Synaptic Regulation of Proopiomelanocortin Neurons Can Occur Distal to the Arcuate Nucleus

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INTRODUCTION

The majority of proopiomelanocortin (POMC) neurons reside in the arcuate nucleus of the hypothalamus where they are well poised to carry out their roles in mediating homeostatic functions as they integrate signals from, and send projections to, the various hypothalamic nuclei and extrahypothalamic sites (Cone 2005). POMC neurons have received much attention recently because of their essential role in the maintenance of normal energy balance. This is clearly evidenced by the fact that the loss of POMC neurons or their peptide products results in increased body weight and obesity (Farooqi and O’Rahilly 2003; Smart et al. 2006; Yaswen et al. 1999). Accordingly, activation of POMC neurons correlates with decreased food intake. Determining how POMC neuron activity is regulated is of particular interest as such knowledge could help elucidate targets for the therapeutic treatment of obesity.

To date, studies examining factors that regulate POMC neuron activity have primarily focused on peptides and rapid neurotransmitters released from terminals in the region of the POMC cell bodies. However, afferent inputs that terminate on dendrites can also play an important role in regulating neuronal activity. In POMC neurons, the anatomical distribution of dendrites has not been well characterized nor has the extent to which POMC neuron dendrites can extend beyond the soma. It has been difficult to determine the orientation of POMC neuron dendrites anatomically because the arcuate nucleus is not a laminated structure. In this study, the tonic release of endogenous cannabinoids from POMC neurons (Hentges et al. 2005) was used as a physiologic sensor to detect where inputs that have cannabinoid receptors terminate on POMC neuron dendrites. The results show that only a specific population of POMC dendrites, extending out of the arcuate nucleus, receives the endocannabinoid-sensitive inhibitory inputs.

METHODS

Animals

Pomc-egfp homozygous mice generated in the inbred C57BL/6J strain (Cowley et al. 2001) were bred with wild-type C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) to produce the mice used in these studies. All mice were housed under controlled temperatures (22–24°C) and a constant 12-h light/dark schedule and given standard chow and tap water ad libitum. All experimental procedures met United States Public Health Service guidelines with the approval of the Institutional Animal Care and Use Committee at Oregon Health and Science University. All brain slices were prepared from 7- to 12-wk-old male mice.

Electrophysiological recordings

Whole cell voltage-clamp recordings were performed as previously described (Hentges et al. 2004). Briefly, 240-μm brain slices were cut in ice-cold oxygenated Krebs solution (in mM: 126 NaCl, 2.5 KCl, 1.2 MgCl2, 2.4 CaCl2, 1.2 NaH2PO4, 21.4 NaHCO3, 11.1 glucose). The POMC neurons were identified by the presence of enhanced green fluorescent protein (EGFP) resulting from the transgene. The internal recording solution contained (in mM) 57 KCl, 70 K+ methyl sulfate, 20 NaCl, 1.5 MgCl2, 0.1 EGTA, 10 phosphocreatine, 2 Mg-ATP, and 0.5 GTP, buffered with 5 HEPES, pH 7.3. All recordings were made at 37°C, and slices were continuously perfused with oxygenated Krebs. GABAergic currents were isolated with the addition of MK801 (0.1 μM, Sigma) and DNQX (10 μM, Sigma) to block N-methyl-D-aspartate (NMDA) and AMPA receptor–mediated glutamatergic currents. Postsynaptic currents were evoked with a 0.5-ms stimulus delivered every 20 s to the slice through a bipolar stimulating electrode. The stimulating electrode was generally placed several

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hundred microns away from the cell to be recorded from dorsal to the POMC-EGFP cells in sagittal slices and lateral or dorsal to the cells in coronal slices. Changing the placement of the stimulating electrode did not affect the cannabinoid responses. Cells were held at −60 mV for recording both evoked and spontaneous currents, and recordings were made using an Axopatch-1D amplifier (Axon Instruments, Union City, CA) and collected using AxographX software (Axograph, Sydney, Australia). In the raw traces of electrically evoked inhibitory responses, Cells were filled with neurobiotin (0.3% through the recording electrode). POMC cells in both sagittal and coronal slices displayed two to four thick primary processes that grossly appeared to be primary dendrites (Fig. 1, G). Lower-magnification images of filled cells display long primary processes in both sagittal and coronal sections (Fig. 1, D and H, respectively). A low-magnification image taken of POMC neurons in the Arc in a sagittal slice is shown in I. Dotted line indicates level at which rostral cuts were made in experiments in which sagittal slices were truncated rostrally. An area ~1 mm rostral and dorsal to the area depicted in I was imaged at high power, and fibers were detected at this distal site. Arrows in J indicate dendrite studded with spine-like protrusions. Inset in J is an enlarged view of 1 of the spines.

Confocal imaging

Adult male Pomp-egfp transgenic mice were perfused transcardially with 10% sucrose by 4% paraformaldehyde in PBS. Brains were collected and postfixed for 18 h and sliced with a vibratome into 100-μm sagittal sections. Sections were mounted onto charged, gelatin-coated slides and coverslipped using Gel/Mount aqueous mounting medium with anti-fading agents (Biomedia, Foster City, CA). Images were collected using an Olympus laser-scanning confocal microscope. An image was obtained every 0.5 μm over 25–50 μm and z-stack reconstructions are presented.

Statistics

Effects of AM 251 or WIN 55212–2 compared with control were analyzed using Student’s t-test. Populations of mIPSCs were compared using Kolmogorov-Smirnov tests. Current/voltage plots were compared by two-way ANOVA. Data were considered significantly different when P < 0.05. The control percentages presented in the bar graphs represent the data gathered at 10 min in the absence of drug as a percent of data gathered at 5 min under baseline conditions. This was done to give a measure of the stability of the eIPSCs or mIPSCs during the 10 min before the perfusion of CB1 receptor agonist or antagonist.

RESULTS

Anatomical distribution of POMC neuron dendrites

Sagittal and coronal brain slices (240 μm; Fig. 1, A and E, respectively) were prepared from transgenic mice expressing EGFP driven by the Pomc promoter. Low-magnification images show the apparently disordered nature of the POMC neuron fibers in either cutting plane (Fig. 1, B and F). The EGFP is freely mobile throughout the cell and fills both axons and dendrites in addition to the soma.

Six to 10 single POMC-EGFP neurons in each cutting plane were filled with neurobiotin (0.3% through the recording electrode). POMC cells in both sagittal and coronal slices displayed two to four thick primary processes that grossly appeared to be primary dendrites (Fig. 1, C, D, G, and H). Thin fibers, likely axons, could be seen branching from a primary dendrite in many of the filled cells in either plane (white arrows in Fig. 1, C and G). The neurobiotin-filled cells in Fig. 1 are confocal images reconstructed over ~50 μm at 1-μm intervals (see supplemental data for three-dimensional reconstructions of filled cells).1 The three-dimensional images show that the primary processes project in all directions from the cell. It was

1 The online version of this article contains supplemental data.
not possible to follow the processes of neurobiotin-filled cells for more than \( \sim 100 \) \( \mu \)m because of both limited filling of the fine processes and the fact that most cells were near the surface of the slice. By filling cells near the surface, the background neurobiotin staining was reduced. Fibers that were cut during the slice preparation were abruptly truncated, whereas other fibers gradually disappeared as they projected deeper into the slice. The dendrites tapered quickly and became fine fibers that often branched into two. Although it was difficult to continuously follow single fibers over long distances, it was possible to see distal fibers that contained the endogenously expressed EGFP and occasional fibers with the neurobiotin fill.

Moving away from the soma, it became difficult to identify whether fibers were axons or dendrites because both were fine and there was no apparent specific orientation to one versus the other, although there were many fibers projecting in the dorsal/rostral direction (Fig. 1J). High-magnification images revealed dendritic-spine like structures on distal POMC fibers, suggesting the dendritic nature of some of the far-reaching fibers (Fig. 1J, arrowheads and inset). Dendrites in Fig. 1J were imaged in an area \( \sim 1 \) mm rostral and dorsal to the rostral arcuate nucleus. In addition to the presence of spine-like protrusions, the fiber indicated with the arrowheads also tapers throughout the field of view, which is another characteristic of dendrites (Craig and Banker 1994).

Immunodetection of axon- or dendrite-specific markers has not been reported in the arcuate nucleus, and in this study, attempts to detect the axonal marker Tau or the dendritic marker MAP2B in hypothalamic slices were unsuccessful. The presence of putative dendritic spines proved a more reliable anatomical indicator than the immunodetection of commonly used dendritic markers.

Properties of POMC neurons in sagittal and coronal slices

The morphology of the POMC neurons indicates that dendrites extend from the soma in each direction. Consistent with an equal distribution of dendrites, there was no difference in input resistance in POMC cells in slices cut in the sagittal or coronal planes (551 ± 36 vs. 613 ± 36 \( \Omega \); respectively, \( n = 14–16 \) cells; \( P = 0.24 \); Fig. 2A). Although not a direct measure, similar input resistance suggests that relatively the same amount of cell membrane remains in each slice orientation. The frequency of mIPSCs was also the same in both sagittal and coronal cutting planes (103 ± 23 and 127 ± 25 on average in a 30-s sweep, respectively; \( P = 0.5 \); \( n = 11–15 \) cells; Fig. 2B). The control current/voltage curves were not different between POMC neurons in coronal or sagittal slices (\( P = 0.81, n = 6 \); Fig. 2C), further indicating that there is not a prominent orientation to the cell surface area (a dendritic arbor for example) or channel localization. Activation of opioid receptors increases potassium conductance in POMC neurons. The peptide agonist [Met]\(^5\) enkephalin (ME, 10 \( \mu M \)) increased the potassium-mediated current in POMC cells in slices cut in the sagittal or coronal planes (\( 36 \) vs. 613 \( \mu M \); \( 10.220.33 \)). Although not a direct measure, similar input resistance suggests that relatively the same amount of cell membrane remains in each slice orientation. The frequency of mIPSCs was also the same in both sagittal and coronal cutting planes (103 ± 23 and 127 ± 25 on average in a 30-s sweep, respectively; \( P = 0.5 \); \( n = 11–15 \) cells; Fig. 2B). The control current/voltage curves were not different between POMC neurons in coronal or sagittal slices (\( P = 0.81, n = 6 \); Fig. 2C), further indicating that there is not a prominent orientation to the cell surface area (a dendritic arbor for example) or channel localization. Activation of opioid receptors increases potassium conductance in POMC neurons. The peptide agonist [Met]\(^5\) enkephalin (ME, 10 \( \mu M \)) increased the potassium-mediated current in recordings from neurons in both coronal and sagittal slices to the same extent (\( P = 0.51, n = 4–6 \); Fig. 2D). Thus the postsynaptic properties of POMC neurons are very similar regardless of the plane of the slice preparation.

**Endocannabinoid inhibition is dependent on the plane of section**

Despite the similarity of postsynaptic properties of POMC neurons in the two cutting planes, there was a clear difference in the presynaptic inhibition of GABA release by endocannabinoids depending on slice orientation. It was previously shown that POMC neurons release endocannabinoids that selectively inhibit presynaptic GABA release onto POMC neurons (Hentges et al. 2005). In that study, all recordings were made from neurons in brain slices cut in the sagittal plane. In this work, the effect of blocking endocannabinoid tone was also examined in slices cut in the coronal plane. Sagittal or coronal sections containing the arcuate nucleus were prepared on alternating days. Similar to the previous result, AM 251 (1 \( \mu M \)) increased the eIPSC in all cells.
tested to an average of 173 ± 15% of control \((P = 0.01, n = 7\) cells; Fig. 3A) in POMC neurons in sagittal slices. However, in coronal sections, there was no effect of AM 251 in any of the eight cells tested \((100 ± 6\% \text{ of control}, P = 0.89, n = 8; \text{Fig. 3C})\) on evoked GABA release or the paired pulse ratio when two stimuli were given 100 ms apart \((P = 0.31 \text{ by paired } t\text{-test})\). There was also no effect of cannabinoind receptor agonist WIN 55,212–2 \((\text{WIN, } 1 \mu\text{M})\) on evoked IPSCs in coronal sections \(\text{(C)}\). Bar graphs below raw traces show compiled data as means ± SE. *\(P < 0.05\).

Sections in the horizontal plane to localize the endocannabinoid-sensitive inputs

Horizontal sections \((240 \mu\text{m})\) through the diencephalon were prepared, and they contained all but the most dorsal region of the arcuate \((\text{Fig. 4A})\). There was no effect of AM 251 on the frequency of mIPSCs in POMC neurons in horizontal sections \((102 ± 3\% \text{ of control}, P = 0.55, n = 6\) cells; Fig. 4, B and C). Because horizontal slices contained the lateral and rostral/caudal extent of the arcuate and other hypothalamic nuclei, it was inferred that the endocannabinoid sensitive

FIG. 4. Loss of endocannabinoid action in horizontal slices. Effect of AM 251 on mIPSC frequency in POMC neurons was examined in horizontal sections. Low-power image in A shows area left intact in horizontal sections containing Arc. There was no effect of blocking endocannabinoid action on number of mIPSCs in POMC neurons in horizontal sections \((B \text{ and } C\). Bar graph in \(C\) shows means ± SE.
inputs must terminate >240 μm dorsal to the section. Considering that coronal sections retain a large extent of dorsal structures, the cannabinoid-sensitive inputs must terminate onto POMC dendrites that are both dorsal and rostral or caudal to the arcuate nucleus.

Sagittal sections with portions removed

To test the possibility that the endocannabinoid-sensitive terminals contact the POMC neurons rostral to the cell body region, the effect of AM 251 was examined in sagittal sections that were truncated rostrally. Sagittal sections were prepared as usual (Fig. 1A shows a slice as normally blocked for making recordings), and before placing in the recording chamber, the slices were cut in a vertical line just caudal to the optic chiasm as depicted by the dotted line in Fig. 1I. This cut was at the very rostral border of the arcuate. The picture in Fig. 5A shows fluorescent POMC neuron cell bodies right up to the cut edge (marked by the asterisk). When the rostral portion of the sagittal slice was removed, there was no effect of blocking endocannabinoid tone with AM 251 on the number of mIPSCs (97 ± 9% of control, P = 0.86, n = 8 cells; Fig. 5, B and C). There was a significant increase in input resistance in POMC neurons recorded in sagittal slices that had the rostral portion removed (551 ± 36 MΩ in full slices compared with 733 ± 49 MΩ in rostrally truncated slices, P = 0.01, n = 16), potentially reflecting a reduction in the area of the cell membrane. The loss of the AM 251 effect was specific to removal of preoptic brain regions because AM 251 increased the proportion of larger mIPSCs (>50 pA) from 250/1,000 to 440/1,000 events.

Examining the kinetic properties of the mIPSCs in intact sagittal slices revealed a mean rise time for the large amplitude mIPSCs (>50 pA) recorded in control solution of 0.237 ± 0.002 ms and a mean half-width of 4.91 ± 0.11 ms. Treatment with AM 251 increased the rise time to 0.432 ± 0.020 ms and increased the time to decay to 50% peak amplitude to 6.40 ± 0.067 ms. The slower rise time and decay after AM 251 may suggest that AM 251 relieves the inhibition specifically at more distal inputs. However, this shift, although statistically significant, is really very minor considering the distribution of rise times and half-widths that range from 0.1 to 2 and 1 to 7 ms, respectively. There is a normal distribution of the rise times and decay rates in both intact and rostrally truncated sagittal sections and thus no strong evidence for distinct populations of synapses that would indicate discrete near and distal populations of inputs like might be expected if inputs where concentrated onto a dendritic arbor. Thus POMC neurons may receive

Loss of selective presynaptic inputs to POMC neurons in rostrally cut sagittal slices

The loss of endocannabinoid inhibition of presynaptic GABA release in sagittal slices with the rostral portion removed suggested that a selective population of terminals was missing. Although the overall frequency of mIPSCs detected in POMC neurons was not significantly different between the sagittal and coronal planes (Fig. 2B), there was a shift in the size distribution of the mIPSCs. Similarly, the rostral cut resulted in a relative increase in the number of smaller amplitude events resulting in a shift to more events in the 20- to 30- and 30- to 40-pA bins (Fig. 5, D and E) and a small decrease in the mean amplitude (41.5 ± 0.9 pA vs. 46 ± 0.9 pA in the intact slice; P < 0.01). A shift in amplitudes was also observed in intact sagittal slices where AM 251 increased the proportion of larger mIPSCs (>50 pA) from 250/1,000 to 440/1,000 events.
inputs onto the soma and dendrites in an evenly distributed fashion.

To determine whether the loss of endocannabinoid inhibition of GABA release was caused by selective removal of cannabinoid receptor–containing terminals, the ability of the CB1 receptor antagonist WIN 55212–2 to decrease the number of mIPSCs was tested in intact and rostrally cut sagittal slices. In sagittal slices, WIN reduced the number of mIPSCs in POMC neurons in all cells tested to an average of 50% of control ($P < 0.001$, $n = 7$ cells) whereas in sagittal slices lacking the rostral portion, WIN inhibited the mIPSCs to a lesser extent (to 73 ± 7% of control, $P = 0.02$, $n = 7$ cells; Fig. 5F). The remaining response to WIN in the slices with the rostral portion removed may reflect that the location of the cut is relatively crude and therefore occasionally some sensitive terminals may remain. Alternatively, the perfusion of agonist may activate some receptors that do not receive enough endocannabinoid tone to be detected when testing the antagonist for the lack of increase with AM 251 but a partial inhibition with WIN in the rostrally truncated slices. Thus the rostral cut eliminated the endocannabinoid inhibition of presynaptic GABA inputs changed the amplitude distribution of mIPSCs and removed many WIN-sensitive terminals (Fig. 5F), suggesting that the rostral cut caused a loss of specific terminals onto POMC neurons.

**Discussion**

These results show that POMC neurons can be regulated by afferent fibers that synapse onto POMC dendrites outside of the arcuate nucleus in a topographically restricted manner. Only portion of the GABAergic inputs is sensitive to endocannabinoid tone, and these inputs terminate in the hypothalamic preoptic region. Thus POMC neurons receive distal inputs and can selectively regulate the inhibitory tone received from a specific subset of inputs through the release of endocannabinoids.

**Distribution of POMC dendrites**

Endocannabinoids are generally released from dendrites onto nearby presynaptic terminals (Soler-Llueva and Sabatini 2006; Wilson and Nicoll 2001), and dendrites are the primary site of synaptic inputs for many types of neurons. Therefore it seems likely that cutting in any orientation other than sagittal must have removed a portion of POMC dendrites necessary to detect the endocannabinoid inhibition of GABA input. In many brain areas, there is an anatomic lamination that makes the orientation of dendrites apparent and often neurons have a clear dendritic arbor. In dense nonlaminated nuclei, there can also be a prominent dendritic arbor, although it can be difficult to see anatomically (Travagli et al. 1996). Functionally, removing a major dendritic arbor would likely result in a reduced number of mIPSCs and changes in current/voltage ($I/V$) curves. No significant difference was observed in either mIPSC number (Fig. 2B) or $I/V$ curves (Fig. 2, C and D) for POMC neurons in coronal versus sagittal sections, suggesting that in each plane, approximately the same amount of afferent input remains.

The morphology of POMC neurons is consistent with the synaptic evidence for the presence of multiple far-reaching dendrites. POMC neurons had two to four primary dendrites extending in various directions regardless of the plane of section. POMC dendrites were found rostral and dorsal to the arcuate several hundred microns from the somata (Fig. 1J). Far-reaching dendrites have been described in unidentified arcuate neurons where anatomically defined dendrites with and without spines were shown to extend ~300 μm (van den Pol and Cassidy 1982). Thus regulation of arcuate neurons may be achieved, at least in part, by synaptic inputs that terminate far from the soma.

**Endocannabinoid regulation depends on the orientation of the slice**

Cutting the hypothalamus in coronal or horizontal sections or removing the rostral portion of sagittal slices eliminated the GABA inputs that express CB1 receptors, and most likely, the area of POMC neuron dendrite that receives those synaptic inputs. By showing that the cutting plane of the slice dramatically changes the cannabinoid actions in the hypothalamus, the results suggest that in vivo POMC neurons receive functionally distinct inputs in a spatially limited fashion. The consequence may be that POMC neurons can inhibit this select subset of GABA terminals through local endocannabinoid actions. The anatomical source of the endocannabinoid-sensitive inhibitory inputs is not known, but the data indicate that these inputs terminate in the hypothalamic preoptic area.

**Implications for the regulation of hypothalamic circuits**

The hypothalamus is a complex brain region that controls reproductive function and a number of critical homeostatic functions including the regulation of body weight. Although the hypothalamic nuclei have been attributed fairly specific roles, it has long been known that there is considerable interaction between the nuclei and extrahypothalamic target sites (Broadwell and Bleier 1976). These data suggest that the interactions within the hypothalamus are not only regulated by axons projecting to target structures but also by inputs that terminate outside of the nucleus containing the cell bodies. Far-reaching dendrites have recently been described in other hypothalamic neurons (Roberts et al. 2006), indicating that regulation of neuron activity occurring at distal sites may be common in the hypothalamus.

In summary, the data presented here show two key findings. First, that distal POMC fibers include dendrites and therefore POMC neuron activity can be regulated at sites beyond the cell body region in the arcuate nucleus. Second, subsets of POMC dendrites can receive selective inputs and endocannabinoids inhibit presynaptic release only at specific dendrites. Distal regulation and site-specific regulation of inputs can now be added to what is already known about the complex regulation of neurons in the hypothalamus.

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