization, gated by cyclic nucleotides (cAMP, cGMP) and is blocked by extracellular Cs\(^+\) and ZD 7288, a selective blocker of I\(_{\text{h}}\) (Harris and Constanti 1995; Ludwig et al. 1998; Santoro et al. 1998; Ghamari-Langroudi and Bourque 2000; Moosmang et al. 2001). I\(_{\text{h}}\) mediates inward rectification of the membrane in response to voltage changes. It has also been functionally implicated in maintaining resting membrane potential (RMP), thereby providing an excitatory drive that contributes to phasic and tonic firing (Doan and Kunze 1999; Ghamari-Langroudi and Bourque 2000). Four different isoforms of the I\(_{\text{h}}\) channel have been cloned, and two different isoforms (HCN1 and HCN3) are highly expressed in rat PVN (Monteggia et al. 2000).

Because I\(_{\text{h}}\) channels and NMU receptors are both present in rat PVN, the goal of the present
Study was to determine the effect of NMU activation on I_h currents and PVN neuron activity. We used a whole-cell patch-clamp method to examine the effects of NMU on rat PVN neurons in vitro.

MATERIALS AND METHODS

Hypothalamic slice preparation. Hypothalamic slices were prepared from P12-14 day old male Wistar rats, as previously described (Shirasaka et al. 2001). All experiments were approved by the Ethics Committee of the Miyazaki Medical College and were in accordance with international guidelines on the ethical use of animals in laboratory experiments. Briefly, the brain was quickly removed and placed into ice-cold artificial cerebrospinal fluid (ACSF) consisting of (in mM) 140 NaCl, 3 KCl, 1.3 MgSO_4, 1.4 NaH_2PO_4, 11 D-glucose, 5 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 2.4 CaCl_2 and 3.25 NaOH. The pH was 7.3, the osmolarity was 290-300 mOsm, and the fluid was bubbled with 100% O_2. Coronal slices were 250 µm in thickness, including PVN, and were prepared using a vibrating brain slicer (DSK-2000; Dosaka, Kyoto, Japan). The slices were incubated for at least 1 h in a chamber filled with equilibrated ACSF at room temperature (24-26°C) before recordings were started.

Electrophysiology. Patch pipettes were made with a puller (PB-7; Narishige, Tokyo, Japan) from thick-wall borosilicate glass (GD-1.5; Narishige). They were filled with a solution consisting of (in mM) 130 potassium gluconate, 10 HEPES, 10 KCl, 1 CaCl_2, 5 EGTA, 1 MgCl_2, 2 Na_2ATP and 0.5 Na_3GTP. The pH was adjusted to 7.2 with KOH. Patch pipette resistances were 5-7 MΩ in the bath, with series resistances in the range of 10-20 MΩ, compensated by 80%. The liquid junction potential (10 mV) was corrected for according to the method described by Neher (1992). Membrane potentials and/or currents were monitored with an Axopatch 200B amplifier (Axon Instruments,
Foster City, CA, USA), acquired through a Digidata 1200 series analog-to-digital interface on a personal computer using Clampex 7.0 software (Axon Instruments). Selected traces were saved to the hard drive of the computer, and all data were saved to a 4.7 GB DVD-RAM.

The membrane potential and current were low-pass-filtered at 1-5 KHz. Whole-cell recordings were made from microscopically identified cells. Once stable recording conditions were obtained, a PVN neuron was identified electrophysiologically as type I (magnocellular) or type II (parvocellular) according to previously established criteria by current-clamp in standard ACSF (Luther and Task, 2000). Type I neurons displayed transient outward rectification, while Type II did not. In voltage-clamp, tetrodotoxin (TTX) was routinely included in external recording solutions to block voltage-gated Na⁺ channels.

**Chemicals.** Reagents included rat NMU-23 (Peptide Institute, Inc. Japan), ZD 7288 (Tocris Cookson, Inc. Ballwin, MO, USA), CsCl (Sigma) and tetrodotoxin (TTX, Sigma). ZD 7288 was prepared as a 50 mM stock solution (in H₂O) and stored at −20°C until use. The other drugs were dissolved in ACSF.

**Data analysis.** Data were analyzed using Clampfit 8.0 (Axon Instruments) and are expressed as mean ± SEM. Iᵢₒ was determined by subtracting Iᵢᵢᵢ from Iᵢss at each hyperpolarizing voltage step using the following equation:

\[ I_{i0} = I_{ss} - I_{ins} \]  

And Iᵢₒ conductance (Gᵢₒ) was estimated as the amplitude of Iᵢₒ measured at various potentials (V) divided by the driving force (V − Eᵢₒ), where Eᵢₒ is the reversal potential of Iᵢₒ (Ghamari-Langroudi and Bourque 2000) as follows:
\[ G_{II} = I_{II}/(V - E_{II}) \] (2)

Differences between mean values recorded under control and test conditions were evaluated using Student’s t-test or one-way ANOVA with Tukey’s post-hoc test. Differences were considered significant at \( P < 0.05 \).

RESULTS

Neuronal membrane properties

A total of 309 PVN neurons (66 type I, 243 type II) were sampled under whole-cell current-clamp conditions. While none of the type I neurons showed a response to NMU, 76 of the type II neurons (31%) were sensitive to NMU. These neurons expressed a depolarizing response approximately 1 minute after application of 100 nM NMU; specifically, they displayed time-dependent inward rectification during the hyperpolarizing pulses (Fig. 1C and D) that was blocked by 70 µM ZD 7288 (Fig. 1B) or 3 mM Cs\(^+\) (not shown). These characteristics are consistent with \( I_{II} \) conductance (Ludwig et al. 1998; Santoro et al. 1998). Further, the responsive neurons exhibited a lack of transient outward rectification in response to a series of depolarizing current pulses delivered at a hyperpolarized membrane potential (Fig. 1A) (Luther et al. 2000).

Effects of NMU on membrane potential

Applications of NMU in concentrations ranging from 10 nM to 1 µM NMU resulted in depolarization and increased firing rate in a concentration-dependent manner in MNU-sensitive PVN neurons when the holding potentials were –60 mV (Fig 2), however, the characteristics of the action potentials were not changed (not shown). The depolarization response appeared approximately 40 s after NMU exposure and peaked at approximately 100 s, with a maximal depolarization range of
1.75 ± 0.47 mV to 7.18 ± 1.21 mV (Fig. 2C). The minimum NMU dose required to elicit an effect on membrane potential was 1 nM, and the maximum dose was approximately 1 µM. The EC$_{50}$ was approximately 70 nM. This response was unaffected by the presence of 0.5 µM TTX + 10 µM CNQX + 10 µM bicuculline (7.18 ± 1.21 mV in the presence of 1 µM NMU, 6.79 ± 1.13 mV in the presence of TTX, CNQX and bicuculline following the application of 1 µM NMU; mean ± SEM, P > 0.05, n = 5).

**Effects of NMU on membrane current**

Application of 1 µM NMU to NMU-sensitive neurons with voltage-clamp at −60 mV produced a negligible inward current (7.5 ± 2.1 pA, n = 16). However, when neurons were held at −60 mV and a series of 1s hyperpolarizing voltage steps from −60 mV to −150 mV, NMU induced a significant increment in steady-state current (I$_{SS}$) at step potentials less than −80 mV (Fig. 3A and B, P < 0.05, n = 7) and instantaneous current (I$_{Ins}$) at step potentials less than −100 mV (Fig. 3A and C, P < 0.05, n = 7). NMU-induced increases in I$_{Ins}$ was not affected by Ba$^{2+}$ (Fig. 4A), but was blocked by ZD 7288 (Fig. 5C) or Cs$^+$ (not shown). Simultaneously, the net reversal potential of the NMU-sensitive component of the fast current (determined in the presence of 100 µM Ba$^{2+}$) was compared with the reversal potential for I$_H$ (also determined in the presence of 100 µM Ba$^{2+}$) (Cardenas et al. 1999).

As illustrated in Fig. 4A and B, the reversal potential was −34.0 ± 2.6 mV (n = 4) under control and NMU conditions. This was similar to the reversal potential of −33.5 ± 2.1 mV (n = 4) obtained with another group of NMU-sensitive neurons for the portion of I$_{Ins}$ which was increased by changing the holding potential from −60 to −80 mV in the presence of Ba$^{2+}$ (Fig. 4C and D). Thus, the increase in I$_{Ins}$ produced by the shift in holding potential likely reflects an increase in tonically open I$_H$ channels.
Blockade of the effects of NMU by ZD 7288 or Cs⁺

In current clamp, NMU-induced depolarization was not affected by TTX (Fig. 5A). However, application of ZD 7288 induced slight hyperpolarization in NMU-sensitive PVN neurons and abolished NMU-induced depolarization (Fig. 5B). NMU increased I_{SS} and I_{Ins} according to hyperpolarizing pulses. To determine whether NMU-induced increase in I_{SS} and I_{Ins} represented enhanced I_H channels, ZD 7288 and Cs⁺ (not shown) were used as I_H channel blockers (Harris and Constanti 1995; McCormick and Pape 1990; Maccaferri et al. 1996; Pape, 1996). ZD 7288 significantly blocked I_{Ins} and NMU-induced increments of I_{Ins} at step potentials less than –80 mV. The I-V relationships were linear in the presence of ZD 7288 (Fig. 5C and D). Further, ZD 7288 blocked I_{SS} and NMU-induced increments of I_{SS} at step potentials less than –80 mV. The I-V relationships were also linear in the presence of ZD 7288 (Fig. 5C and E). This finding indicates that NMU-sensitive neurons have I_H channels, which produce hyperpolarization-activated ZD 7288-sensitive inward currents (I_H) and are activated at RMP, thus producing ZD 7288-sensitive I_{Ins}.

Effects of NMU on I_H

In this study, 1 µM NMU significantly enhanced I_H activity at step potentials less than –80 mV, and the maximal effects were at step potentials of –100 to –120 mV (Fig. 3D). Furthermore, we estimated the effect of NMU on I_H conductance (G_H) (see methods), E_H was obtained in the NMU-sensitive neurons as shown in Fig. 6A: Following a step to –120 mV (1 s duration), the membrane voltage was stepped in the range of –110 to –50 mV (1 s duration, 10 mV increments) (Maccaferri et al. 1996). The plot of the instantaneous current at each test potential yielded the fully
activated I–V relationship, which was linear (Fig. 6B). The extrapolated reversal potential ($E_H$) was $-33.1 \pm 1.8$ mV (Fig. 6B, $n = 5$), similar to that previously reported for $E_H$ in other nervous preparations (McCormick and Pape 1990; Maccaf erri et al. 1996; Pape 1996). The mean $G_H - V$ relations are shown in Fig. 6C. Note that NMU enhanced $I_H$ conductance at step potentials more negative than $-80$ mV ($P < 0.05$, $n = 7$). Furthermore, the modified Bolzmann equation was used as follows:

$$G_{H(V)} = \frac{1}{1 + e^{(V - V_{1/2})/k}},$$

where $G_{H(V)}$ is the fraction of maximal $G_H$ observed at $V$, $k$ is the slope factor, and $V_{1/2}$ is the half-maximal voltage. The mean values were as follows: $V_{1/2} = -110.2 \pm 2.3$ mV, $k = 13.1 \pm 2.0$ in control and $V_{1/2} = -99.5 \pm 3.4$ mV, $k = 11.5 \pm 1.9$ during the application of NMU. These data reveal that NMU produced a significant shift in $V_{1/2}$ to a more depolarized potential (Fig. 6C insert, $P < 0.05$). The slope factor values were not altered by NMU ($P > 0.05$).

**Effects of NMU on the kinetics of $I_H$ activation**

The time course of activation of $I_H$ was obtained from an analysis of the rising phase of the NMU-induced $I_H$ current evoked by hyperpolarizing steps to various voltages. As shown in Fig. 6D, the $I_H$ current traces were fit to a single exponential function of the form $A_t = A_\infty (1 - e^{-t/\tau})$, where $A_t$ is the amplitude of $I_H$ at time $t$, $A$ is the amplitude of $I_H$ at a steady state and $\tau$ is the activation time constant (Ghamari-Langroudi and Bourque 2000). Figure 6D reveals the plots of the means ± SEM ($n = 7$) against voltage steps. The $\tau$ of NMU-sensitive neurons decreased from $800$ ms at $-70$ mV to $100$ ms at $-140$ mV and exhibited fast kinetics. NMU enhanced $I_H$ channels kinetics exhibiting decrements of $\tau$ at step potentials more negative than approximately $-80$ mV ($P < 0.05$ vs. ACSF, $n$
DISCUSSION

This study demonstrated that NMU enhanced $I_{H\text{1}}$ channels activity leading to excitatory responses in a subpopulation of PVN type II neurons.

**Expression of $I_{H\text{1}}$ in PVN NMU-sensitive neurons**

In the present study, NMU-sensitive PVN neurons displayed a time-dependent strong inward rectification during hyperpolarizing pulses (Fig. 1C and D) that was blocked by 70 µM ZD 7288 (Fig. 1B). Further these neurons did not display transient outward rectification. These properties are consistent with $I_{H\text{1}}$ conductance produced by $I_{H\text{1}}$ channels (Ludwig et al. 1998; Santoro et al. 1998, Tasker 1991; Luther et al. 2000; Stern 2001)

**NMU excited PVN NMU-sensitive neurons by enhanced $I_{H\text{1}}$**

NMU evoked small depolarization and increased neuronal excitability. Several previous studies have demonstrated that enhancement of $I_{H\text{1}}$ results in increased neuronal excitability and responsiveness to excitatory input. This occurs via release of neurons from tonic hyperpolarizing synaptic input and via facilitation of action potential triggering by depolarizing input or counter-balancing after-hyperpolarizations following action potentials (Pape and McCormick 1989; Pape 1996; Yagi et al. 1998). The presence of $I_{H\text{1}}$ in magnocellular neurosecretory cells of rat supraoptic nucleus provides an excitatory drive that contributes to phasic and tonic firing (Ghamari-Langroudi and Bourque 2000). $I_{H\text{1}}$ plays a significant role in setting both the RMP and the baseline level of excitability of hippocampal GABAergic interneurons found in the stratum oriens of area CA1 (Lupica et al. 2001).
Several findings in the current study suggest that NMU excites PVN neurons via enhanced $I_{\text{H}}$ channel activity. First, the RMP of NMU-sensitive neurons was approximately $-58 \, \text{mV}$ ($V_h = -60 \, \text{mV}$), and the $E_{\text{H}}$ was approximately $-33 \, \text{mV}$ (Fig. 6B), suggesting that $I_{\text{H}}$ channels are partially active at RMP (Yagi et al. 1998). NMU enhanced the activity of $I_{\text{H}}$ channels at RMP, induced NMU-sensitive neurons depolarizing in current-clamp (Fig. 2), and evoked increments of $I_{\text{ins}}$ by hyperpolarizing steps (Fig. 3C). NMU-induced increment in $I_{\text{ins}}$ was almost completely blocked by ZD 7288 (Fig. 5C), but not by Ba$^{2+}$ (Fig. 4A and B). Thus, this likely reflects an increase in tonically activated $I_{\text{H}}$ (Mayer and Westbrook 1983). Second, in voltage clamp, NMU resulted in an increase of $I_{\text{H}}$ current and enhanced channel kinetics at step potentials less than $-80 \, \text{mV}$. NMU also produced a significant shift in $V_{1/2}$ to a more depolarized potential. Third, ZD 7288 completely blocked NMU-induced depolarization (Fig. 5B) and abolished the effects of NMU on the neurons in voltage clamp (Fig 5C, D and E). Collectively, these data suggest that NMU enhanced $I_{\text{H}}$, resulting in a shift in the membrane potential toward more depolarized levels and firing action potential.

In this study, NMU resulted in depolarization of membrane potential in a dose-dependent manner with an EC$_{50}$ of 70 nM. However, NMU did not induce large depolarization, likely because $I_{\text{H}}$ maintains the membrane potential of neurons within the range necessary for the generation of tonic action potential firing (Ghamari-Langroudi and Bourque 2000; Williams et al. 2002). Recently, it was reported that i.c.v. administration of NMU induced c-fos expression in magnocellular cells (type I) and parvocellular cells (type II) (Ozaki et al. 2002). The expression of the c-fos gene in the PVN should reflect the neural activation either directly or indirectly after i.c.v. administration of NMU (Ozaki et al. 2002). NMU-R2 is expressed in the PVN of the hypothalamus, along the wall of
the third ventricle in the hypothalamus (Howard et al. 2000). In this study, NMU-induced responses in membrane potential were unaffected by TTX + CNQX + bicuculline. This evidence suggests that NMU depolarizes PVN type II neurons via a direct postsynaptic action rather than by indirect modulation of neurotransmission. On the other hand, none of the type I neurons showed a response to NMU. According our data, we suggest that the expression of the c-fos gene in the PVN magnocellular may be mainly via an unknown indirect pathway after i.c.v. administration of NMU.

**Possible mechanism of NMU action**

NMU-R2 is expressed in the PVN of the rat hypothalamus (Howard et al. 2000), and HCN1 and HCN3 mRNA expression is highly enriched in the PVN (Monteggia et al. 2000). It is possible that PVN NMU-sensitive PVN neurons contain I\(\text{H}\) (HCNs) channels and NMU-R2. A key property of neuronal HCN channels is their regulation by neurotransmitters and hormones that act via cAMP, cGMP or intracellular \(\text{Ca}^{2+}\) (Pape 1996). The cAMP and cGMP affect HCN channels by directly interacting with the cyclic nucleotide-binding domain protein of the C-terminus (Ludwig et al. 1998). The increment of intracellular \(\text{Ca}^{2+}\) in thalamic relay cells can result in modifications of I\(\text{H}\) that are similar to those observed following increases in cAMP (Lüthi and McCormick 1998).

NMU-R1 is coupled to phospholipase C stimulation via a \(G_q\)-type G-protein, resulting in the release of the IP second messenger and increased intracellular \(\text{Ca}^{2+}\) in COS-7 cells (Raddatz et al. 2000). NMU-R2-is also coupled to the \(G_q\) family of G proteins in cells, inducing a rapid increase in intracellular \(\text{Ca}^{2+}\) (Shan et al. 2000). In thalamic neurons, transient increases in intracellular \(\text{Ca}^{2+}\) appeared to cause a reversible augmentation of I\(\text{H}\) attributable to the rapid, \(\text{Ca}^{2+}\)-dependent formation of cyclic nucleotides (Lüthi and McCormick 1998). The increment of intracellular \(\text{Ca}^{2+}\) activates
soluble guanylate cyclase, leading to increased cGMP levels (Kuzmiski and Macvicar 2001).

Thus, we propose that NMU binds to NMU-R2 resulting in increased intracellular calcium and cGMP. This leads to an increment in \( I_{H} \) current and neuronal excitation. This response may contribute to activation of autonomic centers in the brainstem and spinal cord that regulate MABP, HR and plasma norepinephrine.
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Figure 1. Electrophysiological properties of PVN NMU-sensitive neurons. A, The neuron displayed time-dependent inward rectification and lacked transient outward rectification (black arrow) in response to a series of depolarizing current pulses delivered at a hyperpolarized membrane potential. B, The neuron displayed inward rectification which was blocked by 70 µM ZD7288. Holding potential was –60 mV. C, The neuron responded to a series of hyperpolarizing current pulses, expressing inward rectification. D, Current-voltage relationships were obtained at the peak voltage (○) and at the steady-state voltage (●) during the hyperpolarizing pulses.

Figure 2. Effects of NMU on PVN NMU-sensitive neurons in current-clamp. A₁, A₂ and A₃ are the responses to 10 nM, 100 nM and 1 µM NMU, respectively (bar, $V_h = –60$ mV). B₁, B₂ and B₃ are the instantaneous spike rates of the neurons in A₁, A₂ and A₃, respectively. NMU elicited increases in firing-action potential in a dose-dependent manner. C, The mean time courses of NMU-induced depolarizations. Note that NMU induced dose-dependent depolarization. (+) $P < 0.05$ vs. ACSF, (*) $P < 0.05$ vs. 10 nM NMU, (#) $P < 0.05$ vs. 100 nM NMU. D, The concentration-response curve of NMU-induced depolarization. The EC₅₀ value obtained from the curve was ~ 70 nM. The number of neurons tested for each concentration is indicated near the bars.

Figure 3. Effects of NMU on PVN NMU-sensitive neuron in voltage-clamp. A, Current traces elicited by a series of 1s hyperpolarizing voltage steps (10 mV decrement, holding potential was –60 mV) in ACSF (control) and during the application of 1 µM NMU. B, Plots of steady-state current ($I_{ss}$) in the control (○), during the application of NMU (●) and the subtraction of NMU from control
(9) against the membrane potential (○ and ● shown in A). C, Plots of instantaneous current (I_{Ins}) in the control (□), during the application of NMU (■) and the subtraction of NMU from control (×) against the membrane potential (□ and ■ shown in A). Note that NMU increased I_{SS} and I_{Ins}. D, Plots of the difference between I_{SS} and I_{Ins} (I_{SS} – I_{Ins} = I_{H}) in the control (Δ), during the application of NMU (▲) against the membrane potential. Note that NMU increased I_{H} at step potentials less than –80 mV. All data are mean ± SEM; n = 7, * P < 0.05 vs. ACSF.

Figure 4. Determination of reversal potentials for the fast current increased by NMU or hyperpolarization in the presence of 100 µM Ba^{2+}. A, Current traces elicited by a series of 100 ms hyperpolarizing voltage steps (10 mV decrement, holding potential was –60 mV) in the presence of 100 µM Ba^{2+} (A₁) and in the presence of 100 µM Ba^{2+} + 1 µM NMU (A₂). B, Plots of fast current amplitude versus command voltage for the currents shown in A₁ and A₂, 100 µM Ba^{2+}; 100 µM Ba^{2+} + 1 µM NMU. Straight lines were fitted to the data points using best fit values for slope and intercept determined by linear regression. The theoretical reversal potential for NMU-increased fast current, indicated by the intersection of 2 lines, was extrapolated to –36 mV. C, Families of currents evoked in the same cell as depicted in A, using 750 ms hyperpolarizing commands ranging from –70 to –120 mV from a V_h of –60 mV (C₁) and –80 mV (C₂), in the presence of 100 µM Ba^{2+}. D, Plots of fast current amplitude vs. command voltage for the current shown in C₁ and C₂, 100 µM Ba^{2+}, V_h = –60 mV; 100 µM Ba^{2+}, V_h = –80 mV. Straight lines were fitted to the data points for V_h values as described for B. The theoretical reversal potential for the hyperpolarization increased fast current was –34 mV, as estimated from straight lines fitted to the data.
Figure 5. Blockade of the effects of NMU by ZD 7288. A, 1 µM NMU (bar) provoked a reversible membrane depolarization accompanied by an increase in firing rate, and the NMU-induced membrane depolarization was unaffected by pre-perfusion with 0.5 µM TTX. B, 0.5 µM TTX, 70 µM ZD 7288 blocked NMU-induced depolarization. C, Current traces elicited by 1s –120 mV hyperpolarizing voltage steps ($V_h = -60$ mV) under ACSF, NMU, ZD 7288, ZD 7288 + NMU and recovery. ACSF, NMU and recovery are shown in C1; ACSF, ZD 7288 and ZD 7288 + NMU are shown in C2. D, Plots of $I_{ins}$ (white arrow shown in C) vs. the command voltage for control (○), 1 µM NMU (■), 70 µM ZD 7288 (△) and 70 µM ZD 7288 + 1 µM NMU (▲). E, Plots of $I_{ss}$ (black arrow shown in C) vs. the command voltage for control (○), 1 µM NMU (●), 70 µM ZD 7288 and 70 µM ZD 7288 + 1 µM NMU (▼). Note that 70 µM ZD 7288 abolished the effects of NMU. All data are mean ± SEM, n = 6.

Figure 6. Effects of NMU on $I_H$. A, The reversal potential of $I_H$ was determined by clamping the NMU-sensitive neuron to –120 mV for 1s and depolarizing in 10 mV at 1 s increments to –50 mV. B, Mean (n = 6) instantaneous currents of $I_H$ (● showed in A) were plotted with respect to membrane potential, and a linear regression was performed. The reversal potential of $I_H$ ($E_H$) was about –33 mV. C, The current ($I_H$) data shown in Fig. 3D were converted into conductance ($G_H$) using the equation $G_H = I_H/(V + 33)$ (V is the test voltage). The solid lines are the best fit through the data points using the Boltzmann equation (ACSF ○; 1 µM NMU ●; n = 7). The mean values were as follows: $V_{1/2} = -110.2 ± 2.3$ mV, k = 13.1 ± 1.9 in the control and $V_{1/2} = -99.5 ± 3.4$ mV, k = 11.5 ± 1.8 during the application of NMU. The insert is bar-graph summarizing effect of 1 µM NMU on half-maximal
voltage \( V_{1/2} \), note that 1 µM NMU significantly reduced \( V_{1/2} \), and elicited a positive shift \( V_{1/2} \) by about 10.8 mV. All data are mean ± SEM. * \( P < 0.05 \) vs. ACSF. D1, \( I_h \) traces evoked by steps to various voltages in the ACSF and during the application of 1 µM NMU. Superimposed on each trace is a monoexponential fit of the data points (a solid line extending to the right). The time constant used in the fits (\( \tau \)) is indicated beside each trace. D2, Plots of the mean ± SEM (n = 7) \( I_h \) activation time constants against voltage steps. Note that NMU enhanced \( I_h \) kinetics at step potentials less than −80 mV. * \( P < 0.05 \) vs. ACSF.