SEX DIFFERENCES IN THE CANNABINOID MODULATION OF AN A-TYPE K+ CURRENT IN NEURONS OF THE MAMMALIAN HYPOTHALAMUS

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Abstract
Cannabinoids regulate biological processes governed by the hypothalamus including, but not limited to, energy homeostasis and reproduction. The present study sought to determine whether cannabinoids modulate A-type K⁺ currents (Iₐ) in neurons of the hypothalamic arcuate nucleus (ARC). Whole-cell patch clamp recordings were performed in slices through the ARC prepared from castrated female and male guinea pigs. Forty percent of guinea pig ARC neurons exhibited a transient outward current that was antagonized by high (mM) concentrations of 4-aminopyridine and (100 nM) rHeteropodatoxin-2. Five of these neurons also were immunopositive for both β-endorphin and the Kv4.2 channel subunit. Bath application of the CB1 receptor agonists WIN 55,212-2 (1 µM) or ACEA (1 µM) selectively induced a rightward shift in the inactivation curve for the Iₐ, significantly increasing the half-maximal voltage without affecting the peak current magnitude, in neurons from female but not male animals. The CB1 receptor antagonist AM251 (1 µM) reversed this action. Collectively, these data reveal that guinea pig ARC neurons, including proopiometanocortin neurons, express a prominent Iₐ that is positively modulated by cannabinoids in a sex-specific way by altering the voltage dependence of its inactivation. The resultant inhibitory effect on this neuronal population may shed some insight into the mechanism(s) by which cannabinoids influence hypothalamic function.
**Introduction**

Cannabinoids affect many regulatory processes controlled by the hypothalamus (Habayeb et al. 2002; Harrold and Williams 2003). For example, cannabinoids stimulate appetite, food-seeking behavior and energy intake (Harrold and Williams 2003; Horvath 2003). Energy homeostasis is regulated in large part by the hypothalamic feeding circuitry comprising the proopiomelanocortin (POMC), neuropeptide Y (NPY) and agouti-related peptide (AGRP)-containing neurons in the arcuate nucleus (ARC; Cowley et al. 2003; Olszewski et al. 2003; Marks and Cone 2003), orexin and melanin-concentrating hormone (MCH)-containing neurons in the lateral hypothalamus (DiLeone et al. 2003), as well as input from the ventromedial nucleus of the hypothalamus (Varma et al. 2000). A likely substrate upon which cannabinoids act to stimulate hyperphagia are the POMC neurons that release the anorexic peptides α-melanocyte-stimulating hormone, β-endorphin and cocaine-amphetamine-regulated transcript (CART) co-expressed in these cells. Chronic cannabinoid exposure alters POMC gene expression in the ARC (Corchero et al. 1997) and the adipostat leptin, which increases excitatory synaptic input onto POMC neurons (Cowley et al. 2003), also reduces hypothalamic levels of endogenous cannabinoids such as anandamide and 2-arachidonylglycerol (Di Marzo et al. 2001). Cannabinoids can alter neuronal excitability via presynaptic inhibition of neurotransmission by amino acid neurotransmitters such as γ-aminobutyric acid (GABA; Vaughan et al. 1999) and glutamate (Shen et al. 1996). Cannabinoids can also inhibit neuronal activity by activating postsynaptic K+ currents. For example, cannabinoids activate an inwardly-rectifying K+ current in AtT20 cells transfected with CB1 receptor (Mackie et al. 1995), and in oocytes co-expressing the CB1 receptor and the G protein-gated, inwardly-rectifying K+ channel GIRK1 (Henry and Chavkin 1995). In hippocampal pyramidal neurons, cannabinoids also positively modulate the depolarization-activated, A-type K+ current (Deadwyler et al. 1995). Despite recent advances in our
understanding of how cannabinoids affect hypothalamic function, the cellular mechanisms through which they regulate processes such as energy homeostasis are largely unknown. This study endeavored to examine the cannabinoid modulation of A-type K⁺ currents (I_A) in hypothalamic neurons from the ARC, in which POMC neurons can be found. All animal procedures described in this study are in accordance with institutional guidelines based on NIH standards.

Female and male Topeka guinea pigs (280-405 g) were obtained from Elm Hill Breeding Labs (Cheimsford, MA, USA), kept under controlled temperature (69-73°F) and light (12 h on: 12 h off), and provided with food and water ad libitum. They were castrated under ketamine/xylazine anesthesia (33 mg/kg & 6 mg/kg, respectively; s.c.) 4-10 d prior to experimentation.

Tetrodotoxin (TTX) with citrate was dissolved in Ultrapure H₂O to a stock concentration of 1 mM. Tetraethylammonium chloride (TEA) was dissolved in UltraPure H₂O to a stock concentration of 500 mM. 4-Aminopyridine (4-AP) and nickel chloride hexahydrate (NiCl₂) were dissolved in UltraPure H₂O to a stock concentration of 100 mM. Arachidonyl-2′-chloroethylamide (ACEA) was dissolved in ethanol to a stock concentration of 1 mM. cis-4-[Phosphomethyl]-2-piperidinecarboxylic acid (CGS 19755; 10 mM) was dissolved in 0.1N NaOH and then diluted to the final volume with UltraPure H₂O. WIN-55,212-2, AM251 and NBQX were dissolved in dimethyl sulfoxide to stock concentrations of 10 mM. Stock solutions of SR 95531, ω-conotoxin MVIIC and rHeteropodatoxin-2 (rHptx2; 10 mM, 10 μM and 100 μM, respectively) were prepared with UltraPure H₂O.

Whole-cell patch recordings in hypothalamic slices were performed as previously described (Wagner et al. 2000; Ibrahim et al. 2003). Neurons exhibiting transient outward tail currents evoked immediately following a hyperpolarizing voltage command (≥ 20 mV) from rest were selected for further analysis. The cells were perfused for 6-7 min with 25 mM TEA, 100 μM 4-AP and 1 μM
TTX to block other depolarization-activated K⁺ channels (except for the Iₐ, which is resistant to TEA and to low concentrations of 4-AP (Storm 1988)), and to isolate the cells from synaptic input impinging upon it. Cells were then subjected to baseline activation and inactivation protocols. The activation of the Iₐ was evaluated by holding the membrane potential at -110 mV, giving 10 mV depolarizing steps (500 msec) starting from -110 mV up to -10 mV, and measuring the resultant peak current that appears at the onset of the various voltage commands. The inactivation of the Iₐ was evaluated by holding the membrane potential at -60 mV and giving 10 mV pre-pulses (500 msec) from -110 to -40 mV, with each pulse followed by a depolarizing test command to -10 mV. The resultant outward current elicited by the depolarizing test command was measured for each of the pre-pulse potentials. After collecting the baseline measurements, slices were perfused with one of the two CB1 receptor agonists WIN 55,212-2 (1µM) or ACEA (1µM) in the presence of TEA, 4-AP and TTX for 7-8 min, and then the activation and inactivation protocols were run again. We assessed CB1 receptor specificity by testing the effect of WIN 55,212-2 on the Iₐ protocols in the presence of the CB1 receptor antagonist AM251 (1 µM). To assess the sensitivity of the Iₐ to antagonism by 4-AP or rHptx2, the protocols occasionally were executed in the presence of higher (3 or 10 mM) concentrations of 4-AP or 100 nM rHptx2. In some experiments, we tested whether cannabinoids presynaptically modulate the Iₐ by blocking the actions of spontaneously released amino acid neurotransmitters at N-methyl-D-aspartate (NMDA), non-NMDA and GABA_A receptors with CGS 19755 (10 µM), NBQX (3 µM) and SR 95531 (10 µM), respectively, in conjunction with blockade of synaptic transmission with TTX. The amplitude and voltage-dependence of the Iₐ were analyzed using p-Clamp and SigmaPlot 8.0 software. We obtained estimates of the half-maximal voltage (V₅₀) and maximal peak current (I_max) from the activation and inactivation curves generated by fitting the data (peak current vs. membrane voltage) to the Boltzmann equation (Deadwyler et al.
1995). If we encountered confounding Ca\(^{2+}\) currents that were \(\geq 10\%\) of the \(I_{\text{max}}\), then we performed the experiments in the presence of 300 \(\mu\)M NiCl\(_2\) and 100 nM \(\omega\)-conotoxin MVIIC (as well as TEA, 4-AP and TTX) to block T-, N- and P/Q-type Ca\(^{2+}\) channels. After recording, some slices were processed for immunohistofluorescence as described previously (Ronnekleiv et al. 1990).

Variance homogeneity was evaluated using Cochran’s C test. Comparisons between treatment groups were performed using the one-way or two-way analysis of variance (ANOVA) followed by the Least Significant Difference (LSD) test. Evaluation of the frequency of occurrence was accomplished using the chi-square contingency test in conjunction with Freeman-Tukey deviate (FTD). Differences were considered statistically significant if the probability of error was less than 5%.

Forty neurons from the ARC of castrated female and male guinea pigs exhibited transient outward currents like those shown in Figures 1-3. We observed this transient outward current in an equivalent percentage of ARC neurons from female (24 of 62 neurons; 39%) and male (16 of 39 neurons; 41%) animals. Eight of these neurons were subjected to immunohistofluorescence, and all eight neurons were immunopositive for the Kv4.2 channel subtype (not shown). By contrast, only one of four (25%) neurons not displaying a transient outward current expressed the Kv4.2 channel subtype (not shown). The somas of seven of these neurons extended into adjacent slides, and this enabled us to subsequently test for \(\beta\)-endorphin immunoreactivity. Sixty-two percent (5 of 8) of these neurons also were immunopositive for \(\beta\)-endorphin, suggesting that all POMC neurons exhibiting the transient outward current also expressed the Kv4.2 channel subtype. In addition, both 4-AP and the Kv4.2 channel blocker rHptx2 markedly attenuated these currents (Figure 1). Figures 2A & 2B show membrane current traces observed during whole-cell patch clamp recordings from castrated female and male guinea pigs used to generate inactivation curves (Figure 2C) from which
the voltage dependence of the inactivation of the transient outward current are assessed. Bath application of the CB1 receptor agonist WIN 55,212-2 (1 µM) produced a rightward shift in the inactivation curve for the transient outward current observed in neurons from ovariectomized females. This was manifest by an increase in the V½ over 10 mV (control: -83.8 ± 2.7 mV vs. WIN 55,212-2: -71.1 ± 3.2 mV; p<.05; Figure 2) with no discernable effect on the Imax (708.9 ± 98.6 pA vs. 564.4± 108.5 pA). This effect was completely blocked by the CB1 receptor antagonist AM251 (1 µM; Figure 2C; n = 4), and was not associated with a change in the holding current or slope conductance (not shown). We observed a similar rightward shift in the I_A inactivation curve with the anandamide analog ACEA (control: -78.2 ± 4.4 mV vs. 1 µM ACEA: -69.3 ± 3.3 mV; n = 5). Antagonism of miniature synaptic currents with CGS 19755 (10 µM), NBQX (3 µM) and SR 95531 (10 µM), in conjunction with the blockade of synaptic transmission by TTX, did not alter this modulatory action of cannabinoids. By contrast, WIN 55,212-2 exerted no such shift in the inactivation curve derived from neurons in orchidectomized male guinea pigs (-76.8 ± 3.5 mV vs. -79.4 ± 4.4 mV; Figure 2), and also did not influence the Imax (811.5 ± 154.1 pA vs.807.9 ± 183.8 pA).

Figures 3A & 3B show membrane current traces, also obtained during recordings in neurons from castrated female and male guinea pigs, used to derive the activation curves (Figure 3C) from which we assessed the voltage dependence of the activation of this current. Contrary to the agonist-induced depolarizing shift observed for the inactivation curve in neurons from ovariectomized females (Figure 2), WIN 55,212-2 was without effect on either the V½ or the I_max of the activation curve (-34.3 ± 1.9 mV vs. -30.3 ± 1.7 mV; 804.0 ± 144.7 pA vs. 664.5 ± 121.6 pA; Figure 3B & 3C). In addition, the indices of activation of the transient outward current observed in neurons from
orchidectomized male guinea pigs were likewise unaltered (-29.4 ± 2.0 mV vs. -26.7 ± 3.4 mV; 879.5 ± 285.3 pA vs. 905.2 ± 305.9 pA; Figure 3).

Taken together, these data indicate that POMC neurons express an I_A, and that there is a sex difference in the positive modulation of this current by cannabinoids. These conclusions are based on the following observations: 1) guinea pig ARC neurons, including those immunopositive for β-endorphin and/or the Kv 4.2 channel subunit express a transient outward current that is sensitive to antagonism by 4-AP and rHptx2 and 2) the CB1 receptor agonists WIN 55-212-2 and ACEA induce a rightward shift in the inactivation curve for this current in female but not male animals. This I_A was prominently displayed in 40% (40 of 101) of ARC neurons, which is slightly above the 30% incidence that we previously reported for neurons in the guinea pig preoptic area (Wagner et al. 2000).

Essentially, the cannabinoid-induced rightward shift in the inactivation curve for the I_A means that less of a hyperpolarizing stimulus is required to remove the ion channel from inactivation. Therefore, more of the channels would be available at subthreshold membrane voltages to provide a transient outward current that would help offset subsequent depolarizing stimuli, and thereby lower neuronal firing rate. Indeed, the I_A promotes membrane repolarization during an action potential and increases the interspike interval between action potentials, thereby reducing action potential duration and firing frequency (Rudy 1988). Future current clamp studies will test the ability of cannabinoids to increase spike latency, and to decrease spike duration and firing frequency, in response to positive, depolarizing test current pulses that immediately follow a series of hyperpolarizing pre-pulses that vary in magnitude. Furthermore, this cannabinoid-induced shift in the voltage dependence of the I_A inactivation is consistent with that previously reported for cultured hippocampal neurons, a process that was attributed to a reduction in intracellular cAMP levels (Deadwyler et al. 1995).
A cannabinoid-induced increase in the Iₐ and the corresponding decrease in the firing rate of POMC neurons would decrease the release of the anorexic peptides α-MSH, β-endorphin and CART co-expressed in these cells. This would account, in part, for the prominent hyperphagia elicited by cannabinoids (Harrold and Williams 2003; Horvath 2003). Moreover, the observed sex difference in the CB1 receptor coupling to the Iₐ in cells integral to hypothalamic feeding circuitry provides some insight into the cellular determinant(s) that may ultimately lead to gender differences in feeding behavior. Indeed, the ability of centrally administered CB1 receptor agonists like CP 55,940 to stimulate consumption of highly palatable foodstuffs is sexually differentiated (Miller et al. 2004). Finally, the sex difference in CB1 receptor/Iₐ coupling observed in the present study is also consistent with reported sex differences in cannabinoid-induced antinociception (Tseng and Craft 2001), locomotion (Tseng and Craft 2001; Wiley 2003) and postural syncope (Mathew et al. 2003).

In conclusion, these results reveal a sex difference in the cannabinoid modulation of the Iₐ in ARC neurons, including POMC neurons. They also impart some insight into the mechanism(s) by which cannabinoids alter energy homeostasis.

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References


Fig. 1 4-AP and the Kv4.2 channel blocker rHptx2 attenuate the $I_A$ in ARC neurons of the guinea pig hypothalamus.  

A, $I_A$ observed in an ARC neuron under baseline control conditions, and in the presence of 3mM and 10mM 4-AP. Currents were generated according to the inactivation protocol.

B, A bar graph illustrating 4-AP inhibition of the $I_A$. Columns represent the mean and vertical lines 1 S.E.M. of the normalized outward current observed in the presence of varying concentrations of 4-AP during the test pulse immediately following the hyperpolarizing pre-pulse to -110 mV.

C, $I_A$ observed prior to and the presence of 100 nM rHptx2. D, A bar graph that illustrates the rHptx2 diminution of the $I_A$. Columns represent the mean and vertical lines 1 S.E.M. of the normalized outward current as described in B.
Fig. 2 CB1 receptor activation alters the voltage dependence of the $I_A$ inactivation in a sex-dependent fashion.  

A, The $I_A$ evoked under baseline control conditions in hypothalamic neurons from female (top) and male (bottom) guinea pigs during the inactivation protocol. The $I_A$ is observed as a transient outward current immediately following delivery of the test pulse (denoted by the arrow).  

B, The $I_A$ evoked in the presence of WIN 55,212-2 (1 µM) in the same neurons using the same inactivation protocol as in A.  

C, Composite inactivation curves for the $I_A$ derived from recordings of female (top) and male (bottom) guinea pig hypothalamic neurons. The Boltzmann equation fits the curves to the corresponding data points. Symbols and accompanying vertical lines represent means $\pm$ S.E.M. of peak currents normalized to the $I_{\text{max}}$ that were observed at the test pulse following a given pre-pulse. The horizontal dashed lines seen in both graphs represent $I/I_{\text{max}} = 0.5 = V_{1/2}$.  

Top graph: three vertical dotted lines intersect the abscissa at different points; representing the $V_{1/2}$ observed under baseline control conditions (center), in the presence of 1 µM WIN 55,212-2 (right) and in the presence of WIN 55,212-2 and the CB1 receptor antagonist AM251 (left). Bottom graph: two vertical dotted lines intersect the abscissa; the one on the right represents the $V_{1/2}$ observed under baseline control conditions, and the one on the left represents the $V_{1/2}$ observed in the presence of 1
μM WIN 55,212-2. *, The estimated V½ derived from hypothalamic neurons in the presence of WIN 55,212-2 that is significantly different (p<.05; one-way ANOVA/LSD) than that observed under baseline control conditions and in the presence of the antagonist.

Fig. 3 WIN 55,212-2 has no effect on the voltage dependence of the I_A activation. A, The I_A evoked under baseline control conditions in guinea pig hypothalamic neurons from female (top) and male (bottom) animals during the activation protocol. The I_A is observed as a transient outward current immediately following pulse delivery (denoted by the arrow). B, The I_A evoked in the presence of WIN 55,212-2 (1 μM) in the same neurons using the same activation protocol as in A. C, Composite activation curves for the I_A derived from recordings of hypothalamic neurons from female (top) and male (bottom) guinea pigs. The curves were generated by fitting the corresponding data points to the Boltzmann equation. Circular symbols represent means and vertical lines 2 S.E.M. of the peak currents observed at the various voltage commands that were normalized to the estimated I_max. The horizontal dashed lines seen in both graphs represents I/I_max = 0.5 = V½. For both graphs, the vertical
dotted line on the left represents the $V_{1/2}$ observed under baseline control conditions, whereas that on the right represents the $V_{1/2}$ observed in the presence of 1 μM WIN 55,212-2.