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Cognition and Behavior

Optogenetic Activation of β -Endorphin Terminals in the Medial Preoptic Nucleus Regulates Female Sexual Receptivity

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Abstract

Estrogen and progesterone (P4) act in neural circuits to elicit lordosis, the stereotypical female sexual receptivity behavior. Estradiol acts through membrane receptors to rapidly activate a limbic-hypothalamic circuit consisting of the arcuate (ARH), medial preoptic (MPN), and ventromedial (VMH) nuclei of the hypothalamus. This initial activation results in a transient but necessary inhibition of lordosis, which appears to be a result of the release of β -endorphin (β -End) from proopiomelanocortin (POMC) terminals onto cells containing the μ -opioid receptor (MOR) in the MPN. To functionally examine the role of the MOR in the hypothalamic lordosis circuit, we transfected a channelrhodopsin (ChR2) adeno-associated virus into POMC cell bodies in the ARH and photostimulated POMC/ β -End axon terminals in the MPN in sexually receptive female Pomc-cre mice. Following estrogen and P4 priming, sexual receptivity was assessed by measuring the lordosis quotient (LQ). Following an initial trial for sexual receptivity, mice were photostimulated during behavioral testing, and brains were processed for MOR immunohistochemistry (IHC). Photostimulation decreased the LQ only in ChR2-expressing Pomc-cre mice. Furthermore, photostimulation of ChR2 in POMC/ β -End axon terminals in the MPN resulted in the internalization of MOR, indicating activation of the receptor. Our results suggest that the activation of the MOR in the MPN is sufficient to attenuate lordosis behavior in a hormone-primed, sexually receptive female mouse. These data support a central role of MOR in female sexual behavior, and provide further insight into the hypothalamus control of sexual receptivity.

Key words: β -endorphin; estradiol; lordosis; μ -opioid receptor; POMC

Significance Statement

The μ -opioid receptor (MOR) has been shown to modify lordosis, the female sexual receptivity behavior. Estrogen and progesterone (P4) facilitate lordosis, though the initial effects of estradiol in the arcuate nucleus of the hypothalamus (ARH) inhibit the behavior through MOR activity in the medial preoptic nucleus (MPN). The present study uses optogenetics to directly stimulate proopiomelanocortin (POMC)/ β -endorphin (β -End) axonal terminals in the MPN in sexually receptive female mice to examine the effects of MOR activity on lordosis. Photostimulation of these terminals is sufficient to activate and externalize the MOR, and to attenuate lordosis behavior in otherwise sexually receptive mice. Our results provide further evidence of an ARH to MPN projection that, by activating MOR, is inhibitory to lordosis behavior.

Introduction

Innate motivated behaviors are crucial for the survival of the individual (e.g., eating, drinking) or the species (e.g., reproduction). Reproduction is dependent on the sexual receptivity of the female, which in many species is regulated by ovarian steroids at specific times. Lordosis, female sexual receptivity behavior, is a reflexive behavior in which the female arches the spine, elevates the hind quarters, and lifts the tail and head to allow the male to intromit (Beach, 1948; Pfaff et al., 1994). In cycling females the release of ovarian hormones tightly regulates receptivity through the priming of specific neural circuits along with appropriate sensory cues (Micevych and Sinchak, 2007), to ensure that lordosis occurs in response to mounting by a conspecific male (Micevych and Ulibarri, 1992; Micevych and Sinchak, 2007) at a time in the cycle that maximizes the potential for reproductive success. While the manifestation of such a behavior involves many sites along the neuraxis, a few critical circuits have emerged that are involved in the production of lordosis behavior, including mesolimbic and hypothalamic- limbic circuits (Micevych and Sinchak, 2007; Micevych and Meisel, 2017).

The circuit consisting of the arcuate nucleus (ARH), medial preoptic nucleus (MPN), and ventromedial nucleus (VMH) of the hypothalamus has been shown to be vital for the expression of lordosis (Sinchak and Micevych, 2001; Micevych and Meisel, 2017; Fig. 1). A subset of neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons located in the ARH express estrogen receptor (ER) α (Sar et al., 1990; Simonian et al., 1999) and are involved in reproduction (Cheung and Hammer, 1995; Cheung et al., 1995). Indeed, estradiol initially acts in the ARH to activate both NPY and POMC neurons. Previous work has indicated that estradiol treatment activates NPY-Y1 receptors on POMC/ β -endorphin (β -End) neurons within the ARH (Mills et al., 2004).

The POMC population within the ARH is highly heterogeneous, both in physiology and function (Cheung and Hammer, 1995; Ibrahim et al., 2003; Hentges et al., 2009; Campbell et al., 2017; Lam et al., 2017), and this heterogeneity makes parsing out the different roles of these neurons difficult. POMC neurons span the rostral-caudal extent of the ARH and project to various nuclei throughout the hypothalamus and surrounding regions (Sinchak and

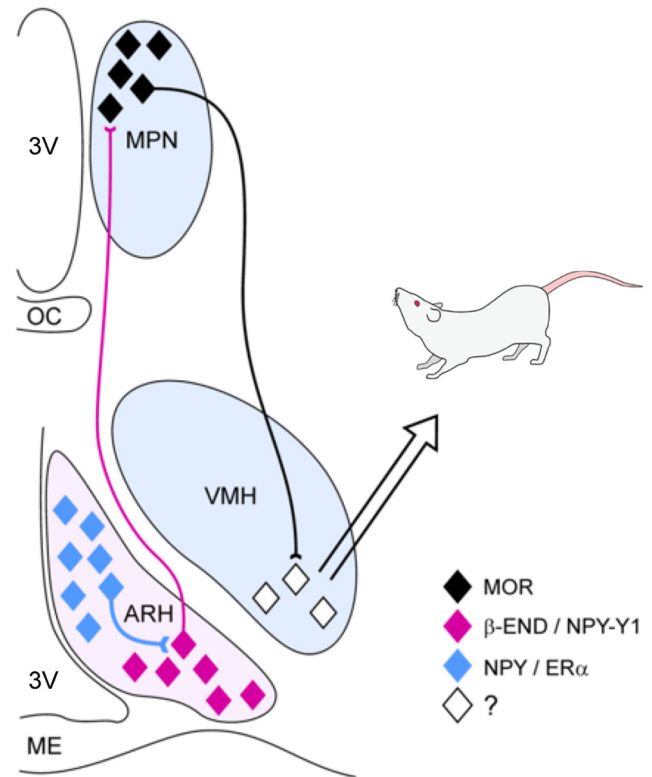


Figure 1. Hypothalamic lordosis circuit. Estradiol acts initially on NPY neurons in the ARH, which project to and activate POMC/ β -End neurons. These neurons project to the MPN where the release of β -End activates and internalizes MORs. When these receptors are internalized, lordosis is attenuated. Neurons from the MPN project further to the VMH, where signals from other circuits are integrated before projecting to lower brain regions and ultimately to the spinal motor neurons responsible for the manifestation of lordosis behavior. 3V, 3rd ventricle; OC, optic chiasm; ME, median eminence. Figure adapted from Micevych and Christensen (2012), with permission.

Micevych, 2003; Smyth, 2016). The rostral ARH POMC population that is involved in reproduction appears to be separate from that involved in food intake, distinguished by distinct morphologic properties (Cheung and Hammer, 1995), as well as physiologic properties (Ibrahim et al., 2003). These reproductively important POMC neurons project to the medial portion of the MPN, where there is a concentration of cells that express μ -opioid receptors (MORs; Simerly and Swanson, 1986; Cheung and Hammer, 1995; Eckersell et al., 1998; Mills et al., 2004).

MORs have been implicated in regulating sexual receptivity in rodents (Torii et al., 1999; Sinchak and Micevych, 2001; Acosta-Martinez and Etgen, 2002a; Mills et al., 2004; Micevych et al., 2017), mediated by the release of the endogenous opioid peptide β -End, which has a high affinity for MOR (Birdsall and Hulme, 1976). Activation of MORs can be monitored by examining their cellular location; MOR-immunoreactivity (MOR-ir) localized primarily on the cell surface indicates receptors that have not been activated, while activated MOR are rapidly internalized and appear in the cytoplasm. The initial actions of estradiol within this circuit result in a transient but necessary

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Table 1. Experimental group parameters

Group	Virus	Hormone replacement	<i>n</i>
Group 1	ChR2	EB+P4	7
Group 2	Control	EB+P4	6
Group 3	ChR2	Oil	4
Group 4	Control	Oil	4
Group 5	ChR2	EB+P4	4

Presented are the groups of mice used in this study, including AAV, hormone treatment, and number of mice per group. All hormones were dissolved in safflower oil and delivered subcutaneously 2 h before lights off. Mice that did not receive hormone replacement instead received an equal volume of safflower oil only.

inhibition of lordosis, through the activation and internalization of MOR in the MPN (Eckersell et al., 1998; Sinchak and Micevych, 2001; for review, see Micevych et al., 2017).

The estradiol activation of MOR is dependent on ER α (Micevych et al., 2003). MOR activation is out of phase with sexual receptivity throughout the estrous cycle (Sinchak and Micevych, 2003); when receptors are engaged lordosis is inhibited, and reversal of this activation by progesterone (P4) allows lordosis behavior (Sinchak and Micevych, 2001; for review, see Micevych and Meisel, 2017). MPN neurons project to the VMH (Simerly and Swanson, 1988), the final integrative hub for lordosis behavior within the hypothalamus. From here, projections innervate lower brain regions and eventually the spinal motor neurons to produce the behavior (for review, see Micevych et al., 2015; Micevych and Meisel, 2017).

We tested whether optogenetic activation of β -End terminals only in the medial MPN would lead to the activation/internalization of MOR and inhibition of sexual receptivity. To this end, channelrhodopsin (ChR2)-expressing AAVs were inserted into Pomc-cre neurons in the ARH, followed by selective photostimulation of the β -End-releasing terminals in the MPN in hormone-primed, sexually receptive female mice, and lordosis behavior was examined.

Materials and Methods

Animals

Adult [postnatal day (P)60] female Pomc-cre mice [originally JAX #005965 (Balthasar et al., 2004) and #010714 (McHugh et al., 2007)] from our colony, and adult (P60) male C57/Bl6J from Charles River (Charles River Laboratories, Inc.) were used for all experiments. All female mice received ovariectomies and fiber optic cannulae (see below, Surgeries). Mice were randomly assigned to one of four groups: (1) ChR2-AAV + hormone replacement, (2) control AAV + hormone replacement, (3) ChR2-AAV + oil control, or (4) control AAV + oil control. An additional control group was cre-positive mice that received the ChR2-AAV and hormone replacement, but in which the fiber optic cannulae was incorrectly placed. See Table 1 for group descriptions and Table 2 for the number of mice of each strain per group. All animal procedures were performed in accordance with the regulations of the University of California-Los Angeles Chancellor's Animal Research Committee.

Table 2. Transgenic strains used in study

Group	JAX #005965	<i>n</i>	JAX #010714
Group 1	6		1
Group 2	4		2
Group 3	2		2
Group 4	2		2
Group 5	4		0

Two strains of Pomc-cre mice were used in this study; provided are the number of each strain of mouse used in each experimental group (described in Table 1).

Surgeries

Adult (P60) female Pomc-cre mice were anesthetized under isoflurane and transfected with an AAV expressing ChR2 (AAV1.CAGGS.Flex.ChR2-tdTomato.WPRE.SV40; Addgene catalog #18917-AAV1) or the control virus (AAV1.CAG.Flex.tdTomato.WPRE.bGH; Addgene catalog #28306-AAV1). AAVs were delivered bilaterally into the ARH (from bregma; AP: -1.50 , ML: ± 0.25 , DV: -5.50) with a 5- μ l Hamilton syringe (Hamilton Company, #7634-01) equipped with a 32-G removable needle (Hamilton Company, #7803-04), via World Precision Instruments Ultra-MicroPump (World Precision Instruments, Inc., UMP3-3), at a rate of 30 nl/min for a total volume of 300 nl per side. AAVs were allowed five weeks to incubate to result in full fiber expression in the MPN before behavioral testing. Following injections, a custom-made ferrule fiber (200- μ m core diameter, 240- μ m outer diameter, Doric Lenses) was implanted into the MPN (from bregma; AP: 0, ML: ± 0.35 , DV: -4.75) and fixed on the skull with dental cement (Parkell, Metabond). Two weeks before behavioral testing, mice were bilaterally ovariectomized (ovx), to allow sufficient time for the loss of endogenous hormones.

Hormone replacement

Mice received subcutaneous injections of 17 β -estradiol benzoate (EB) and P4 dissolved in safflower oil, 2 h before lights out, over a 3-d period to mimic the estrous cycle. On day 1, mice received 20- μ g EB, on day 2, 10- μ g EB, and on day 3, 250- μ g P4. Control mice received injections of equivalent volumes of safflower oil only.

Behavioral and optogenetic testing

Lordosis behavior was tested 2 h after lights off on the third day of hormone injections. Before running experimental behavior tests, each female was exposed to a sexually experienced male once before behavior testing as training. Data from three subsequent sets of experimental behavioral tests were collected and averaged per test per animal. Approximately 15 min before behavior testing, sexually experienced adult male mice were placed in individual Plexiglas testing arenas to acclimate. Immediately before being placed in the arena with the male, the optogenetic patch cord (Doric Lenses) was attached to the implanted fiber optic cannula via a zirconia mating sleeve (Doric Lenses). The patch cord remained attached for the duration of the behavior test. A single round of behavioral testing consisted of two interactions with a male mouse, each interaction lasting for 10

Table 3. Primary antibodies

Antibody	Dilution	Source
Rabbit anti-MOR	1:4000	NeuroMics, #RA10104
Rabbit anti-cFOS	1:5000	Abcam, #ab190289
Rabbit anti-POMC	1:8000	Phoenix Pharmaceuticals Inc., #H-029-30

Provided is the species in which the antibody was raised, dilution of antibody in microliters, and commercial source of primary antibodies used.

mounts by the male. If the male ejaculated, the female was moved to a different arena with a different male to continue testing. For the first interaction of the behavior test, each female mouse was subject to a pre-test to determine sexual receptivity, measured by the lordosis quotient (LQ; the number of times a female displays lordosis/10 mounts by a male \times 100). If the mouse displayed a LQ $<$ 50 it was considered unreceptive, and there was no further testing that day. Each test began when the female was placed in the cage and lasted until the male mounted the female 10 times. Following the pre-test, the female was removed from the arena and the laser was switched on for at least 5 min before the second test, to allow for release of the peptide and engagement of MORs before being placed back in the arena with the male. Photostimulation (ChR2, 473 nm, 20 Hz, 20-ms pulses, 1–3 mW/mm²) was applied for the duration of second interaction with the male. Behavior tests were recorded using a Yi Action Camera (XiaoYi Technology Co., LTD) and scored by observers blinded to experimental condition. Following the final behavior test mice were perfused 60 min after removal from the arena. For each animal, the results of the three pre-tests were averaged to a single LQ score, as were the results of the three photostimulation tests.

Perfusion, removal, and sectioning

Mice were transcardially perfused with cold 0.9% saline, followed by cold 4% paraformaldehyde (PFA) in Sorrenson's buffer (pH 7.4). Brains were removed and post-fixed in the same PFA solution for 24 h, then switched to 30% (w/v) sucrose in phosphate buffer overnight before being flash frozen in hexanes cooled on dry ice. Brains were sectioned 25 μ m coronally using a Leica cryostat (Leica Biosystems, CM1950) and stored in a cryoprotectant solution at -20°C until used for immunohistochemistry (IHC).

IHC

Sections containing the MPN were processed for MOR IHC, and sections containing the ARH were processed for cFOS IHC to verify POMC/ β -End neuronal activation, via tyramide signal amplification (TSA) process. Sections

were washed in tris-buffered saline (TBS; pH 7.4) on a rotating table for 30 min at room temperature (RT). Sections were transferred to a solution of 0.5% H₂O₂ in TBS for 10 min, then washed again in TBS for 30 min. Sections were then blocked in a solution of 2% normal goat serum (NGS; Equitech-Bio, #SG30-0500) and 0.03% Triton X-100 (Sigma-Aldrich, #X100-100ML) in TBS for 1 h at RT. Sections incubated for 1 h at RT in a 1% NGS/0.03% Triton X-100 solution, containing either rabbit anti-MOR or rabbit anti-cFOS (Table 3), before being placed on a rotating table at 4°C for 48 h. Sections were then washed in a solution of 0.03% Triton X-100 in TBS (TBST) for 30 min at RT, then transferred to a solution containing biotinylated goat anti-rabbit antibody, at 1:300 dilution, in TBST for 1 h. Sections were washed in TBST for 30 min as before, then incubated in a TBST solution containing 0.2% Solution A and 0.2% Solution B (VectaStain ABC HRP kit, #PK-4000, Vector Laboratories) for 1 h at RT. Sections were rinsed in TBS for 30 min, then transferred to a solution containing 0.6% biotinylated tyramide in a borate buffer. Sections were washed in TBS as before, then incubated in a TBST solution containing Streptavidin conjugated to Alexa Fluor 488 (1:2000; Table 4). Sections were rinsed a final time in TBS, mounted onto SuperFrost slides (Fisher Scientific, #12-550-15). Once dry, slides were applied with mounting medium containing DAPI (DAPI Fluoromount-G, Southern Biotech, #0100-20) before being coverslipped and sealed with nail polish. Slides were stored in the dark at -20°C until imaging.

To verify that neurons infected by the AAVs expressed POMC, IHC was performed using an antibody against POMC (1:8000; Table 3). Sections containing the ARH were washed 3 \times 10 min in TBS and blocked in a solution of TBS/2% NDS/0.3% Triton X-100 for 1 h at RT. Sections were incubated in a solution containing rabbit anti-POMC at 4°C overnight. Sections were again washed at RT as before, then transferred to a solution containing the fluorescent secondary antibody donkey anti-rabbit Alexa Fluor 488 (1:2000; Table 4) and allowed to incubate for 2 h at RT. Sections were washed a final time before being mounted onto SuperFrost slides as above. See Tables 3, 4 for dilutions and commercial source of antibodies used.

Imaging

Images were obtained with a Zeiss LSM880 (Zeiss Blue software, Zeiss) using the 405, 488, and 561 laser lines, with appropriate emission filters to prevent optical bleed through. Sections containing the MOR in the MPN were imaged with a 63 \times oil objective (NA 1.4), employing an optical zoom of 4.7 for a final magnification of \sim 300. Three cells expressing MOR-ir in the dorsal part of the medial MPN were imaged at three Z-planes spanning the

Table 4. Secondary antibodies

Antibody	Dilution	Source
Biotinylated goat anti-rabbit IgG	1:300	Vector Laboratories, #BA-1000
Streptavidin Alex Fluor 488	1:2000	Molecular Probes, #S-11223
Alexa Fluor 488 donkey anti-rabbit IgG	1:2000	Jackson ImmunoResearch, #711-545-152

Provided is the type of secondary antibody including species in which it was raised, dilution of antibody in microliters, and commercial source.

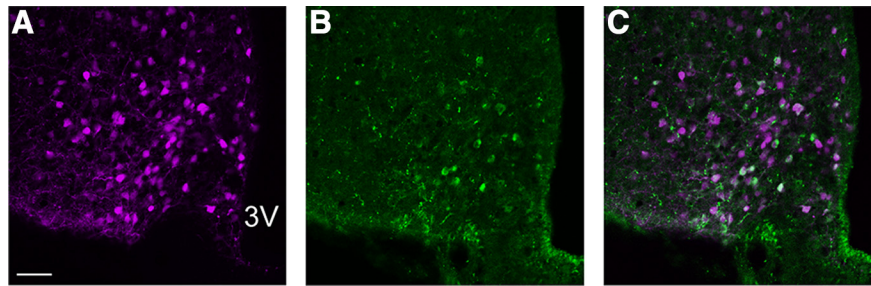


Figure 2. POMC expression in tdTomato-labeled cell bodies. IHC was run to confirm that tdTomato was expressed in POMC neurons in the ARH. **A**, tdTomato in magenta. **B**, Rabbit anti-POMC in green. **C**, Merged image showing tdTomato and POMC-ir. 3V, 3rd ventricle. Scale bar = 50 μm .

depth of the cell. Cells in the ARH expressing cFOS, and those labeled with the antibody against POMC, were imaged using a 10 \times (NA 0.45) or 20 \times (NA 0.75) objective to assess colocalization of cFOS-ir or POMC-ir and tdTomato.

Data analysis

Images were analyzed using Imaris software (Imaris 9.2.1, Bitplane, Oxford Instruments Group). Previous studies (Dournaud et al., 1998; Eckersell et al., 1998; Sinchak and Micevych, 2001, 2003; Mills et al., 2004) have indicated a correlation between receptor internalization and the number of MOR-ir structures. To analyze MOR-ir and internalization, the Surfaces feature was used. A ROI containing only the cell of interest was analyzed, with a threshold for MOR-ir set at 1 SD above mean background fluorescent intensity, per image. The minimum size threshold to further exclude nonspecific for a labeled structure was set at 0.1 μm . Images were analyzed for the area of the image that contained MOR-ir, and divided by the total area of the image, to obtain the percentage of the image expressing MOR-ir. Each of the three Z-planes per cell was analyzed and averaged to find the total area covered per analyzed cell, and this process was repeated for the three total cells imaged per animal and averaged.

Colocalization of cFOS-ir and tdTomato, as well as POMC-ir and tdTomato, was analyzed using ImageJ (Schneider et al., 2012). The total number of cells in the ARH expressing tdTomato only, cFOS-ir or POMC-ir only, and both cFOS-ir or POMC-ir and tdTomato was counted. Additionally, the percentage of all cFOS-labeled cells expressing tdTomato, and the percentage of total tdTomato expressing cFOS or POMC, was analyzed. Images were optimized by adjusting the brightness uniformly across all pixels.

Statistics

Statistics for behavioral and imaging data were analyzed using a one-way ANOVA or a two-way ANOVA, followed by Tukey's multiple comparisons tests, as appropriate. All statistics were analyzed using GraphPad Prism version 7.03 for Windows (GraphPad Software). Significance for all analyses was set at $p \leq 0.05$, and all values are expressed as mean \pm SEM.

Results

AAV expression and fiber optic placement

POMC cell bodies were localized along the entire rostral-caudal extent of the ARH, in agreement with previous studies (Finley et al., 1981; Cheung and Hammer, 1995; Smyth, 2016). Along with projections to the MPN, fibers expressing tdTomato projected to various nuclei within the hypothalamus, amygdala, and bed nucleus of the stria terminalis. Fibers rarely innervated the VMH. Transfection of POMC neurons was confirmed using IHC along with fluorescence microscopy, as both the ChR2-AAV and the control virus were conjugated to tdTomato. IHC was used to verify that neurons infected with tdTomato did in fact express POMC. In agreement with Padilla et al. (2012), we found that not all neurons that expressed tdTomato also expressed POMC-ir. In the present study, $\sim 30\%$ of the tdTomato-positive neurons in the ARH also expressed POMC-ir, while nearly 70% of neurons expressing POMC-ir co-expressed tdTomato (Fig. 2).

Only mice in which both cell bodies in the ARH and axonal fibers in the MPN expressed tdTomato were included in the study. Cre-positive mice with no tdTomato expression were excluded from the experimental analyses. All mice showed expression of tdTomato in cell bodies in the ARH (Fig. 3A), and all mice expressed labeled fibers in the MPN (Fig. 3B).

In the MPN, fiber optic cannulae placement was histologically confirmed. A group of mice ($n = 4$) expressing ChR2 had misplaced fiber optic cannulae, and these animals were used as an additional control group (group 5; Table 1). All mice underwent a pre-test to determine sexual receptivity. Mice receiving hormone replacement with a pre-test LQ of <50 were excluded from the study. As expected, mice receiving oil only, without hormone replacement, were never receptive.

Optogenetic stimulation of β -End in the MPN reduced sexual receptivity

A two-way ANOVA followed by Tukey's multiple comparisons post-test was conducted to compare the effect of virus and photostimulation on the LQ, and analysis showed a significant interaction ($F_{(2,28)} = 3.96$, $p = 0.03$; Fig. 4). There was a significant main effect of viral treatment ($F_{(2,28)} = 7.73$, $p = 0.002$). See Table 5 for pre-test

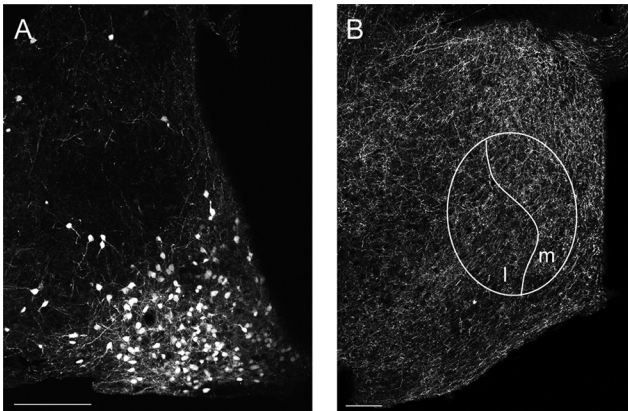


Figure 3. ChR2 expression in ARH and MPN. A representative image showing (A) soma and fiber expression of ChR2-tdTomato in the ARH, and (B) fiber expression in the MPN in the same animal. The MPN is shown at the level containing the medial (m) and lateral (l) subdivisions. 3V, 3rd ventricle. Scale bars = 100 μ m.

group LQ means and SEM and Table 6 for group means and SEM following photostimulation.

Tukey’s multiple comparisons test indicated that there was no difference in pre-test LQ between any of the three groups (group 1 vs group 2, simple main effect, $p = 0.94$;

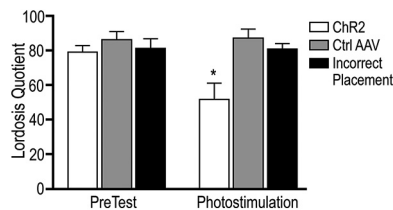


Figure 4. Photostimulation of ChR2 in the MPN significantly attenuates the LQ. A two-way ANOVA followed by Tukey’s multiple comparisons test indicated a significant interaction between virus type and behavior test on the LQ ($F_{(2,28)} = 3.96, p = 0.03$). Photostimulation of ChR2-expressing fibers in the MPN significantly reduced the LQ as compared to all other conditions (denoted by *). There was no difference in initial sexual receptivity between any of the three groups. In Pomc-cre mice expressing ChR2 in β -End terminals, photostimulation significantly attenuated lordosis (photostimulation, white bar) as compared to the pre-test without photostimulation (pre-test, white bar; simple main effect $p = 0.01$). Photostimulation of ChR2 (white bar) also significantly reduced the LQ as compared to both mice with the control virus (gray bar, simple main effect $p = 0.001$) and mice with incorrectly placed fiber optic cannulae (black bar, simple main effect $p = 0.03$). Photostimulation of ChR2 (photostimulation, white bar) furthermore significantly reduced the LQ as compared to the pre-test LQ of both mice receiving the control virus (pre-test, gray bar, simple main effect $p = 0.002$) and those with incorrectly placed fiber optic cannulae (pre-test, black bar, simple main effect $p = 0.03$). Mice that received the control AAV exhibited no difference ($p > 0.99$) in LQ between the pre-test and during photostimulation, nor did mice expressing ChR2 but with incorrect placement of the fiber optic cannula ($p > 0.99$). There was no difference in LQ between mice that received the control virus and those with ChR2 but incorrect placement of fiber optic cannulae in any condition. Values are expressed as mean \pm SEM.

Table 5. Mean LQ during pre-test

Group	Mean	SEM
Group 1	79.9	3.3
Group 2	86.8	4.5
Group 5	82.0	5.2

Presented is the mean and the SEM of the pre-test LQ score for each of the groups that received hormone replacement.

group 1 vs group 5, simple main effect, $p = 0.99$; group 2 vs group 5, simple main effect, $p = 0.99$; Fig. 4). Analysis of simple main effects revealed that overall, mice expressing ChR2 with properly placed fiber optic cannulae showed significant attenuation of the LQ as compared to all other conditions (Fig. 4). Compared with the pre-test LQ, photostimulation of fibers expressing ChR2 in the MPN significantly reduced the LQ (simple main effect, $p = 0.01$; Fig. 4) in those mice expressing ChR2 with correct cannula placement (group 1). Pomc-cre mice that received the control virus (group 2) showed no difference in LQ between the pre-test and during photostimulation (simple main effect, $p > 0.99$; Fig. 4), nor did those mice that expressed ChR2 but in which the fiber optic cannula was misplaced (group 5; simple main effect, $p > 0.99$; Fig. 4).

During photostimulation, mice expressing ChR2 (group 1) had significantly lower LQ scores than those expressing the control virus (group 2; simple main effect, $p = 0.001$; Fig. 4), a difference of $\sim 35\%$, similar to that seen between the pre-test and photostimulation conditions of ChR2 mice alone. Furthermore, only when the fiber optic cannulae were correctly positioned did photostimulation in mice expressing ChR2 attenuate the LQ (group 1 vs group 5, simple main effect, $p = 0.03$; Fig. 4). Photostimulation did not result in a difference in LQ between mice with the control virus (group 2) and those with ChR2 but incorrectly placed cannulae (group 5; simple main effect, $p = 0.98$; Fig. 4).

Finally, photostimulation in mice expressing ChR2 with properly placed fiber optic cannulae (group 1) significantly attenuated the LQ as compared to the pre-test condition of both mice with the control virus (group 2, simple main effect, $p = 0.002$) and those mice with incorrect fiber optic placement (group 5, simple main effect, $p = 0.03$). All mice, regardless of viral injection, that received subcutaneous oil injections without hormones were uniformly non-receptive (LQ = 0; data not shown).

MOR is internalized in MPN neurons following photostimulation of β -End terminals

A one-way ANOVA followed by Tukey’s multiple comparisons test detected a significant difference in receptor

Table 6. Mean LQ following photostimulation

Group	Mean	SEM
Group 1	52.6	9.0
Group 2	88.0	4.8
Group 5	81.4	3.0

Presented is the mean and the SEM of the LQ score during photostimulation for each of the groups that received hormone replacement.

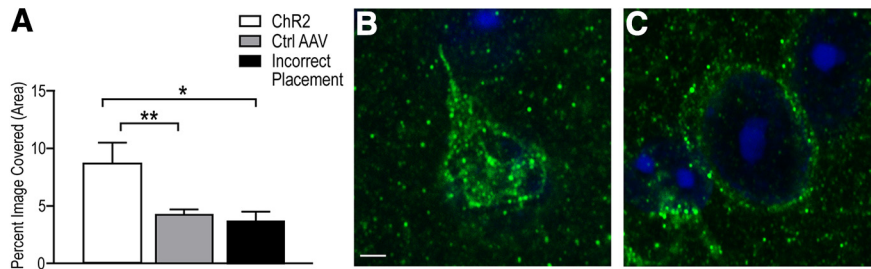


Figure 5. Photostimulation of ChR2 in β -End terminals in the MPN internalizes the MOR. Photostimulation induced the internalization of MOR within cells in the MPN. **A**, A one-way ANOVA indicated that there was a significant difference in receptor internalization between groups ($F_{(2,11)} = 5.15, p = 0.03, r^2 = 0.48$). Tukey’s multiple comparisons test indicated that mice with ChR2 in β -End terminals in the MPN showed a greater percentage of the area of the image covered by MOR-ir (mean, $8.7 \pm 1.8\%$) as compared to both mice that received the control AAV (mean, $4.3 \pm 0.4\%$; $*p = 0.05$) and those with ChR2 but incorrect fiber optic placement (mean, $3.7 \pm 0.4\%$, $**p = 0.04$). Images show DAPI (blue) and MOR (green) in a representative cell from group 1 (**B**) and group 2 (**C**). Scale bar = $2 \mu\text{m}$. All values expressed as mean \pm SEM.

internalization ($F_{(2,11)} = 5.15, p = 0.03, r^2 = 0.48$; Fig. 5) measured as the percentage of the area of the image expressing MOR-ir. Pomc-cre mice expressing ChR2 (group 1) showed an increase in receptor internalization (mean, $8.7 \pm 1.8\%$) as compared to both mice with the control virus (group 2; mean, $4.3 \pm 0.4\%$; $p = 0.05$) and mice with incorrectly placed cannulae (group 5; mean, $3.7 \pm 0.8\%$, $p = 0.04$) in response to photostimulation. There was no difference in receptor internalization between group 2 and group 5 ($p = 0.94$).

cFOS is activated in tdTomato-expressing soma in the ARH

Following behavioral testing, cFOS-ir was evaluated to determine neuronal activation of ARH POMC neurons. In all mice, cFOS-ir occurred only in a subset of POMC neurons in the ARH.

In response to photostimulation, mice with ChR2 trended toward expressing a greater percentage of cFOS in tdTomato-positive neurons (mean, $41.3 \pm 9.6\%$) than did mice with the control virus (mean, $15.7 \pm 6.2\%$), although the effect was not statistically significant (unpaired t test, ChR2 vs control AAV, $t_{(5)} = 2.021, p = 0.09$; Fig. 6). Furthermore, cFOS-ir occurred almost exclusively in POMC neurons.

Discussion

In this study, we selectively activated the POMC population involved in sexual receptivity behavior by injecting

a ChR2-AAV into the ARH of Pomc-cre mice and subsequently photostimulating the β -End-releasing terminals in the MPN. We found that the photostimulation of these terminals attenuated lordosis behavior in hormone-primed, sexually receptive mice. These results support the hypothesis that activation of MOR is involved in the inhibition of lordosis behavior seen following estradiol administration (Eckersell et al., 1998; Sinchak and Micevych, 2001; Mills et al., 2004). Furthermore, the data suggest that activation of these receptors is capable of over-riding hormonal signals that would otherwise facilitate lordosis.

Only Pomc-cre mice expressing ChR2 showed a significant decrease of the LQ in response to photostimulation compared with mice in all other conditions, including their own pre-test. Interestingly, this inhibition of lordosis behavior was comparable to that seen in mice with a global MOR knock-down (Sinchak et al., 2005), which is a strong indication that an important modulatory aspect of sexual receptivity is mediated through MOR activation in the MPN. The role of MOR in the lordosis circuit has been supported by pharmacological studies indicating that the application of β -End (Wiesner and Moss, 1984; Torii et al., 1996, 1999) or other MOR agonists (Sinchak and Micevych, 2001; Acosta-Martinez and Etgen, 2002a,b) reduces the expression of lordosis. Blocking MOR activation/internalization with antagonists is sufficient to block the estradiol-induced MOR inhibition of sexual re-

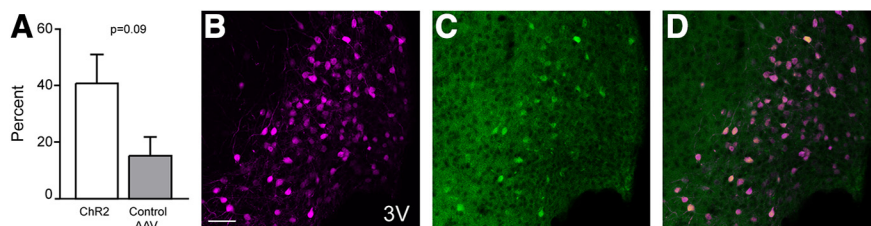


Figure 6. cFOS expression in the ARH is increased following photostimulation. IHC for cFOS was run to confirm activity in POMC neurons in the ARH. **A**, Following photostimulation, cFOS trended toward greater expression in neurons in mice with ChR2 than those that had the control virus (ChR2 mean, $41.3 \pm 9.6\%$; control AAV mean, $15.7 \pm 6.2\%$; $p = 0.09$), though this effect was not statistically significant, likely due to a lack of statistical power. tdTomato in magenta (**B**), cFOS in green (**C**), and merged image with both tdTomato and cFOS (**D**) in a representative mouse expressing ChR2 in the ARH. Double-labeled neurons appear yellow. Scale bar = $50 \mu\text{m}$. 3V, 3rd ventricle. All values expressed as mean \pm SEM.

ceptivity (Wiesner and Moss, 1984), even in the presence of an agonist (Sinchak and Micevych, 2001).

In gonadally intact rodents, estradiol from maturing ovarian follicles activates the ARH – MPN circuit, inhibiting lordosis (for review, see Micevych and Meisel, 2017). To coordinate the expression of lordosis behavior with ovulation, P4 relieves the MOR-mediated inhibition, allowing sexual receptivity to be synchronous with ovulation maximizing reproductive potential. In the present experiment, ovx mice were treated with estradiol and P4 to mimic the sequential ovarian hormonal stimulation that produce a sexually receptive female. Under these conditions, MOR are not activated and are localized primarily on the cell membrane. As expected, only mice in group 1 (Table 1) showed a decrease in the LQ in response to photostimulation (Fig. 4), and this inhibition of behavior was coincident with an increase in MOR internalization (Fig. 5), which follows receptor activation (Sinchak and Micevych, 2001; Micevych et al., 2003; Al-Hasani and Bruchas, 2011), not seen in any other group. Mice that remained sexually receptive had MOR on the cell surface, an indication that these receptors were not activated.

While receptor activation and internalization are two separate events, internalization has been established as an assay for receptor activation (Eckersell et al., 1998; Sinchak and Micevych, 2003; Mills et al., 2004). MOR is a G protein-coupled receptor (Manglik et al., 2012), coupled to inhibitory G proteins (Al-Hasani and Bruchas, 2011). MOR agonists (i.e., morphine, endomorphin-1) activate these receptors in the MPN, which results in their internalization (Sinchak and Micevych, 2001; Micevych et al., 2003), and inhibits lordosis behavior (Sinchak and Micevych, 2001), but so does estradiol treatment (Mills et al., 2004). Furthermore, estradiol decreases the firing rate of neurons in the medial preoptic area (Bueno and Pfaff, 1976), a region that includes the MPN and has a high concentration of MOR (Hammer, 1984). Our data here show that the specific activation of these receptors is sufficient to attenuate lordosis behavior. Taken along with the initial inhibitory actions on this circuit, the data suggests that estradiol acts in part on an inhibitory circuit to constrain sexually receptive behavior. Ultimately, MOR is a key component of the circuit controlling lordosis behavior following estradiol treatment (Eckersell et al., 1998; Sinchak and Micevych, 2003; Mills et al., 2004).

Furthermore, in gonadally intact rodents, the internalization of MOR varies across the estrous cycle. Receptor density is highest during diestrus, when the animal is unreceptive, and lowest during proestrus (Hammer, 1984, 1990). The estradiol-induced internalization of MOR is regulated by ER α . In mice with a global knock-out (KO) of ER α (ER α KO), estrogen does not induce the internalization of MOR, though estradiol does induce receptor internalization in ER β KO mice (Micevych et al., 2003). However, these ER α KO mice still exhibit MOR internalization in response to direct MOR agonists (Micevych et al., 2003), indicating that the MOR is still functional. Additionally, ER α is found in a subset of POMC neurons (Morrell et al., 1985) and estradiol regulates the expression of the β -End peptide (Priest and Roberts, 2000). Like MOR,

β -End fluctuates across the estrous cycle (Barden et al., 1981; Wardlaw et al., 1982).

Following estradiol, P4 relieves the opioid-induced inhibition and MORs are no longer activated, as indicated by their presence on the cell surface, thereby allowing the display of lordosis behavior (Sinchak and Micevych, 2001). This is confirmed by the observation that in all mice except for those that expressed ChR2 and received photostimulation (group 1; Table 1), P4 was sufficient to allow MOR to return to the cell surface, and these mice displayed lordosis. Our finding that photostimulation of ChR2 in the MPN resulted in the internalization of the receptor and inhibition of lordosis, even with P4 in the system, supports previous findings that the activation of MOR is powerful enough to inhibit the hormonal facilitation of lordosis.

Importantly, mice with ChR2 and correct fiber optic placement showed a statistically significant decrease in the LQ compared with those mice with ChR2 but where the cannulae were not positioned in the medial MPN (Fig. 4). The intensity of laser light decreases in an approximately linear fashion as it propagates from the source (Britt et al., 2012). Of the mice with incorrect placement, the average distance of placement was ~ 1.5 mm away from the MPN. Using the Predicted Irradiance Values calculations provided by the Diesseroth Lab (<https://web.stanford.edu/group/dlab/cgi-bin/graph/chart.php>), the irradiance value falls from 7.95 mW/mm $^{-2}$ immediately at the source to 0.06 mW/mm $^{-2}$ at a distance of 1.5 mm. This decrease in photostimulation was not sufficient to stimulate POMC terminals to release β -End to either activate/externalize MORs or impact lordosis behavior. There was a significant difference ($p = 0.04$) in MOR internalization between ChR2-expressing mice with correct optical fiber placement and incorrect placement.

POMC IHC was used to verify that tdTomato expression occurred in POMC neurons. POMC-ir was colocalized with the fluorescent reporter tdTomato (Fig. 2), but a large number of neurons expressed tdTomato alone without POMC-ir, findings similar to Padilla et al., 2012. Within the ARH, tdTomato was expressed in the majority of cell bodies as indicated by the fluorescent reporter tdTomato, but many of these labeled neurons did not express Alexa Fluor 488. However, cFOS-ir occurred only in a subset of these POMC neurons (Fig. 6), rather than the entire population. This likely represents activation of only the reproductively-relevant POMC neurons. While the increase in cFOS-ir likely occurred at least in part in response to mounting by the male (Pfaus et al., 1993), it is possible that at least some of the activation occurred in response to photostimulation in the MPN (Stamatakis and Stuber, 2012), as cFOS-ir in the ARH trended toward being greater in mice with ChR2 than with the control virus.

Both the optical stimulation parameters and MOR internalization provide a level of certainty that the effect on lordosis behavior was due to release of β -End in the MPN. Although we did not formally measure β -End release (i.e., microdialysis; Al-Hasani et al., 2018), stimulation with 20 Hz has been demonstrated to release neuropeptides, in

contrast to a lower frequency stimulation (i.e., 2 Hz) that releases amino acid transmitters (Arrigoni and Saper, 2014). In the present study, 20 Hz stimulation activated POMC/ β -End terminals in the MPN. We expected that this stimulation would produce a rapid release of either GABA or glutamate, transmitters expressed in POMC neurons (Hentges et al., 2009), followed by longer lasting peptide-mediated response. However, to ascertain that β -End was released, we assessed MOR internalization – an indicator of receptor activation. As expected, following stimulation the pattern of MOR internalization was coincident with inhibition of lordosis and consistent with previous pharmacological studies that indicated that opiate and endogenous opioid activation of MOR in the MPN inhibits lordosis (Pfaus and Pfaff, 1992; Eckersell et al., 1998; Torii et al., 1996, 1999; Sinchak and Micevych, 2001; Mills et al., 2004; Sinchak et al., 2005; Long et al., 2017). At this point, we cannot determine whether the release of amino acid transmitters from POMC terminals also affects the lordosis response.

Overall, several converging lines of evidence suggest that it is β -End release that accounted for the behavioral results: (1) photostimulation was effective only in mice that both expressed ChR2 and had correct fiber placement; (2) cFOS-ir was localized in a subset of POMC-expressing ARH neurons; (3) the decrease in lordosis behavior was coincident with increased MOR internalization in the MPN.

The present study is a refinement of previous methodologies designed to investigate the role of the β -End in reproduction. The use of optogenetics allows for highly specific activation of β -End terminals only in the MPN, rather than pharmacological studies which may inadvertently affect receptors in regions outside of the MPN. The MOR (along with delta and kappa opioid receptors) are found throughout the brain (Mansour et al., 1987, 1988; Erbs et al., 2015). Indeed, the MOR is found in circuits both within and outside of the hypothalamus (Mansour et al., 1987, 1988) that may directly and indirectly affect reproduction. For example, intracerebroventricular infusion of MOR agonists and/or antagonists (Pfaus and Pfaff, 1992) may affect opioid receptors throughout the brain and even the periphery, depending on their half-life (Pardridge, 2011). Moreover, peripheral administration of MOR agonists (Wiesner and Moss, 1984) likely have effects on non-reproductive functions that may confound experimental interpretation. In this study, we specifically activated only those β -End terminals that expressed ChR2 in the MPN, thereby reducing the potential for unwanted non-specific effects on the MOR, and other opioid receptors.

The full expression of lordosis requires both MOR activation (Sinchak et al., 2005) and a period of behavioral inhibition (for review, see Micevych et al., 2017). Our results indicate that photostimulation of ChR2 in POMC/ β -End terminals in the MPN is sufficient to both attenuate lordosis and activate/externalize the MOR in a hormone-primed, sexually receptive female mouse. Thus, taken with previous studies, our data confirm the important

inhibitory role that the MOR plays in regulating lordosis behavior.

While humans do not exhibit lordosis as a reproductive reflex, the use of opioids has been linked to the inhibition of reproductive function in humans (Vuong et al., 2010; Grover et al., 2014) and suggests an inhibitory role for these peptides in reproduction between species. Given that much of the hypothalamic circuitry for homeostatic and reproductive functions has been conserved within mammals through evolution (Xie and Dorsky, 2017), the further study of the role of opioids in reproduction provides a window into the hypothalamic control of reproduction.

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