Neuroscience 262 (2014) 70–82

α-MSH EXERTS DIRECT POSTSYNAPTIC EXCITATORY EFFECTS ON NTS NEURONS AND ENHANCES GABAERGIC SIGNALING IN THE NTS

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Abstract—The central melanocortin system plays an essential role in the regulation of energy balance. While anorexigenic effects of α-melanocyte-stimulating hormone (α-MSH) acting in the nucleus of the solitary tract (NTS), a critical medullary autonomic control center, have been established, the cellular events underlying these effects are less well characterized. In this study, we used whole-cell patch-clamp electrophysiology to examine firstly whether α-MSH exerts direct postsynaptic effects on the membrane potential of rat NTS neurons in slice preparation, and secondly whether α-MSH influences GABAergic signaling in the NTS. In normal artificial cerebrospinal fluid, perfusion of α-MSH (500 nM) resulted in a depolarization in 39% of cells ($n = 16$, mean 6.14 ± 0.54 mV), and a hyperpolarization in 22% of cells ($n = 9$, −6.79 ± 1.02 mV). Studies using tetrodotoxin to block neuronal communication revealed α-MSH exerts direct depolarizing effects on some NTS neurons, and indirect inhibitory effects on others. A third subset of neurons is simultaneously directly depolarized and indirectly hyperpolarized by α-MSH, resulting in a net lack of effect on membrane potential. The inhibitory inputs influenced by α-MSH were identified as GABAergic, as α-MSH increased the frequency, but not amplitude, of inhibitory postsynaptic currents (IPSCs) in 50% of NTS neurons. α-MSH had no effect on the frequency or amplitude of miniature IPSCs. Furthermore, pharmacological blockade of $GABA_A$ and $GABA_B$ receptors, and physical removal of all synaptic inputs via cellular dissociation, abolished hyperpolarizations induced by α-MSH. We conclude α-MSH exerts direct, postsynaptic excitatory effects on a subset of NTS neurons. By exciting GABAergic NTS neurons and presynaptically enhancing GABAergic signaling, α-MSH also indirectly inhibits other NTS cells. These findings provide critical insight into the cellular events underlying medullary melanocortin anorexigenic effects, and expand the understanding of the circuitries involved in central melanocortin signaling. **Crown Copyright © 2013 Published by Elsevier Ltd. on behalf of IBRO. All rights reserved.**

Key words: brainstem, nucleus of the solitary tract, α-melanocyte-stimulating hormone, melanocortins, electrophysiology, GABA.

INTRODUCTION

The medullary nucleus of the solitary tract (NTS) is a critical autonomic center which regulates numerous aspects of food intake and energy balance, ranging from the initial detection of food in the stomach to feelings of satiation, through the integration of multiple central and peripheral inputs (for reviews see Berthoud, 2008; Grill and Hayes, 2012). Among the many peptides expressed by the NTS is pro-opiomelanocortin (POMC), the precursor for melanocortins such as α-melanocyte-stimulating hormone (α-MSH) (Palkovits et al., 1987; Bronstein et al., 1992). The NTS is one of only two central sites in which POMC is expressed, the second, larger population of POMC neurons being located in the arcuate nucleus of the hypothalamus (Jacobowitz and O'Donohue, 1978; Cone, 2005; Huo et al., 2006). Both groups of POMC cells provide α-MSH innervation to NTS neurons, where α-MSH then acts via the melanocortin-4 receptor (MC4R) to ultimately modulate autonomic processes (Cone, 2005; Zheng et al., 2010). For example, injection of the long-lasting melanocortin-3 and -4 receptor (MC3/4R) agonist melanotan II (MTII) into the fourth ventricle (4V) or the dorsal vagal complex (DVC, including dorsal motor nucleus of the vagus and the NTS) causes potent decreases in food intake by decreasing meal size, and also results in decreases in body weight. Conversely, 4V or DVC administration of the MC3/4R antagonist SHU9119 induces increases in food intake and body weight, underscoring the physiological significance of melanocortin effects in the brainstem (Grill et al., 1998; Williams et al., 2000; Zheng et al., 2005). More targeted studies have revealed the medullary MC3/4Rs which mediate these anorexigenic effects may be located in the NTS, as microinjection of SHU9119 into the NTS abolishes α-MSH-mediated decreases in food intake (Zheng et al., 2010). In addition to direct effects of melanocortins on the regulation of energy balance, brainstem melanocortin signaling is also essential in mediating anorexigenic effects of other feeding-related peptides,

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Abbreviations: 4V, fourth ventricle; aCSF, artificial cerebrospinal fluid; DVC, dorsal vagal complex; EGTA, ethylene glycol tetraacetic acid; EPSC, excitatory postsynaptic current; GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPSC, inhibitory postsynaptic current; KA, kynurenic acid; MC3R, melanocortin-3 receptor; MC4R, melanocortin-4 receptor; mIPSC, miniature inhibitory postsynaptic current; MTII, melanotan II; NTS, nucleus of the solitary tract; POMC, pro-opiomelanocortin; RT-PCR, reverse transcription polymerase chain reaction; TTX, tetrodotoxin; α-MSH, α-melanocyte-stimulating hormone.

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http://dx.doi.org/10.1016/j.neuroscience.2013.12.039

as 4V administration of SHU9119 attenuates the decreases in food intake induced by leptin and cholecystokinin, for example (Fan et al., 2004; Zheng et al., 2010). The autonomic effects of melanocortins in the brainstem also extend beyond the control of feeding, as 4V and DVC injection of MTII has been shown influence water intake (Grill et al., 1998; Williams et al., 2000), and microinjection of α-MSH or MTII into the NTS alters heart rate, blood pressure (Pavia et al., 2003), gastric motility, and gastric tone (Richardson et al., 2013).

Although the physiological outcomes of MC3/4R activation in the brainstem and NTS are well documented, less well characterized are the cellular effectors and local circuitry through which melanocortins act in the NTS to ultimately exert effects on autonomic function. A previous study by Wan et al. (2008) reported presynaptic effects of α-MSH on vagal glutamatergic signaling to the NTS, as assessed by changes in the frequency of excitatory postsynaptic currents (EPSCs) and in the amplitude of EPSCs evoked by electrical stimulation of the solitary tract (Wan et al., 2008). Studies from our laboratory using post-hoc single cell reverse transcription polymerase chain reaction (RT-PCR), however, have revealed the presence of postsynaptic MC4Rs in NTS neurons (Hoyda et al., 2009; Mimee et al., 2012). Thus in light of our previous findings, the present study was undertaken to explore a potential postsynaptic site of action for α-MSH in the NTS. To this end, we performed whole-cell current-clamp recordings to examine whether α-MSH can act postsynaptically to directly influence the membrane potential of neurons in the NTS. Furthermore, while it has been established that α-MSH influences excitatory glutamatergic signaling in the NTS (Wan et al., 2008), we examined whether it can also affect inhibitory γ-aminobutyric acid (GABA) transmission in the NTS using whole-cell voltage-clamp recordings.

EXPERIMENTAL PROCEDURES

Preparation of slices for electrophysiological recording

Coronal slices containing the NTS were prepared daily from unanesthetized, 21–28 day-old male Sprague–Dawley rats (Charles River, Quebec, Canada). Rats were decapitated and their brains rapidly placed in ice-cold carbogenated slicing solution made of (in mM): 87 NaCl, 2.5 KCl, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 1.25 NaH_2PO_4, 25 glucose, 75 sucrose bubbled with 95% O_2/5% CO_2. A region of brainstem containing the NTS was isolated and 300-μm coronal sections were cut with a vibratome (Leica, Nussloch, Germany). Slices were then incubated at 32 °C in artificial cerebrospinal fluid (aCSF) made of (in mM): 124 NaCl, 2.5 KCl, 20 $NaHCO_3$, 2 $CaCl_2$, 1.3 $MgSO_4$, 1.24 KH_2PO_4, 10.5 glucose saturated with 95% O_2/5% CO_2 for a minimum of 1 h prior to electrophysiological recording. All procedures were in accordance with the ethical criteria established by the Canadian Council on Animal Care and were approved by the Queen's University Animal Care Committee.

Preparation of dissociated NTS neurons

Rat coronal brain slices were obtained as described above, and the NTS was identified and microdissected from each slice using a dissection microscope. After a 30-min incubation at 30 °C in Hibernate solution (Brain Bits, Springfield, IL, USA) containing 2 mg/mL papain (Worthington Biochemical, Lakewood, NJ, USA), dissected NTS fragments were washed three times and then gently triturated with Hibernate solution supplemented with B27 (Life Technologies, Carlsbad, CA, USA). The dissociated NTS neurons were collected in a pellet by centrifugation at 200*g* for 8 min at 4 °C. Neurons were then resuspended in Neurobasal A medium supplemented with B27, 0.5 mM L-glutamine, and 100 U/ml penicillin–streptomycin (all Life Technologies), aliquoted to 35-mm glass-bottomed culture dishes (MatTek, Ashland, MA, USA), and left to adhere to the dishes for approximately 3 h in a 5% CO_2, 37 °C incubator. Following adhesion, approximately 2 mL of supplemented Neurobasal A medium was added to the dishes, which were then maintained for up to 4 days in the incubator.

Electrophysiology

Brain slices were transferred to a recording chamber continuously perfused at a flow rate of 1.5–2 ml/min with carbogenated aCSF warmed to approximately 32 °C. Neurons were visualized at 40x magnification with an upright differential interference contrast microscope (Scientifica, East Sussex, United Kingdom). Borosilicate glass electrodes (World Precision Instruments, Sarasota, Florida, USA) were pulled on a Sutter Instruments P97 flaming micropipette puller and filled with an intracellular solution made of (in mM): 125 potassium gluconate, 10 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 5.5 EGTA, 10 HEPES, 2 NaATP (pH 7.2 with KOH) for current-clamp recordings. A high-chloride intracellular recording solution was used for voltage-clamp recordings examining inhibitory postsynaptic currents (IPSCs), made of (in mM): 140 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 5.5 EGTA, 10 HEPES, 2 NaATP (pH 7.2 with KOH). Electrodes had a resistance of 3–5.5 MΩ when filled with the intracellular solution. After obtaining a high-resistance seal (minimum 1 GΩ), brief suction was used to rupture the membrane and obtain whole-cell access. Whole-cell recordings were made with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, California, USA) sampled at 10 kHz, filtered at 2.4 kHz using a Micro 1401 interface and data were collected with Spike 2 software for current-clamp recordings and Signal 4 software for voltage-clamp recordings for offline analysis (Cambridge Electronic Devices, Cambridge, UK). Neurons selected for experimentation had action potentials with an amplitude of at least 60 mV and a stable baseline membrane potential. All solutions were applied to slices via bath perfusion. Response to α-MSH in current-clamp configuration was determined by

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