Supplementary Material & Methods

Ovariectomy, hormonal priming and training of female mice

For the ovariectomy, adult females received an intraperitoneal (IP) injection of medetomidine 1mg/kg ten minutes before gas anesthesia induction (4% isoflurane). Mice were then maintained under 1 – 2 % isoflurane on a heating pad during the surgery. Females were shaved on each flank and the surgical area was disinfected with vetedine soap (Vetoquinol) and povidone iodine solution 10%. On each flank, a small incision (0.5-1cm) was made on the skin. After proper dilaceration of the conjunctive and adipose tissue, the muscular plane was incised. The ovary was then exposed outside the abdominal cavity and the uterine horn was sutured. We then cut the tissue between the suture and the ovary to remove it. The horn was put back in the abdominal cavity and the muscular plane and the skin sutured with absorbable thread. Females then received a subcutaneous injection of meloxicam 20mg/kg [1] at 1mg/mL [2] for pain relief and an IP injection of atipamezole 1.5mg/kg [3] to reverse anesthesia. Mice were monitored daily for one week after surgery and injected with 20mg/kg meloxicam if any behavioral pain signs were noticed. After ovariectomy, mice were allowed to recover for 1-2 weeks. Hormonal priming was used to induce receptivity as previously described [4]. Briefly, ovariectomized females received subcutaneous injections of 10µg Estradiol Benzoate (E2) 48h and 50µg of Progesterone (P4) 7h before the behavioral test. E2 and P4 were dissolved in 100µL of sesame oil and injected subcutaneously. All females used with our experimental males were trained for sexual behavior with other stimulus males to increase their lordosis behavior [5]. This training consisted in 3 sessions of sexual behavior (1 each week on 3 consecutive weeks). During these sessions, ovariectomized, hormonally-primed females were introduced in a male's home cage to allow for at least 20 min of copulatory behavior. For male sexual behavior training and experimentation, we exposed male mice to sexually experienced ovariectomized females with induced estrus by hormonal priming. Receptivity of the female was checked before using them as stimulus for all sexual behavior experiments.

Perfusion and slice preparation

Mice were habituated for 2 hours in the behavioral room and then an unfamiliar male (for 10 min) or a receptive female intruder (for 30 min) was introduced in their home cage. After the social interaction, the intruder was removed and the test mice were kept in darkness until perfusion. 100 min after the intruder introduction, mice were deeply anesthetized with a solution of ketamine (300mg/kg) and medetomidine (3 mg/kg) and transcardially perfused with a cold phosphate buffer saline solution (PBS, 52mM Na_2PO_4, 17mM KH_2PO_4, 150 mM NaCl, pH 7.2) for 1 min followed by a cold 4% PFA solution for 5 min. Brains and VNOs were collected, post fixed overnight in 4% PFA at 4°C and dehydrated in a 30% sucrose solution for 48h. All solutions were diluted in PBS. Dehydrated brains were snap frozen in cold isopentane (-30°C) and embedded in tissue-tek OCT™ compound (Sakura, 4583). They were then coronally cut on a Leica CM 3050S cryostat at a 25µm thickness. Serial free-floating brain sections were kept in cold PBS with 0.1% sodium azide until mounted on SuperFrost plus glass

slides. Once dry, the sections were stored at -80°C. Expression of virally injected constructs was confirmed by mCherry expression under a fluorescence microscope.

Egr1 and HA-Tag immunohistochemistry

The brain sections were incubated in a saturation solution (5% normal donkey serum (NDS), 0.3% Triton X-100 solution in PBS) for 2 hours at room temperature (RT). Sections were then incubated overnight (4°C) with rabbit-anti-Egr1 monoclonal antibodies (Cell Signaling, clone 15F7, diluted at 1/200) or mouse-anti-HA monoclonal antibodies (BioLegend, clone 16B12, diluted at 1/1000) in the saturation solution. After several washes in PBS, the sections were incubated for 2 hours at RT with Donkey-anti-Rabbit secondary antibodies Alexa 488 conjugated (Molecular Probes, 711-545-152) diluted at 1/500 in the saturation solution. The sections were finally counterstained with Hoechst (life technologies, 1/1000) for 5min. Slides were mounted with Fluoromount-G™ medium and imaged with a Confocal microscope (Zeiss LSM 780).

In situ* Hybridization - *Cyp19a1

In situ hybridization was performed with the RNAscope™ multiplexed Fluorescent v2 kit (ACDBio) following the manufacturer's protocol. Briefly, the brain sections were incubated in a hydrogen peroxide solution (5%) for 10 min, then with a target retrieval buffer (ACDBio) for 5 min at 95°C. The sections were permeabilized after an incubation with the Protease 3 for 30min at 40°C, and were then incubated (2h at 40°C) with a mouse specific Cyp19a1 probe (reference 430821) to specifically target Cyp19a1 mRNA. The signal was then amplified with successive incubations in the amplification solutions (ACDBio) followed by a tyramide signal amplification step (TSA Biotin Kit, reference NEL749A001KT, Akoya Biosciences). The signal was then revealed by incubation with a streptavidin conjugated Cy2 diluted at 1/500 (Jackson ImmunoResearch, reference AB_2337246)

Confocal images normalization and cell classification

All confocal images were z-normalized with a custom Python script (see G-Node repository DOI: 10.12751/g-node.7sl3ks). Transformed images were then loaded in Qupath v0.5.0 [6]. Each region of interest (ROI) was established following the Paxinos and Franklin mouse brain atlas and individual cells within the ROIs were detected based on the Hoechst staining. These data were then loaded in R and analyzed with a custom script (DOI: 10.12751/g-node.7sl3ks). For every analyzed brain region, detected cells were considered Egr1 positive if the mean value of its fluorescence (green channel) in the nucleus was above 3 standard deviations (SD) (except the mPOA (2SD)). In one batch of animals (from all groups) we needed to lower to 2.5 this threshold because of a lower overall staining. For the VNO, due to highly variable background levels, Egr1 positive cells were manually counted. Cells were considered mCherry

positive if they had an overall (cytoplasm + nucleus) mean value of fluorescence (red channel) above 2 SD.

Stereotaxic surgery

Socially experienced animals received an intraperitoneal (IP) injection of medetomidine 1mg/kg ten minutes before gas anesthesia induction (4% isoflurane). Males were shaved on the head and the surgical area was disinfected with vetedine soap (Vetoquinol) and povidone iodine solution 10%. Mice were placed on the stereotaxic frame and maintained on a heating pad under 1 – 2 % isoflurane during the surgery. Animals were then injected with a viral solution in the corresponding brain region as described in the Materials and Method section. After the surgery, animals received a subcutaneous injection of meloxicam 20mg/kg [1] at 1mg/mL [2] for pain relief and an IP injection of atipamezole 1.5mg/kg [3] to reverse anesthesia. Mice were monitored daily for one week after surgery and injected with 20mg/kg meloxicam if any behavioral pain signs were noticed.

References

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