Neuroscience 159 (2009) 83–93

5-HT INHIBITION OF RAT INSULIN 2 PROMOTER Cre RECOMBINASE TRANSGENE AND PROOPIOMELANOCORTIN NEURON EXCITABILITY IN THE MOUSE ARCUATE NUCLEUS

K. HISADOME,[a] M. A. SMITH,[a,b] A. I. CHOUDHURY,[b] M. CLARET,[b] D. J. WITHERS[b] AND M. L. J. ASHFORD[a]*

[a]*Biomedical Research Institute, Ninewells Hospital and Medical School, University of Dundee, Dundee, DD1 9SY, Scotland, UK*

[b]*Centre for Diabetes and Endocrinology, Rayne Institute, University College London, London, WC1E 6JJ UK*

Abstract—A number of anti-obesity agents have been developed that enhance hypothalamic 5-HT transmission. Various studies have demonstrated that arcuate neurons, which express proopiomelanocortin peptides (POMC neurons), and neuropeptide Y with agouti-related protein (NPY/AgRP) neurons, are components of the hypothalamic circuits responsible for energy homeostasis. An additional arcuate neuron population, rat insulin 2 promoter Cre recombinase transgene (RIPCre) neurons, has recently been implicated in hypothalamic melanocortin circuits involved in energy balance. It is currently unclear how 5-HT modifies neuron excitability in these local arcuate neuronal circuits. We show that 5-HT alters the excitability of the majority of mouse arcuate RIPCre neurons, by either hyperpolarization and inhibition or depolarization and excitation. RIPCre neurons sensitive to 5-HT, predominantly exhibit hyperpolarization and pharmacological studies indicate that inhibition of neuronal firing is likely to be through 5-HT$_{1F}$ receptors increasing current through a voltage-dependent potassium conductance. Indeed, 5-HT$_{1F}$ receptor immunoreactivity co-localizes with RIPCre green fluorescent protein expression. A minority population of POMC neurons also respond to 5-HT by hyperpolarization, and this appears to be mediated by the same receptor-channel mechanism. As neither POMC nor RIPCre neuronal populations display a common electrical response to 5-HT, this may indicate that sub-divisions of POMC and RIPCre neurons exist, perhaps serving different outputs.

Key words: 5-HT, hypothalamus, K^+ channel, RIPCre, POMC, 5-HT$_{1F}$ receptor.

The CNS control of food intake involves complex interactions between circulating hormones, nutrients, neuropeptides, monoamines and other neurotransmitters. These act at a variety of hypothalamic areas (including the paraventricular nucleus (PVN) and the lateral (LHA) and medial hypothalamic areas) to modulate orexigenic and anorexigenic neural pathways (Broberger, 2005). At least two populations of neurons within the arcuate nucleus (ARC) of the hypothalamus contribute to the central circuitry that controls energy homeostasis. These neurons make up part of the melanocortin pathway, which consists of cells containing neuropeptide Y (NPY) along with the endogenous melanocortin antagonist, agouti-related protein (AgRP) and cells containing alpha-melanocyte stimulating hormone (α-MSH) and other proopiomelanocortin (POMC) derived peptides (Ellacott and Cone, 2004; Cone, 2005). These neurons are key targets for the hormones leptin and insulin, the actions of which effect an anorexigenic output (Niswender et al., 2004).

Food intake is accompanied by changes in the release of monoamines in the hypothalamus (Schwartz et al., 1990), and sympathomimetic drugs (e.g. *d*-fenfluramine) have long been used to reduce food intake and appetite (Ioannides-Demos et al., 2005). Consequently, pharmacological manipulation that results in enhancement or inhibition of 5-HT synaptic transmission reduces and increases food intake, respectively, in animals and humans (Halford et al., 2005). Electrophysiological recordings from neurons of transgenic mice expressing green fluorescent protein (GFP) under the control of the POMC promoter demonstrate that 5-HT depolarizes arcuate POMC neurons (Heisler et al., 2002), an action also observed for leptin (Cowley et al., 2001; Choudhury et al., 2005). There are at least 14 different 5-HT receptor subtypes and many are present at significant levels in the hypothalamus, notably 5-HT$_{1B}$, 5-HT$_{1F}$, 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$ and 5-HT$_7$ (Hoyer et al., 2002). However, it is still unclear exactly which 5-HT receptor subtypes contribute to modulation of activity in the hypothalamic circuits that sub-serve long-term control of food intake and energy expenditure. In addition, the underlying mechanisms by which 5-HT receptor activation alters the electrical activity of these ARC neurons are unknown. Studies, using selective 5-HT receptor subtype agonists and antagonists, have demonstrated both hyperphagic and hypophagic responses in animal studies. Unfortunately, many of these ligands lose their receptor selectivity at higher concentrations, resulting in some uncertainty over receptor subtype identity in relation to changes in food intake. Nevertheless, there are two main subtypes proposed as key mediators of the anorexigenic action of 5-HT, the 5-HT$_{1B}$ and 5-HT$_{2C}$ receptors (Ramos et al., 2005). Neurons expressing 5-HT$_{1B}$ (Makarenko et al., 2002) and 5-HT$_{2C}$ receptors (Clemett et al., 2000) are present in hypothalamic feeding centers (i.e. PVN, LHA

*Corresponding author. Tel: +44-1382-636702; fax: +44-1382-667120.
E-mail address: m.l.j.ashford@dundee.ac.uk (M. Ashford).

Abbreviations: AgRP, agouti-related protein; ARC, arcuate nucleus; GFP, green fluorescent protein; LHA, lateral hypothalamic area; NPY, neuropeptide Y; PBG, 1-phenylbiguanide; POMC, proopiomelanocortin; PVN, paraventricular nucleus; RIPCre, rat insulin 2 promoter Cre recombinase transgene; TEA, tetraethylammonium chloride; TTX, tetrodotoxin; α-me 5-HT, α-methyl 5-HT; α-MSH, alpha-melanocyte stimulating hormone; ΔVm, change in membrane potential; 5-CT, 5-carboxamidotryptamine; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino) tetralin.

0306-4522/09
doi:10.1016/j.neuroscience.2008.12.003

and ARC), but are also found in brain areas not implicated in energy homeostasis (Hoyer et al., 2002). The 5-HT_{2C} receptor knockout mouse demonstrates increased food intake and obesity (Tecott et al., 1995) and the hypophagic action of *d*-fenfluramine is attenuated in the 5-HT_{1B} knockout mouse (Lucas et al., 1998). However, global 5-HT_{1B} and 5-HT_{2C} receptor knockout mice also develop physiological abnormalities such as seizures, anxiety and aggression (Tecott et al., 1995; Ramboz et al., 1996). These observations highlight the multi-functional role of the serotonergic system, but also raise the question whether the feeding behaviors observed are due to the lack of a given receptor in the energy regulatory centers and/or in areas associated with other physiological or pathological outcomes. In addition, although histochemical and *in situ* hybridization studies demonstrate that many 5-HT receptor subtypes are located in energy regulatory centers, these data provide no information about receptor-mediated alteration of neuronal function, most importantly in relation to changes in neuronal excitability.

Recent studies have indicated that another population of arcuate neurons, defined by GFP expression driven by the rat insulin 2 promoter Cre recombinase transgene (RIPCre), which are distinct from NPY/AgRP and POMC neurons, are involved in the regulation of body weight and energy homeostasis (Cui et al., 2004; Choudhury et al., 2005). Thus, we have examined the actions of 5-HT on the electrical activity of this population of arcuate neurons and show that they respond to 5-HT in a heterogeneous manner with the majority of responding neurons displaying hyperpolarization and reduced excitability.

EXPERIMENTAL PROCEDURES

Hypothalamic slice preparation

As previously described (Choudhury et al., 2005; Smith et al., 2007) we have used 2 Cre recombinase transgenic lines, RIPCre and POMCCre and inter-crossed these with the ZEG indicator mouse to generate mice with GFP expression in selective hypothalamic neuronal populations. All procedures conformed to the UK Animals (Scientific Procedures) Act 1986, and were approved by our institutional ethical review committee. Every effort was made to minimize the number of animals used and their suffering. RIPCreZEG and POMCCreZEG mice (8–16 weeks old) were killed by cervical dislocation; the brain was rapidly removed and submerged in an ice cold slicing solution containing (in mM) KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 28, $CaCl_2$ 0.5, $MgCl_2$ 7, D-glucose 7, ascorbate 1, pyruvate 3 and sucrose 235, equilibrated with 95% O_2, 5% CO_2 to give a pH of 7.4. Hypothalamic coronal slices (350 μm), containing the ARC, were cut using a Vibratome (St Louis, MO, USA), transferred and kept at room temperature (22–25 °C) in an external solution containing (in mM) NaCl 125, KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 25, $CaCl_2$ 2, $MgCl_2$ 1, D-glucose 10, D-mannitol 15, ascorbate 1 and pyruvate 3, equilibrated with 95% O_2, 5% CO_2, pH 7.4.

Electrophysiology

Individual arcuate neurons were identified by epifluorescence and differential interference contrast optics using an upright Zeiss Axioskop-2 FS plus microscope. Slices were continually perfused with a modified external solution (0.5 mM $CaCl_2$ and 2.5 mM $MgCl_2$, no ascorbate and pyruvate) at a flow rate of 5–10 ml/min and a bath temperature of 33 °C. For high potassium experiments, the normal external solution was replaced with a solution containing (in mM) NaCl 130, KCl 20, $CaCl_2$ 0.5, $MgCl_2$ 2.5, D-glucose 10, D-mannitol 15, Hepes 10, pH 7.4. Patch-clamp recordings were performed using borosilicate patch pipettes (4–8 MΩ) filled with an internal solution containing (in mM) K-gluconate 130, KCl 10, EGTA 0.5, Hepes 10, NaCl 1, $CaCl_2$ 0.28, $MgCl_2$ 3, Na_2ATP 3, tris-GTP 0.3, phosphocreatine 14 (pH 7.2). Whole-cell series resistance (Rs) was compensated using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) in current (I_{fast}) and voltage-clamp modes (Rs: 30–60 and 10–30 MΩ respectively). Voltage and current commands were manually or externally driven using PClamp 9.2 software and injected into neurons via the patch-clamp amplifier. Under current clamp, hyperpolarizing current pulses (between −5 and −20 pA, at a frequency of 0.05 Hz) were used to monitor input and series resistance at resting membrane potentials. In addition, input resistance was calculated from I–V relationships evoked from a holding potential of −70 mV (±5–30 pA, 0.5 s pulse duration). Voltage clamp recordings of transient voltage-dependent potassium (I_A) conductance and the delayed and inward rectifying potassium conductances were performed as described in Smith et al. (2007). Whole cell currents and voltages were filtered at 5 and 2 kHz respectively, and digitized at 50 kHz using Pclamp 9.2 software. All data were stored un-sampled on digital audiotape for off-line analysis using Clampex 9.2 or Igor pro. Membrane potentials were either replayed un-sampled on an EasyGraph TA240 chart recorder (Gould, Ballainvilliers, France), or digitized and imported into Abode illustrator for illustration purposes.

Drugs were added to the external solution and applied to slices via the perfusion system or locally applied using a broken tipped pipette (~4 μm diameter) positioned above the recording neurons, as previously described (Choudhury et al., 2005). At least 10 min of stable control data were recorded before the application of any drug, and antagonists were applied for at least 10 min prior to challenge with agonist. Neuronal integrity was determined by biophysical and gross anatomical assessment, as described previously (Smith et al., 2007; Claret et al., 2007).

Immunocytochemistry

Hypothalamic sections (30 μm) from paraformaldehyde perfused brains were processed as previously described (Choudhury et al., 2005). Primary antibodies used were rabbit polyclonal antibodies raised to the C- or N-terminal domains of the 5-HT_{1F} receptor and were obtained from MBL International (MA, USA; cat No. LS-3344 and LS-3338, respectively). Slices were incubated with primary antibody (1:300 dilution) for 48 h at 4 °C, following which they were incubated with anti-rabbit secondary antibody conjugated to Alexa Fluor 549 (1:800 dilution) for 1 h. RIPCre-GFP expression and 5-HT_{1F} receptor localization were detected using a confocal microscope (BioRad MRC 100).

PCR

A 475 base pair fragment encoding a region of the 5-HT_{1F} receptor was detected by PCR from mRNA extracted from mouse hypothalamus using the following primers: forward GGAAGCTGAGTTGAGATGATGGC, reverse CACGTACAACAGATGATGTCG.

Chemicals

Kynurenic acid, (+) bicuculline, tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), barium chloride, 5-HT, 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT), SB 242084 and CGS 12066B were purchased from Sigma-Aldrich (Dorset, UK). α-Methyl 5-HT (α-me 5-HT), 5-carboxamidotryptamine (5-CT), ketanserin, methiothepin, SB 204741, BW 723C86, CP 93129, SB 224289, L 694247 and BRL 54443 were obtained from Tocris Bioscience (Bristol, UK), tetrodotoxin (TTX) from Alomone Laboratories, Ltd. (Jerusalem, Israel), and zacopride from Professor B. Costell (University of Bradford). All drugs were dissolved in saline immediately before use.