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Neuromedin U receptor-2 mRNA and HCN channels mRNA expression in NMU-sensitive neurons in rat hypothalamic paraventricular nucleus

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Abstract

We have characterized the neuromedin U (NMU)-sensitive neurons in the rat paraventricular nucleus (PVN) using whole-cell patch-clamp recordings and single-cell reverse transcription-multiplex polymerase chain reaction (single-cell RT-mPCR). Following completion of whole-cell recording, the NMU-sensitive neurons were examined for oxytocin (OT), vasopressin (VP), and corticotrophin-releasing hormone (CRH) mRNA expression using single-cell RT-mPCR. Of the NMU-sensitive neurons ($n = 23$), 82% expressed OT mRNA, 9% expressed VP mRNA, 9% did not express the detected specific phenotypes mRNA. Further, the NMU-sensitive neurons (23/23) predominantly expressed NMU-receptor 2 (NMUR-2) mRNA, co-expressed HCN1 channel mRNA, HCN2 channel mRNA, and HCN3 channel mRNA but not HCN4 channel mRNA. These results suggest that NMU modulates the function of the PVN putative parvocellular neurons and is involved in the regulation of OTergic and VPergic neurons by enhanced HCN ion channels activity via NMU-receptor 2.

Keywords: Neuromedin U; PVN; Single-cell RT-mPCR; Oxytocin; Vasopressin; Patch-clamp

Neuromedin U (NMU) is a neuropeptide that is present in the gut and central nervous system [1,10] and has potent effects on smooth muscle. The two receptors for NMU, NMU-R1, and NMU-R2, are G-protein-coupled receptors (GPCRs) [6]. NMU-R2 is confirmed predominantly to the paraventricular nucleus (PVN) of the rat hypothalamus, while low levels of NMUR-1 mRNA are present in a variety of brain tissues [5,6,15]. Recent reports have demonstrated that intracerebroventricular (i.c.v.) administration of NMU to rats enhances c-Fos expression in neurons containing these peptides [12], while plasma levels of oxytocin (OT) and vasopression (VP) are increased [13]. We previously reported that i.c.v. administration of NMU can provoke an increase in mean arterial blood pressure (MABP), heart rate (HR), and plasma norepinephrine [2]. Furthermore, NMU stimulates the release of corticotrophin-releasing hormone (CRH) from hypothalamic explants in vitro, and the administration of NMU into PVN has acute effects on stress-related behaviors in rats [18]. In addition, we have previously demonstrated that NMU selectively depolarizes the subpopulation of PVN parvocellular neurons via enhancement of the I_H current [14]; therefore, in the present study, the effects of NMU on neuronal excitability, the expression of NMU mRNA receptors, and the relationships between the NMU-sensitive neurons and the genotypes are examined through the whole-cell patch-clamp and single-cell RT-mPCR methods.

Hypothalamic slices were prepared from P12–14-day-old male Wistar rats, as previously described [14]. Harvesting of cytoplasm and reverse transcription was carried out as previously described [7]. First-strand cDNA was synthesized for 1 h at 42 °C. The first multiplex-PCR was performed as a hot start in a final volume of 30 μl containing 4.5 μl cDNA, 100 pmol of each primer, and 0.3 mM each of dNTP, 3 μl

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10× PCR buffer, and 3.5 U HotStarTaq DNA Polymerase in a Gene Amp PCR system 9700 with the following cycling protocol: (1) 15 min at 95 °C; (2) 30 cycles of 1 min at 94 °C, 1.5 min at 57 °C, and 2 min at 72 °C; (3) 10 min at 72 °C; and (4) holding at 4 °C. The nested-PCR amplifications were carried out with 2.5 μl of the first PCR product in individual reactions using the following modifications: 3.0 U HotStarTaq DNA Polymerase and 0.2 mM dNTP. The second round was as follows: (1) 15 min at 95 °C; (2) 35 cycles of 45 s at 94 °C, 1 min at 56 °C, and 1 min at 72 °C; (3) 10 min at 72 °C; and (4) holding at 4 °C. Nested primer sequences were as follows: GAPDH (accession no. NM_017008) external sense: 5′-GATGGTGAAGGTCGGTGTG, external antisense: 5′-GGGCTAAGCAGTTGGTGGT; GAPDH internal sense: 5′-TACCAGGGCTGCCTTCTCT, internal antisense: 5′-CTCGTGGTTCACACCCATC (361 bp); OT (accession no. NM_012996) external sense: 5′-ACACACCAGAAGAGGGCATC, external antisense: 5′-GTCAGAGCCAGTAGGCCAAG; OT internal sense: 5′-AGGGCCTTTGGTAGAGCAGT, internal antisense: 5′-GAGCTCAAAAGGGACACAGC (416 bp); VP (accession no. NM_016992) external sense: 5′-ACCTCTGCCTGCTACTTCCA, external antisense: 5′-AGCCAGCTGTACCAGCCTAA; VP internal sense: 5′-CCAGAACTGCCCAAGAGG, internal antisense: 5′-GCTTCCGCAAGGCTTCTG (217 bp); CRH (accession no. NM_031019) external sense 5′-AGAAGAGAGCGCCCCTAAAC, external antisense: 5′-GTTGCTGTGAGCTTGCTGAG; CRH internal sense: 5′-CAACCTCAGCCGATTCTGAT, internal antisense: 5′-CCTCAGAAGGTGGAAGGTGA (330 bp); NMUR-1 (accession no. NM_023100) external sense: 5′-GAGACTGTCTGCCTGGCTTC, external antisense: 5′-CCAATGGGACACCATACTCC; MUR-1 internal sense: 5′-TCCAAGCCAAGTCTGTGATG, internal antisense: 5′-CGTTCCCTATCTCGAAGCTG (376 bp); NMUR-2 (accession no. NM_022275) external sense: 5′-GACAGCCCTCTTCGAGACTG, external antisense: 5′-TTGCAGGTAGGGGAGACAAC; NMUR-2 internal sense: 5′-TTCGAGACTGTGTGCTTTGC, internal antisense: 5′-GTTTGGTGACTGTGCAGGTG (250 bp); HCN1 (accession no. NM_053375) external sense: 5′-CTGACATGCGCCAGAAGATA, external antisense: 5′-GATTGGAGGGATCGCTTGTA; HCN1 internal sense: 5′-CAACTTCAACTGCCGGAAAC, internal antisense: 5′-CCTTGGTCAGCAGGCATATT (254 bp); HCN2 (accession no. AF247451) external sense: 5′-TCATCGTGGAGAAGGGAATC, external antisense: 5′-GGCAGTTTGTGGAAGGACAT; HCN2 internal sense: 5′-ACTACGCATCGTGCGTTTC, internal antisense: 5′-CGTGCCCAATGAACATAGC (419 bp); HCN3 (accession no. NM_053685) external sense: 5′-TCGGACACTTTCTTCCTGCT, external antisense: 5′-TGACTCATGGCCTTGAACAG; HCN3 internal sense: 5′-TTCCTGGTGGACCTGATTTC, internal antisense: 5′-CACAGCAGCAACATCATTCC (269 bp); HCN4 (accession no. NM_021658) external sense: 5′-ATCGTGGTGGAGGACAACA, external antisense: 5′-CCGATGAACATGGCATAGC; HCN4 internal sense: 5′-GGAGACTCGCATTGACTCG, internal antisense: 5′-AGCCAGACGTCAGACATGC (405 bp). To investigate the presence and size of the amplified fragments, 10 μl aliquots of PCR products were separated by electrophoresis in an agarose gel (2%) and visualized by ethidium bromide staining. All individual PCR products were verified several times by direct sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit and the Applied Biosystems (ABI, Foster City, CA, USA) PRISM 310 Genetic Analyzer. Sequence comparison was carried out using the BLAST database. The poly(A) + RNA was prepared from the fresh hypothalamus of 13-day-old Wistar rats using the Micro-to-Midi Total RNA Purification System (Invitrogen). Reverse transcription was performed with 250 μg of the poly(A) + RNA as described above. The RNA was diluted and used as a positive +reverse transcriptase (+RT) or negative (−RT) control for the PCRs

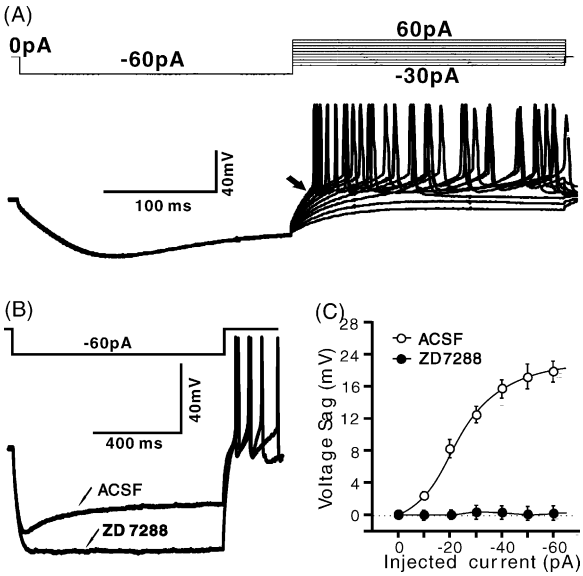

Fig. 1. Electrophysiological properties of PVN NMU-sensitive neurons. (A) The NMU-sensitive neuron displayed time-dependent inward rectification and lacked transient outward rectification (black arrow) in response to a series of depolarizing current pulses delivered at a hyperpolarized membrane potential. (B) The neuron displayed inward rectification (sag), which was blocked by 70 μM ZD7288 in response to a 60 pA hyperpolarizing current pulse. Holding potential (Vh) was −60 mV. (C) Summary of the intracellulary injected hyperpolarizing current amplitude versus the depolarizing 'sag' in ACSF (○) and 70 μM ZD 7288 (●), $n = 6$.

Under current clamp, 23 of 75 PVN parvocellular neurons were identified as NMU-sensitive neurons as previously described [14]. The NMU-sensitive 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), identified as putative parvocellular neurons [9,17]. Further, the responsive neurons also displayed time-dependent inward rectification during the hyperpolarizing pulses (Fig. 1A–C) blocked by 70 μM ZD 7288 (Fig. 1B and C) or 3 mM Cs^+ (not shown). These characteristics are consistent with I_H conductance [8,16], indicating that hyperpolarization-activated, cyclic nucleotide-gated