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PRESYNAPTIC ACTIONS OF OPIOID RECEPTOR AGONISTS IN VENTROMEDIAL HYPOTHALAMIC NEURONS IN ESTROGEN- AND OIL-TREATED FEMALE MICE

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Abstract—Estrogens act upon ventromedial hypothalamic (VMH) neurons, and their effects on female arousal and sexual behaviors mediated by VMH neurons involve several neurotransmitters and neuromodulators. Among these are opioid peptides which might be predicted to oppose estrogenic action on VMH because they tend to decrease CNS arousal. Spontaneous excitatory postsynaptic currents were recorded from VMH neurons from 17β-estradiol- (E, 10 μg/0.1 ml) or oil-treated control ovariectomized (OVX) mice using whole-cell patch-clamp techniques. To examine the impact of opioidergic inputs, recordings of neurons from both treatment groups were obtained in the presence of the general opioid receptor agonist methionine enkephalin-Arg-Phe (MERF, 3 μM), or μ-receptor specific agonist [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO, 1 μM). Compared with oil, E treatment for 48 h significantly increased the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) without affecting their amplitude. MERF and DAMGO each abolished this E effect, causing significant reductions in sEPSCs. The effect of MERF was abolished by naltrexone (general opioid receptor antagonist, 3 μM) and the effect of DAMGO by D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH$_2$ (CTAP) (μ-opioid receptor selective antagonist, 1 μM); in contrast, κ- and δ-opioid receptor agonists, U69593 (300 nM) and [D-Pen^2,D-Pen^5]-enkephalin (DPDPE, 1 μM) respectively, had little effect on the sEPSCs compared with DAMGO. To consider presynaptic vs. postsynaptic effects of opioids, miniature excitatory postsynaptic currents (mEPSCs) were investigated in E- and oil-treated VMH neurons and opioid receptor antagonist effects on mEPSCs were observed. Both MERF and DAMGO reduced the frequency of mEPSCs, but had no effect on their amplitude. Our findings indicate that opioids suppress excitatory synaptic transmissions in VMH neurons primarily through μ-receptors and could thereby decrease sexual arousal in mice.

Key words: VMH, opioid receptor, patch-clamp, arousal, lordosis.

Among three identified major opioid receptors (μ-, δ- and κ-receptors), the μ-receptor is the principal receptor responsible for narcotics addiction and reward seeking behaviors. Typical G-protein-coupled receptors (GPCRs), μ-receptors are highly expressed in several brain regions (Hawkins et al., 1988; Mansour et al., 1988; Besse et al., 1990). Their physiological functions include regulation of pain and analgesia, respiratory and cardiovascular functions, intestinal transit, feeding, mood, thermoregulation, hormone secretion and immune functions (Dhawan et al., 1996). In addition, μ-receptors are known to be involved in steroid hormone–regulated sexual behaviors (Forsberg et al., 1987; Pfaus and Gorzalka, 1987a,b; Vathy et al., 1991; Pfaus and Pfaff, 1992).

The most intensively studied sex behavior, displayed by female rodents in a laboratory setting, is estrogen-dependent lordosis (Pfaff et al., 1994). Lordosis has served as a very useful model to examine the mechanisms of opioid action during female reproduction. For instance, exogenous opiates such as morphine and morphiceptin inhibit lordosis, depending on method of administration as well as on concentration used (Pfaus et al., 1986; Vathy et al., 1991). Infusion of low doses of endogenous opioid peptide β-endorphin into a steroid-primed female ovariectomized (OVX) rat's third ventricle (Wiesner and Moss, 1984, 1986), lateral ventricles (Pfaus et al., 1986; Pfaus and Gorzalka, 1987b), or medial preoptic area (mPOA) (Sirinathsinghji, 1986) inhibits lordosis behavior. The opposite effect was achieved, i.e. lordosis behavior was facilitated, when naloxone was administered centrally and this effect displayed dose and site specificity (Pfaus and Gorzalka, 1987a). Many of the brain regions involved in the hormonal regulation of lordosis behavior contain opioid peptides as well as opioid receptors. Infusion of a potent μ-receptor agonist into ventromedial hypothalamic nuclei (VMH) and mPOA inhibited lordosis (Acosta-Martinez and Etgen, 2002a).

While a primary function of opioids is related to pain regulation, they also reduce generalized CNS arousal (Pfaff, 2005). Thus, one opportunity in the current study is to examine the potential impact of a generalized arousal (Garey et al., 2003) neurochemical system (μ-receptor signaling) on neurons concerned with specifically sexual arousal.

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Abbreviations: ACSF, artificial cerebrospinal fluid; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH$_2$; DAMGO, [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; E, 17β-estradiol; GPCRs, G-protein-coupled receptors; mEPSC, miniature excitatory postsynaptic current; MERF, methionine enkephalin-Arg-Phe; mIPSC, miniature inhibitory postsynaptic current; mPOA, medial preoptic area; OVX, ovariectomized; R-ACSF, regular artificial cerebrospinal fluid; S-ACSF, sucrose-based artificial cerebrospinal fluid; sEPSC, spontaneous excitatory postsynaptic current; TTX, tetrodotoxin; U69593, κ-opioid receptor agonist; VMH, ventromedial hypothalamic nuclei.

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We hypothesized that μ-receptor signaling would oppose estrogenic actions on hypothalamic neurons. Indeed, in female rats, estrogens also oppose μ-receptors inhibitory effects on sex behavior regulating neurons (Eckersell et al., 1998; Micevych et al., 2003; Sinchak and Micevych, 2001). But relations between estrogens and opioid systems are complex; estrogen treatment of OVX animals can increase μ-receptor levels (Martini et al., 1989; Hammer, 1990; Weiland and Wise, 1990; Joshi et al., 1993; Piva et al., 1995; Quinones-Jenab et al., 1997).

Two important electrophysiological actions of opioids are: first, they hyperpolarize cells by increasing membrane K^+ conductance; and second, they inhibit synaptic transmission by reducing voltage-dependent Ca^{2+} currents. Estrogens have been reported to modulate the coupling of μ-receptor to a K^+ channel (Cunningham et al., 1998). Twenty-four hour pretreatment of OVX guinea pigs with estrogen decreases the potency of the μ-receptor agonist [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) to hyperpolarize hypothalamic arcuate neurons (Kelly et al., 1992). Since lordosis is fostered by increased electrical activity in VMH (Pfaff, 1999), we tested the hypothesis that a general and a μ-receptor selective agonist would decrease synaptic transmission to VMH neurons. That would be important because, in turn, that decrease would be expected to decrease VMH neuronal excitability and thus lead to decreased sexual arousal. We concentrated on the posterior ventrolateral part of the VMH because a high percentage of these neurons express estrogen receptors (Pfaff and Keiner, 1973), and destruction of these neurons abolishes lordosis behavior (Pfaff and Sakuma, 1979).

EXPERIMENTAL PROCEDURES

Animals and slice preparation

Four-week-old OVX female Swiss-Webster mice (n=42) were treated with either 17β-estradiol (E, 10 μg/0.1 ml; Sigma, St. Louis, MO, USA), s.c. per day, a physiologically active dose for eliciting lordosis behavior (Kow et al., 1994) or sesame oil for two consecutive days prior to patch-clamping. This age was appropriate because at this stage females (but not males) respond well to ovarian hormones by increasing lordosis (Kow et al., 2005). Hypothalamic slices containing VMH were prepared as described previously (Kow and Pfaff, 1995). Briefly, on the day of recording, mice were deeply anesthetized with urethane solution (16 mg/kg BW), and decapitated. Brains were promptly removed to oxygenized (95% O_2 and 5% CO_2, Matheson Tri-Gas, Parsippany, NJ, USA) ice-cold sucrose-based artificial cerebrospinal fluid (S-ACSF). Coronal slices (250 μM thick) containing VMH were prepared on a 1000 plus sectioning system (Vibratome, St. Louis, MO, USA), using S-ACSF solution consisting of (mM): 210 sucrose, 3.5 KCl, 1 $CaCl_2$, 4 $MgCl_2$, 1.25 NaH_2PO4 and 10 D-glucose. Slices were then transferred into a regular artificial cerebrospinal fluid (R-ACSF) solution consisting of (mM): 126 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 D-glucose, 1.25 MaH_2PO4 and 26 $NaHCO_3$. The pH of both solutions was adjusted with NaOH to 7.3, and osmolarity was 300–310 mOsmol/l. They were maintained in R-ACSF for 45 min before whole cell patch-clamp and recordings were done at room temperature. Artificial cerebrospinal fluid (ACSF) was saturated with 95% O_2 and 5% CO_2.

All animals had food and water available *ad libitum*. All handling procedures of mice followed a protocol approved by the Rockefeller University's Animal Care and Use committee (IACUC) in accordance with the Animal Welfare Act and the DHHS "Guide for the Care and Use of Laboratory Animals." Every effort was made to minimize the number of animals used and their suffering.

Drugs

Methionine enkephalin-Arg-Phe (MERF; 3 μM) is an opioid heptapeptide that is an agonist for μ-, δ-, and κ-opioid receptors. It was used to test general opioid agonist effects on spontaneous excitatory postsynaptic currents (sEPSCs). The selective μ-receptor agonist DAMGO (3 μM) was used to test μ-receptor mediated effects. Specificity of MERF- or DAMGO-mediated effects was tested by using general opioid receptor antagonist naltrexone (3 μM) or specific μ-receptor antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP) (1 μM) respectively. When recording miniature inhibitory (mIPSCs) or excitatory (mEPSCs) postsynaptic currents, tetrodotoxin (TTX) 0.5 μM/cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX) 10 μM or TTX/bicuculline (40 μM) was added to the ACSF solution accordingly. κ- And δ-receptor specific agonists, U69593 (300 nM) and [D-Pen^2, D-Pen^5]-enkephalin (DPDPE; 1 μM) respectively, were used as well to examine their involvement in synaptic transmissions. All reagents were purchased from Sigma (St. Louis, MO, USA), except for bicuculline, which was from Tocris (Ellisville, MO, USA). All drugs were diluted to final concentration in a fresh ACSF solution on the day of patch-clamp recordings.

Patch-clamp recordings

Whole cell patch-clamp recording was performed using the MultiClamp 700 B amplifier (Axon Instruments, Foster City, CA, USA). One slice was placed in the recording chamber and neurons were visualized on a video monitor with a Nikon E600FN microscope (Morrell, Melville, NY, USA). While in the recording chamber, each slice was perfused constantly with ACSF at 2 ml/min, at room temperature. Whole cell recordings were made with a glass pipette filled with a solution containing (mM): 140 K-gluconate, 10 Hepes-K, 0.6 $NaHCO_3$, 2.0 Mg-ATP, 2.0 Na_2-ATP, 0.3 Na-GTP, 8.3 sucrose, 2 $MgCl_2$-6 H_2O, 1 $CaCl_2$ and 5 EGTA (pH: 7.3 and osmolarity 280 mOsmol/l). A borosilicate glass pipette (Warner Instruments Inc., Hamden, CT, USA) was constructed on using a Narishige PP-830 vertical electrode puller (Narishige, Greenvale, NY, USA). While in the bath, tip resistance was 4–5 MΩ, series resistance 10–15 MΩ, and the holding potential was −70 mV. Data were collected by using PCLAMP 9.2 and analyzed by CLAMPFIT software (Axon Instruments). Spontaneous synaptic and mIPSC/EPSCs were analyzed by using MINIANALYSIS software (Synaptosoft, Decatur, GA, USA).

Statistics

Data were analyzed using Clampfit 9.2. software (Axons Instruments) and plotted with GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). Values are presented as mean±S.E.M. Each neuron served as its own control when agonists were assessed, i.e. in each neuron the effect of any drug was assessed before and after use of an agonist and was analyzed using the Student's *t*-test or one-way analysis of variance (ANOVA) followed by Turkey's post hoc tests when appropriate. The effects of E were also measured using the Student's *t*-test. Any neuron showing a change of more than 30% due to drug administration was classified as a responsive neuron. Those that never attained a 30% change were classified as non-responsive neurons and data from these neurons were not included in the data analyses. In all cases significance set at $P<0.05$.

RESULTS

Electrophysiological recordings were obtained from 126 VMH neurons. Almost all recordings were obtained from