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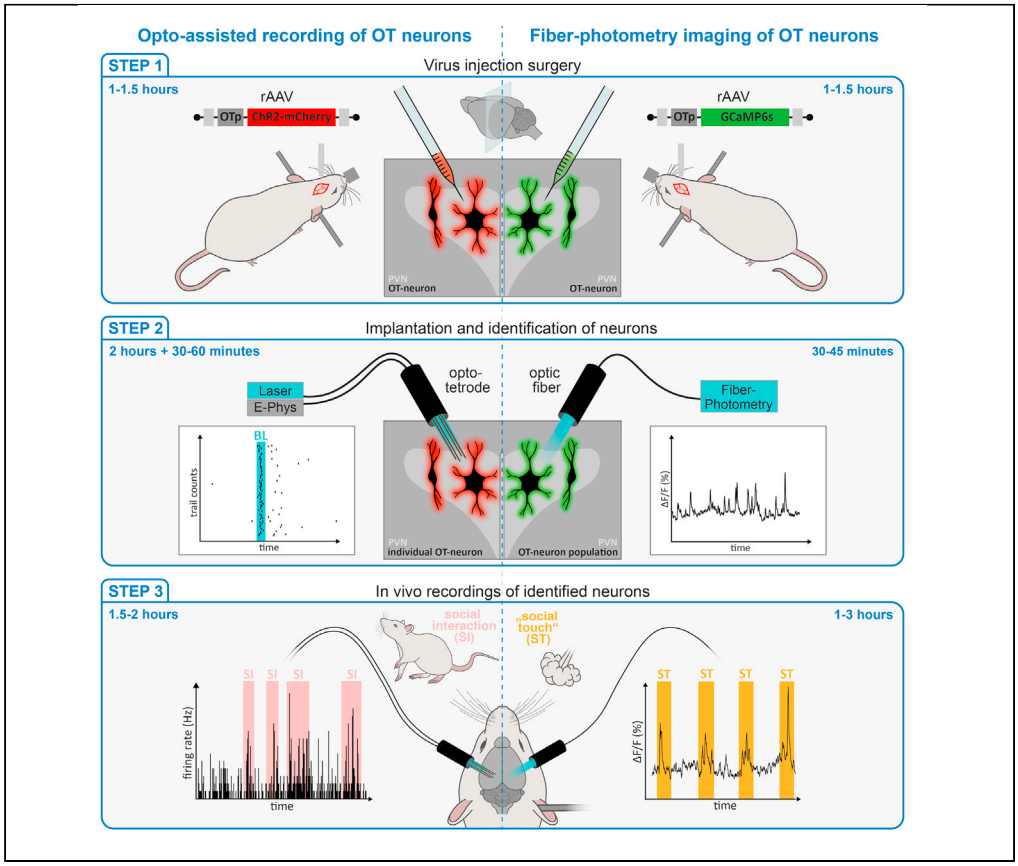
Viral vectors for opto-electrode recording and photometry-based imaging of oxytocin neurons in anesthetized and socially interacting rats



Here, we present a step-by-step protocol to target, record, and manipulate the activity of oxytocin neurons in awake rats. The protocol includes a procedure to record the activity of oxytocin neurons from awake and socially interacting rats using opto-electrodes for simultaneous electrophysiological recording and virally based cell-type-specific opto-tagging with Channelrhodopsin 2. Furthermore, we illustrate a procedure for optically guided implantation of optic fiber and imaging of oxytocin neuron population activity expressing calcium indicator GCaMP6s with the fiber photometry technique.

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Highlights

A detailed protocol for targeting of oxytocin neurons with viral vectors

Protocol allows recording from optogenetically tagged oxytocin neurons

Monitoring oxytocin neurons activity during social interactions

Imaging of oxytocin neuron population activity with fiber photometry

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Viral vectors for opto-electrode recording and photometry-based imaging of oxytocin neurons in anesthetized and socially interacting rats

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SUMMARY

Here, we present a step-by-step protocol to target, record, and manipulate the activity of oxytocin neurons in awake rats. The protocol includes a procedure to record the activity of oxytocin neurons from awake and socially interacting rats using opto-electrodes for simultaneous electrophysiological recording and virally based cell-type-specific opto-tagging with Channelrhodopsin 2. Furthermore, we illustrate a procedure for optically guided implantation of optic fiber and imaging of oxytocin neuron population activity expressing calcium indicator GCaMP6s with the fiber photometry technique.

For complete details on the use and execution of this protocol, please refer to Tang et al., 2020.

BEFORE YOU BEGIN

The neuropeptide oxytocin (OT) is involved in the regulation of behaviors essential to life, including socialization, and is produced by specialized endocrine neurons in the hypothalamus, mostly localized in the paraventricular (PVN) and supraoptic (SON) nuclei. Recording the activity of OT-producing neurons during naturalistic behaviors is critical to understanding their circuit-specific functions in a living brain.

Selective recording of OT neuronal activity during social behavior has only recently been made possible (Carcea et al., 2021; Resendez et al., 2020; Tang et al., 2020) thanks to the employment of recombinant adeno-associated viral (rAAVs) vectors that achieve specific expression of transgenes in OT neurons, in combination with electrophysiology or calcium imaging techniques. The two methods that we use for cell-type specific recording in freely moving animals are extracellular recordings of action potentials combined with optogenetic tagging (opto-tagging) (Lima et al., 2009) and imaging of genetically encoded calcium indicators (GECIs) (Chen et al., 2013), such as GCaMP6s, with fiber photometry.

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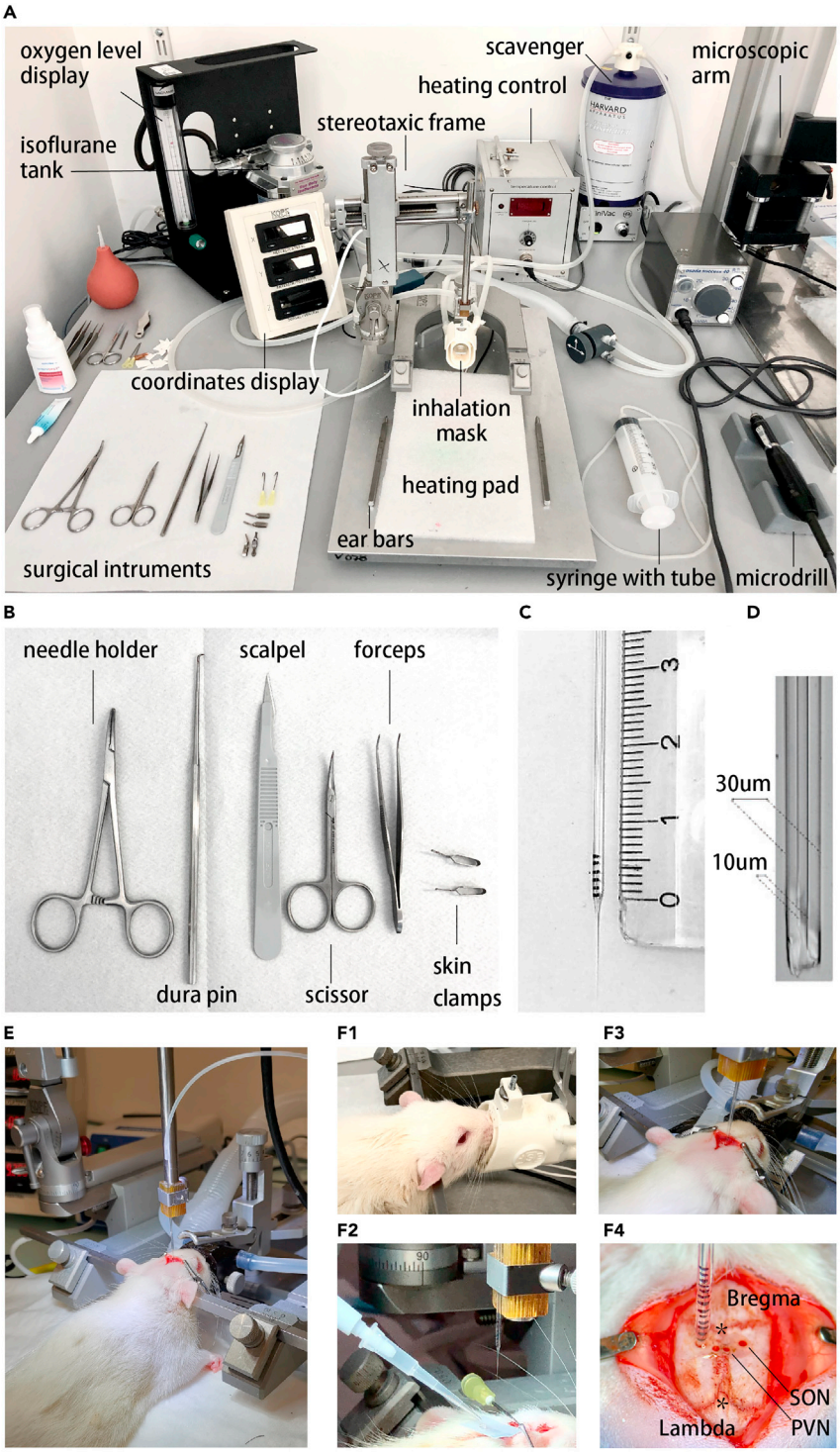

Figure 1. Surgery set-up and tools for viral vectors delivery

Equipment needed to perform delivery of viral vectors.

(A) Surgery table is equipped with a stereotaxic frame with heating pad, isoflurane delivery system, ventilation tubes, gas scavenger, high-speed drill, syringe with tube for injection, binocular surgical microscope and surgical instruments.

(B) Surgical instruments include a needle holder, a fine-tip pin for penetration of dura, a scalpel, scissors, forceps, and skin clamps.

Figure 1. *Continued*
(C) Glass capillary for viral vector delivery with 1 mm marks drawn on the side to calibrate injected volume (1 mark = 70 nL).
(D) Microscopic view of the glass pipette tip. After cutting, the diameter of the tip should be measured under the microscope. (20–50 μm outer diameter).
(E) Depiction of the surgical setup when calibrating the stereotaxic coordinates prior to viral injection.
(F1) Depiction of the head firmly secured with ear bars with the nose positioned in the inhalation mask. (F2) Setup used to transfer the viral solution to a sterile Parafilm square before loading it into the glass pipette. (F3) Insertion of the glass capillary into the target brain area (the PVN or SON). (F4) Clean craniotomy area with glass pipette inserted in the brain (SON) for viral injections. Bregma and Lambda are marked with symbols.

Note: We have used this protocol in wild type female Wistar rats (age 8–16 weeks), but it could likely be generalized to other strains, sex, or age without major modifications.

Viral delivery of gene of interest in oxytocin neurons

Timing: 1–1.5 h (3–4 weeks before the recording experiment)

The goal of this preparation is to deliver 300 nL of rAAV solution (titer 10^9–10^{10} vg/mL, dissolved in saline) into the PVN and SON to express transgenes specifically in OT neurons via a small OT promoter sequence incorporated into the vector (for details, see Knobloch et al., 2012).

Required tools (Figures 1A–1C):

Setup	Surgical instruments	Surgical equipment	Injection equipment
• Heating pad (37°C) • Stereotaxic frame • Oxygen tank • Isoflurane dispenser • Scavenging system • Induction chamber • Microscope	• Needle holder • Scalpel • Forceps • Bone scraper • Scissors • Suturing needle and thread • Drill	• Carprofen (50 mg/mL) or meloxicam in 1 mL syringe • Cotton swabs • Wound disinfectant • Eye and nose cream (bepanthen) • Razor • Betadine • 0.9% saline solution • Lidocaine • Sterile PBS	• Virus (at −80°C) • Glass pipette • Small parafilm square • 50 mL syringe with small tube

Note: To ensure minimal tissue damage, glass pipettes should be at least 10 mm long with a tip diameter between 20 and 50 μm that enlarge up to 150–200 μm at their "shoulder" (Figure 1D). In this protocol we use 10 μL glass pipettes (IntraMARK, BRAND) and a P-97 pipette puller with the following parameters: TEMP 650; PULL 30; VEL 70; TIME 200. We then cut the pipette tip with fine scissors and check the tip diameter under the microscope. Optimal parameters may vary between pullers and pipette type.

1. Induce general anesthesia by inhalation of isoflurane gas at 3% concentration and progressively lower the concentration to 1–2% (0.2 L/min).
   a. To achieve full analgesia, administer a subcutaneous injection of both bupivacaïne (2 mg/kg) and an NSAID drug, such as meloxicam (2 mg/kg) following veterinary recommendations.
   b. Periodically monitor the animal's respiration rate throughout the surgery and adjust the isoflurane concentration accordingly.
   c. 24 h after surgery, supplement the water with meloxicam (2 mg/kg) for *ad libitum* use during the recovery process.

⚠ CRITICAL: Appropriate regulations for experiments with rats must be followed. The procedure must comply with animal surgery guidelines, including the use of aseptic surgical techniques to prevent possible infection as well as the use of appropriate anesthesia

and analgesia to minimize pain. The present experiments were approved by the Committee on Animal Health and Care of the government of Baden-Württemberg in Karlsruhe under the licenses 35-9185.81/G-102/17 and 35.9185.81/G-193/20.

2. Place the rat on a heating pad to maintain a constant body temperature of 37°C. Shave the fur from the scalp and then fix the head in the stereotaxic frame with two ear bars and a nose clamp equipped with an inhalation mask (Figure 1F1).
3. Before starting the surgery:
   a. Apply eye ointment to prevent the animal's eyes from drying out.
   b. Clean the scalp with betadine.
   c. Check for the absence of the withdrawal reflex to a foot pinch.
   d. Make sure to use sterilized surgery tools (Figure 1E).
4. Make an incision (1–1.5 cm) with a surgical blade along the midline of the scalp.
   a. Remove the periosteal soft tissue within the incised area.
   b. Clean the surface of the skull with sterilized 0.9% saline solution.
   c. Bregma and Lambda should be clearly visible after cleaning.

⚠ CRITICAL: Ensure that Bregma and Lambda are level in the horizontal plane. The dorsal/ventral difference should be less than 70 μm. For optimal accuracy, also verify that the dorsal/ventral difference between two symmetrical points on the skull (e.g. ±2 mm lateral from the midline) is minimal.

5. Mark the position of the center of the craniotomy with a thin marker pen (1.8 mm posterior to Bregma and 0.35 mm lateral for the PVN, 1.2 mm posterior to Bregma and 1.8 mm lateral for the SON) (Figure 1F4).
6. Perform the craniotomy (0.5 mm circular shaped hole) above the target region using a high-speed drill and a 0.6 mm drill tip with ball nose. If excessive bleeding occurs, please refer to 'problem 1' for potential solutions.

⚠ CRITICAL: Cautious drilling should be used when targeting the PVN to avoid piercing the numerous blood vessels located near the midline. In case of bleeding, gently apply pressure to the hole using a hemostat and a cotton tip until it stops flowing. Apply sterilized phosphate-buffered saline (PBS) solution or sterile saline to clean the excess blood and prevent further bleeding.

7. Fix the glass pipette to the stereotaxic arm and secure the small tube onto the top-end of the glass pipette to create an airtight seal. This seal will allow for accurate aspiration or dispensation of the viral solution using negative or positive pressure, respectively.
   a. Place a sterile square of Parafilm onto the head of the rat and use a standard laboratory pipette to transfer the required volume of viral solution (plus extra) onto it.
   b. Avoid aspirating air while transferring the viral solution to the parafilm square (Figure 1F2).
   c. Load the glass pipette with the viral solution while being careful not to damage the tip or aspirate air.
8. Move the glass pipette tip to the appropriate coordinates relative to bregma.
   a. Insert the glass pipette into the brain through the previously drilled hole until reaching the desired depth (DV = −8.0 for PVN and −9.25 for SON).
   b. Slowly inject 300 nL of the virus (100 nL/min) followed by a 3 min wait period to allow for adequate diffusion.
   c. Retract the pipette very slowly for the first 500 μm (10 μm/s) and then increase to a normal speed (100 μm/s) until the pipette is clear of the skull (Figures 1F3 and 1F4).

⚠ CRITICAL: For the PVN, the glass pipette might bend before going through the dura and the vein. This is especially true when using longer, more flexible pipette tips (below

the shoulder of the glass pipette). Therefore, in order to successfully insert the pipette without breaking the tip, the length of the pipette tip should be shortened to the point at which it will go through without breaking while still being long enough to reach the target site. We recommend injecting the SON first, as it requires longer pipettes. The pipette tip might get clotted, in this case lift up the tip a bit and apply a strong pressure in the syringe, sometimes it helps to unblock the clot. Alternatively, you can use a cotton swab soaked in warm saline to apply warmth to the pipette tip as this will often cause the blood to release. Use another dry cotton swab to absorb the excess saline and release blood. Otherwise, it should be cut with small scissors and the zero-point has to be readjusted to Bregma.

9. Protect the virus injection holes with silicone elastomers (Kwik-SilKS, a bio-compatible surgical adhesive) to avoid tissue hyperplasia. This step is essential for the facilitation of opto-electrode implantation after 3 weeks.
10. Close the wound using surgical sutures, apply lidocaine and disinfect the sutured wound before placing the rat in a heated recovery cage. Return the rat to the home cage once it has recovered from anesthesia.
11. Check the animal's health status and administer analgesics (for example: carprofen (5 mg/kg) once daily for 48–72 h) following surgery. If the animal still shows signs of pain, administer additional analgesics, such as metamizol (100 mg/kg up to 3 times a day), Tramadol, Rimadyl, etc., as needed. Always follow local animal facility guidelines when choosing which drugs to administer.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-oxytocin (PS38) (1:1000)	Prof. H. Gainer	N/A
Anti-oxytocin (T-5021) (1:1000)	Peninsula Laboratories	N/A
Anti-Ds-Red (1:2000)	Clontech	Cat#632397
Anti-Synaptophysin (1:1000)	Abcam	Cat#ab32127
Anti-GFP (1:1000)	Abcam	Cat#13970
Anti-cfos (1:500)	Cell Signaling Technology	Cat#9F6
Anti-Cre (1:1000)	NOVAGEN Healthcare	Cat#69050
Hydroxystilbamidine (Flurogold) (1:1000)	Santa Cruz	CAS 223769-64-0
Bacterial and virus strains*		
rAAV-OTp-ChR2-mCherry	Lab-made	N/A
rAAV-OTp-GCaMP6s	Lab-made	N/A
CAV2-Cre	Montpellier vectorology platform(PVM)	N/A
rAAV-OTp-DIO-Gi-mCherry	Lab-made	N/A
rAAV-OTp-DIO-Gq-mCherry	Lab-made	N/A
rAAV-OTp-DIO-GFP	Lab-made	N/A
rAAV-OTp-Venus	Lab-made	N/A
Chemicals, peptides, and recombinant proteins		
Phosphate-buffered saline (PBS)	Gibco	11510546
Paraformaldehyde (PFA)	Sigma-Aldrich	24849719
Dental cement (Paladur)	Heraeuse Kulzer	N/A
Dental cement (UV glue)	3M™ RelyX™	Unicem 2
Carprofen (Rimadyl)	Zoetis	779-358
Meloxicam (Metacam)	Boehringer Ingelheim Vetmedica GmbH	QM01AC06
Wound disinfectant (vet-sept)	Livisto	799-414
Eye and nose cream (Bepanthen)	Bayer Vital GmbH	01578681
Silicone Elastomer	World Precision Instrument	KWIK-SIL™

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Betadine	Meda Pharma	FRCM045967
0.9 % Saline solution	B. Braun	SIN 9481 P
Lidocaine	Aspen	03839482
Isoflurane	Cp-pharma	798-932
Dafalgan	Bristol-Myers	2933893
Rapidocain	Sintetica	456315
Ketamine	Sigma-Adrich	1867-66-9
Xylazine	Sigma-Adrich	7361-61-7
Triton X-100	Sigma Aldrich	9036-19-5
Normal goat serum (NGS)	Thermo Fisher Scientific	R37624
Experimental models: Organisms/strains		
Rat: Wistar (female, 8 weeks, 200–260 g)	Charles River	N/A
Software and algorithms		
MATLAB	MathWorks	2016
Offline Sorter	Plexon Inc.	v4
Open Ephys to Plexon converter	Open Ephys	ephys2plx
Video analysis (subtitle)	Arctime	N/A
Auto behavior analysis	Noldus	*EthoVision* XT
DAQ (electrophysiology and fiber photometry)	OpenEphys GUI	0.2.7
MATLAB code used for the analysis of fiber photometry data	Tang et al., 2020	Supplementary Software section
Other		
Opto-Silicon Probe (oDrive mounted)	NeuroNexus	A1x32-Poly3-10mm-50-177-OH32LP-21mm
Opto-Silicon Probe (acute recording)	NeuroNexus	A1x32-Poly3-10mm-50-177-OA32LP
Tungsten wires	California Fine Wire Company	Stablohm 675
Skull screws	Wegertseder	DIN 84 M1x2 (1074-100)
Optic Fiber (opto-tetrodes)	Thorlabs	CFMLC12L10
Optic Fiber (fiber photometry)	Thorlabs	CFMLC15L10
Pulse generator	A.M.P.I.	Master9
Electrophysiology recording system	Open Ephys	Open Ephys Acquisition Board
Low-autofluorescent fiber patch cord (fiber photometry)	Doric Lense	MFP_400/440/1100-0.37_2m_FC-ZF2.5_LAF
Opto-tetrodes (microdrive)	Neuralynx	VersaDrive-8 Optical
Laser System (blue light 473 nm)	DreamLasers	SDL-473-LM
Glass pipettes	IntraMARK Brand	708707
Needle holder	Fine Science Tools	12001-13
Scalpel handle	Fine Science Tools	10003-12
Scalpel blade	Fine Science Tools	10023-00
Forceps	Fine Science Tools	11063-07
Scissors	Fine Science Tools	14061-10
Skin clamps	Fine Science Tools	18052-01
Dura pin	Fine Science Tools	10059-13

*All virus titers are between 10^9 and 10^{10} vg/mL, viruses were cloned and produced by our lab, thus viral maps are available upon request.

STEP-BY-STEP METHOD DETAILS

Steps 1–36 outline how to perform photo-assisted recording of oxytocin neurons in freely moving rats.

Chronic implantation of an opto-electrode and microdrive

Timing: 2 h

OT neurons reside mainly in the PVN and SON together with other neuronal cell types, making them difficult to target during *in vivo* electrophysiology experiments. This issue can be solved by

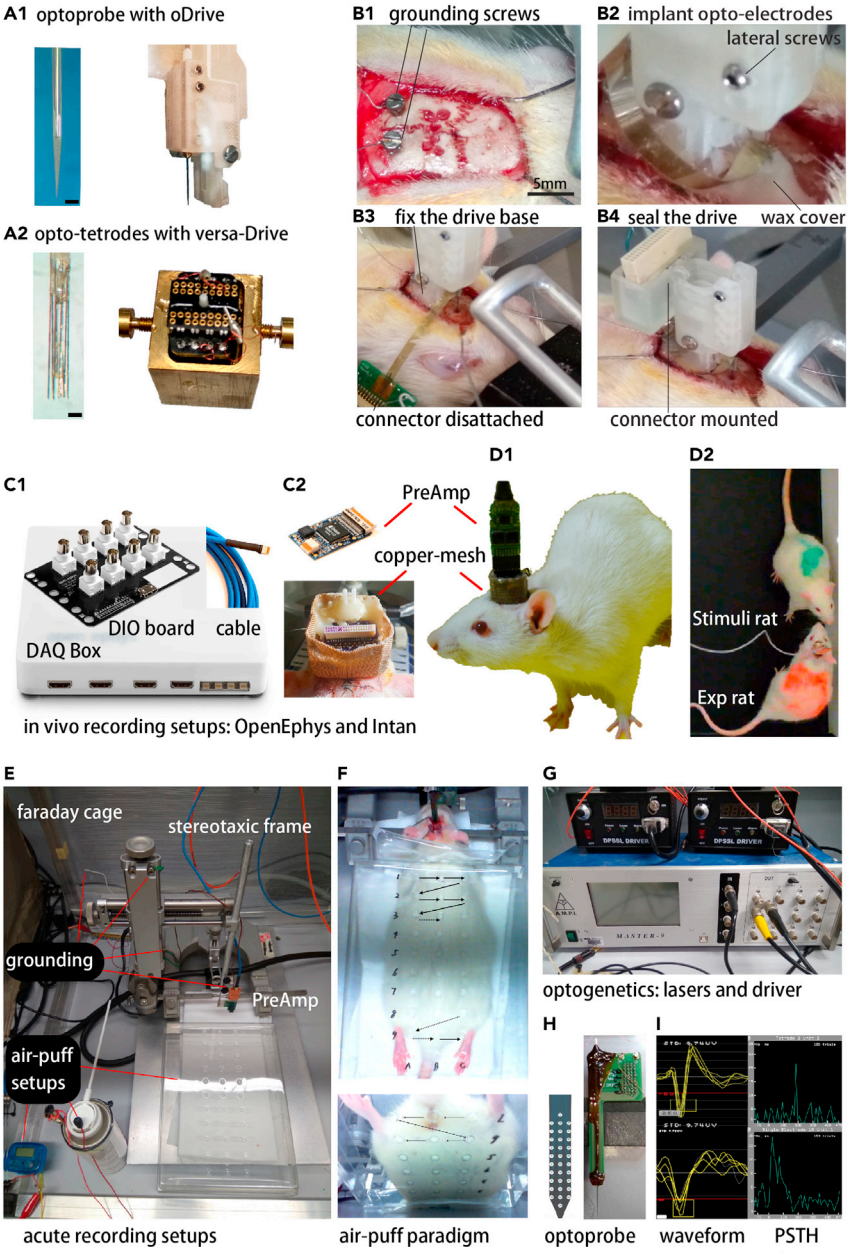

Figure 2. Implantation of opto-electrodes and photo-assisted recording of oxytocin neurons

(A1) Silicon probe electrode inserted into a single-axis optic fiber integrated with a microdrive (opto-electrode). Left panel is the magnified view of anopto-electrode tip (~ 100 μm in diameter, black bar = 150 μm). Right panel is the microdrive (oDrive, Neuronexus) with the integrated opto-electrode fully retracted. (A2) Tungsten-wire tetrodes mounted with eight independent shuttle drives (VersaDrive-8 Optical, Neuralynx) surrounding an optic fiber in the middle (opto-tetrode). Left panel is the magnified view of the opto-tetrode (~ 300 μm in diameter, black bar = 150 μm). Right panel is the VersaDrive-8 in the assembly jig.

(B1) Surgical steps for the implantation of the microdrive: two ground/reference screws are implanted in the posterior part of the skull and craniotomy holes are drilled above the target brain areas. Scale bar, 500 μm. (B2) Use pre-heated wax to seal the implantation hole and the naked opto-electrode shank, then apply a 3D-printed plastic cover around the probe implantation site and fix it with UV glue. Finally, suture the skin, put a copper mesh around the whole implanted drive and solder the grounding wires to the copper mesh to form a mini-faraday cage (see C2). (B3) Mount the microdrive on the stereotaxic arm with the adapter, loosen the lateral screws to free the optic fiber (see text) before implantation. Then align the probe tip to Bregma zero point and move it above the target coordinates, and

Figure 2. *Continued*

slowly implant it half way before the mirodrive base touches the skull. Fix the microdrive base on the skull first with UV-glue, then apply dental cement. (B4) Mounting of the attached omnetics connector onto the microdrive.

(C1) Electrophysiology data acquisition system (DAQ, OpenEphys), digital input/output board, and Smartlink connecting cable. (C2) Pre-amplifier (top) and a close-up picture of the mini-faraday cage mounted on the rat's head (bottom).

(D1) Picture of a rat implanted with an opto-tetrode mounted on the microdrive and protected with a copper mesh for chronic recording. (D2) Video-frame of the implanted experimental (Exp) rat connected to the DAQ during social interaction with a conspecific (Stimuli rat).

(E) Full setups for acute recording and optogenetics with an opto-electrode (in anesthetized rats) and somatosensory stimulation with 'airpuffs'. It is necessary to ground every potential source of electrical noise to the faraday cage using a grounding hub. This includes the stereotaxic frame arms, probe adapter, preamplifier, and power supplies of any nearby devices.

(F) Picture of an anesthetized rat during acute recording covered with a transparent box with a grid of holes for 'airpuff' stimulations on the skin in various body regions. Arrows depict sequential order of stimulation sites.

(G) Impulse generator (Master-9) and diode-pumped solid-state (DPSS) lasers (DreamLaser) for blue light optogenetic stimulation.

(H) Opto-electrode for acute recording and photo-stimulation (Neuronexus).

(I) Two representative single units monitored in real-time during implantation (waveforms on the left) and PSTH of the units aligned to blue light pulses (histograms on the right). The upper panel shows a putative interneuron displaying indirect activation as indicated by a 180 ms delay in the onset of increased spiking activity following the blue light pulses. The lower panel shows a putative OT neuron as it responds to blue light pulses with a very short (<10 ms) delay.

employing viral tools that enable the expression of particular transgenes in the desired cell population. Here we describe a procedure to implant an opto-electrode, which is an optic fiber coupled to a recording electrode, into the target brain region to allow for optogenetic tagging (opto-tagging) of the OT neuron population.

> *Note:* The term 'opto-electrode' used in this text will refer to an optic fiber coupled to either a basic silicon electrode (Figure 2A1) or a tetrode (Figure 2A2), both of which have been tested using this protocol. Both types have different strengths and limitations, therefore the choice of opto-electrode will depend on the specific goals of the study.

This approach combines the extracellular recording of neural activity with the selective excitation of OT neurons via the cell-type specific expression of channelrhodopsin2 (ChR2), an excitatory light-sensitive ion-channel activated by a specific wavelength of light ($\lambda \sim 470$ nm). In this approach, the opto-electrode is accompanied by a microdrive (oDrive, Neuronexus or Versa-8 optical, Neuralynx) which will be fixed to the top of the skull following implantation of the opto-electrode. The microdrive is shielded from physical damage and electrical interference by using a protective copper mesh, which facilitates chronic recording in freely moving rats.

1. For surgery preparation, follow steps 1–5 of the viral injection section in the "before you begin" section after making note of the following modifications:
   a. Make the incision on the skin at least 2 cm long and use four bulldog skin clamps to ensure adequate exposure of the entire Bregma–Lambda region, which is required for successful implantation of the opto-electrode/microdrive system.
   b. Clean the skull around the implantation site with 2% hydrogen peroxide (H_2O_2) to prevent micro-bleeding before drilling any extra holes.
   c. Avoid leakage of H_2O_2 into the holes designated for viral injection.
2. Using the high-speed drill, make six holes (0.5 mm diameter) in the skull around the implantation site. Install two stainless-steel screws (0.6 mm diameter) pre-soldered with silver wires (ground and reference) in the two most caudal holes (Figure 2B1). Install regular stainless-steel screws in the remaining four holes.
3. Cover the six screws with a thin layer of UV glue (RelyX Unicem 2 Self-Adhesive Resin Cement) for better anchoring on the skull surface.

a. Make sure not to apply any glue to the Bregma point and implantation site as they have to remain intact for the following steps.
b. Use micro-drills (HSS mini milling cutters 0.5 × 40 mm ball nose) to enlarge the PVN injection hole for the implant, and remove the debris with a rubber suction bulb.
c. Penetrate the dura with a fine needle or insect pin.
d. If bleeding occurs, apply gentle pressure with a micro hemostatic sponge. If excessive bleeding occurs, please refer to 'problem 1' for potential solutions.

Note: If there is tissue hyperplasia around the craniotomy hole from the previous viral injection, carefully clean it with sterilized PBS solution, remove excess tissue or protective gel with fine tweezers, and then enlarge the hole with the drill to obtain the desired diameter (0.5–1 mm); the implantation site must be clean and white brain tissue must be visible for the success of this protocol.

4. Propel the microdrive to extend the pre-mounted opto-probe or opto-tetrodes up to half of the driver's full length (e.g., 10 mm out of 20 mm, Figure 2A1). Then attach the microdrive to the stereotaxic arm with an adaptor and verify that the opto-electrode shank is perfectly aligned to the vertical bars on the stereotaxic frame in all directions.

⚠ CRITICAL: The Neuronexus oDrive is designed with lateral optic fiber fixation screws (Figure 2B2). Carefully loosen them first before propelling the probes. This step can only be done before implanting the probe, as any physical contact will damage the probe and brain tissue once it has been implanted. Neglecting this step can also cause the optic fiber to break while propelling the probe.

⚠ CRITICAL: If using any other customized opto-tetrodes, it is important that the optic fiber tip is placed 300–500 μm above the tetrode's recording sites (Figure 2A2). A higher distance will not allow for optogenetic activation, whereas a smaller distance will generate strong photo-electrical noise during opto-stimulation. We recommend using a 200 μm fiber cannula with a 0.37 NA (numerical aperture) to ensure sufficient light coverage and penetration of the recording area. The conductance of each channel should be measured before implantation. We recommend gold-plating the tetrode tips and checking before implantation that the impedance of each electrode is 0.3–0.5 MΩ. If large photo-electrical artifacts from opto-stimulation are detected on the recording electrodes, please refer to 'problem 2' for potential solutions.

Note: When working with tetrodes, it is recommended to apply a drop of sterilized PBS onto the tetrodes as the superficial tension of the liquid drop will keep the wires close together and thereby facilitate easier implantation.

5. Mount the microdrive with the opto-electrode onto the stereotaxic arm with an adapter.
   a. Repeat the stereotaxic calibration of the skull with respect to Bregma using the tip of the opto-electrode (be careful not to touch the skull) and then position the shank above the craniotomy hole (AP: −1.8 mm, ML 0.35 mm for PVN).
   b. For non-tetrode opto-electrodes, position the probe medial and the optic fiber lateral to the skull midline.
   c. In all cases, make sure the opto-electrode shank will fit in the craniotomy hole before attempting implantation.
6. Slowly insert (10 μm/s) the opto-electrode into the brain tissue until reaching a point above the target depth (DV −7.5 mm).
   a. If bleeding occurs during insertion, pause and wash with PBS for 1 min before continuing (see 'problem 1' for potential solutions).

b. If the electrodes bend upon entering the brain (usually due to incomplete puncturing of the dura), retract the electrodes from the brain tissue, wash with PBS thoroughly, adjust the position 100 μm anterior or posterior and then re-attempt the implantation.

Note: When working with tetrodes, wax should be applied prior to fixation of the drive base on the skull because of the short distance between the drive base and electrodes.

7. Seal the probe shank and implantation hole with heated wax.
   a. Place some solid wax around the electrodes, then carefully melt it with the tip of a soldering gun (80°C–100°C); alternatively, place solid wax in a beaker and use heat to melt it, then draw it into a syringe and quickly apply it to the electrodes implantation site before it turns solid again.
   b. Carefully remove any excess wax that remains on the skull surrounding the craniotomy hole (Figure 2B2). The purpose of the wax is to seal the hole in the event of bleeding and to prevent dental cement from contacting the electrodes, which would prevent movement of the microdrive.
8. Fix the base of the microdrive onto the rat's skull (Figure 2B3) with UV glue (3M RelyXUnicem 2) and apply dental cement (Paladur, Heraeus Kulzer; interior layer 1:2, outer layer 1:1 liquid:solid component ratio) around the microdrive base and the six anchor screws to achieve a firm and solid implant.
9. After the dental cement has solidified, mount the Omnetics connector onto the microdrive (Figure 2B4).
10. Prepare a custom-made square-shaped copper mesh to protect the microdrive and the opto-electrodes from physical damage (e.g., due to self-grooming).
    a. Solder the wires from ground and reference screws to the copper mesh to build a mini-faraday cage around the implanted head-stage (Figure 2C2).
    b. Seal the copper mesh all around the microdrive with solder.
    c. Fix the copper mesh to the skull with dental cement.
    d. If neural recordings display high amounts of electrical noise and/or a low signal-to-noise ratio, please refer to 'problem 3' for potential solutions.

⚠ CRITICAL: Carefully prevent any leakage of soldering material onto the rat's skin. The copper mesh helps to decrease electrical noise from the environment. In order to prevent artifacts from the animals movements, the ground wires must be firmly soldered to the copper mesh and secured to the skull with dental cement.

11. Let the dental cement dry for 10 min and then place the rat in a heated recovery cage without any objects that can potentially damage the implants during awakening.
12. After recovery, place the rat in a single-housing cage with a high ceiling to avoid damage to the head stage (e.g., remove any obstacle in the cage that can potentially hit the implanted opto-electrode). If the implanted head-stage becomes detached or damaged, please refer to 'problem 4' for potential solutions.
13. Monitor the health status of the rat every day for one week after the implantation, administer analgesic via the drinking water (e.g., Dafalgan 500 mg/L, tablets, store at room temperature (15°C–25°C)). Rats typically return to normal behavior 3 days after surgery. However, if the rat continues to frequently scratch the surgical site, it is recommended to administer additional analgesic (e.g., Rapidocain 30 mg/kg, i.p., stock concentration 10 mg/mL, store at room temperature), as needed.

⚠ CRITICAL: optic fiber tips are extremely vulnerable, especially during the connection with the fiber patch cord. Make sure it is well protected after surgery. For the Neuronexus oDrive, secure it with lateral screws; for customized opto-tetrodes, it is recommended

to fix the optic fiber ferrule, and only mount the tetrodes to the microdrive to allow them to move independently (see Figures 2A1 and 2A2).

Optically assisted identification of oxytocin neurons

Timing: 30–60 min

Once the opto-electrode is correctly placed in the target region, short pulses of light are used to evoke time-locked responses in the neurons which are expressing the light-sensitive opsin (ChR2) and to distinguish the neuronal population of interest from other neighboring neurons.

14. Starting from day 3 following implantation until day 7, the experimenter should handle the rat for 15 min/day for habituation to the connecting procedure.
15. Turn on the laser 30 min before starting the recording to preheat the system. Adjust the laser power to have a stable output power of 10–20 mW/mm^2 (after coupling the optic patch cord to the laser). Measure the power of each optic fiber in advance with an optical power meter (e.g., PM100D, Thorlabs) (Figure 2G).

⚠ CRITICAL: Handling of the laser system (Class 3B for visible-light lasers with output power > 5 mW) can be hazardous for eye exposure and must be used by instructed personnel only. Precautions when handling the laser include keeping the beam away from the eye or face and using laser safety glasses or goggles designed for the specific wavelength of the laser. Also, remember that reflections of mirrors, glasses, and shiny surfaces can be just as hazardous as the direct beam. Do not wear watches or jewelry when handling the laser beam.

Note: A damaged patch cord is another reason for failed optogenetics stimulation. It is important to make sure both tips of the fiber patch cord are perfectly polished and no light leakage from the fiber cable occurs. The coupling efficiency should be above 90% from the light source.

16. Connecting procedure of the implanted rat to the recording system:
    a. Gently lock the rat's head with one hand.
    b. Connect the optic fiber to the fiber patch cord and the Omnetics connector with the pre-amplifer (always align the connector and the pre-amplifer correctly with the printed logo "Omnetics" on the same side).
    c. Attach the recording cable (SPI ultra-thin wire, Intan) to the recording system and connect the fiber patch cord to the laser.
    d. Place the connected rat into a clean and empty cage (Figure 2D1).

⚠ CRITICAL: Handling of the animal is crucial to reduce the stress caused by the connecting procedure. Unhabituated rats are very likely to damage the opto-electrodes; well-handled rats should sit calmly on the experimenter's arm. If the animal displays signs of stress during handling despite habituation, wait until the rat calms down before proceeding to any further steps. Alternatively, animals could be lightly anesthetized (3–5 s) in the isoflurane chamber prior to connecting the cables, note however, that this has to be permitted by the animal welfare licenses. If the rat has lost or broken the implanted head-stage, please refer to 'problem 4' for potential solutions.

17. Set the parameters of the triggering device (e.g., Master-9, A.M.P.I.) to activate the laser (wavelength 473 nm, DreamLasers) in 10 ms pulses with different inter-pulse intervals (stimulation at 1 Hz, 5 Hz, or 10 Hz with 300, 1500, and 3000 pulses, respectively) (Figure 2G).

18. Turn on the data acquisition system (DAQ), link the laser TTL output to the digital input of the DAQ board, and start the electrophysiology recording and the stimulation sequence (Figure 2C1).
19. Visualize the light-evoked response of the recorded single units online in the raster mode (aligned to the laser TTL signals).
    a. Check if some neurons are activated by the light pulses, which provides an indication that the electrodes have been correctly implanted in the target region.
    b. Afterwards, in the offline mode, analyze the sorted spikes in a peri-stimulus time histogram (PSTH) aligned to the onset of the light pulses. Only units that show a clear increase (+3 standard deviations compared to the average of a duration of 100 ms during pre-stimulation, peak response within 10 ms delay) in response to the light pulses are labeled as ChR2-expressing OT neurons (Figure 2. I lower panel, also see Figure 4. A with red marker and Figure 4C).
    c. If no action potentials (spikes) are detected with the recording electrodes in the target region (PVN), please refer to 'problem 5' for potential solutions.
    d. If no neurons respond to the blue laser stimuli or have a long delay and/or jittered response, please refer to 'problem 6' for potential solutions.

Note: OpenEphys GUI (starting from version 0.2.7) has a built-in online PSTH function, it is useful to visualize the real-time laser response of any recorded units.

20. Save the channel number and firing properties of the optically responding cells for easier identification in the analysis (Figures 2H and 2I).

Note: When using silicon probes, the channel arrangement illustrated in the channel map provided by the manufacturer has to be converted depending on the type of adaptor and preamplifier used. We recommend to check carefully the channel arrangement before implanting the probe by inserting it slowly, using the stereotaxic arm, into sterile PBS solution and visualizing the noise level of each channel in real time on the recording acquisition system. When electrodes are inserted into the PBS solution the noise level of the corresponding channel should decrease due to the higher conductivity of the solution compared to air.

21. If no units were found responding to the laser stimuli, propel the microdrive 50–100 μm and wait a few hours before re-doing the steps above. Do not propel the microdrive more than 200 μm per day. Please refer to 'problem 5' for potential solutions if the problem persists.

⚠ CRITICAL: Only neurons displaying a response to the light pulses with a short latency (less or equal to 10 ms) can be considered a primarily responding neuron. Other units showing responses, but with higher latencies are likely to be secondary responses, therefore not specific to the cell type of interest (Figure 2. I upper panel, also see Figure 4, A borderless panel).

Recording of identified oxytocin neurons during social behavior

Timing: 1.5–2 h

After the identification of light responding cells (opto-tagging), it is important to perform the *in vivo* recordings with experiment protocols as soon as possible, usually within 3–7 days, to maximize the likelihood of acquiring the signal from the same opto-tagged neurons. The following protocol can be used for extracellular recording in freely-moving rats and social interaction of novel conspecific rats.

22. Bring the experimental rat and the stimulus rats into the recording room at least 30 min prior to the recording, to allow the rats to habituate to the new environment (this might vary depending on your experimental design).
23. Place the experimental rat, already connected to the neural recording system, into the recording chamber (open field arena, square 60 × 60 cm) and start the recording session (electrophysiology recording and video camera need to be synchronized, e.g., by TTL outputs or LEDs).
24. Let the rat freely explore the open field arena for 10 min. This session will serve as a 'baseline'. Gently remove the rat from the arena and place it into an empty clean cage for an interval of 15 min.
25. For social interaction, place the experimental rat and a conspecific naïve rat (in our case, female Wistar rat, 8–16 weeks old, 220–300 g) in opposite corners of the recording chamber at the same time and let them interact for 10 min (Figure 2D2).

Note: Always thoroughly clean the arena with 70% ethanol followed by distilled water prior to the experiment to remove any odour that may alter the normal behavior of the rats. Use low illumination (< 20 Lux) in the recording chamber to avoid stress for the animals.

26. Track the rat's locomotion with an automated tracking software (e.g., Ethovision XT11, Noldus) and manually annotate social interaction events.
27. Remove both rats from the recording chamber and place them in separate cages.
28. Repeat the steps 17–21 at the end of the recording session to confirm the identity of opto-tagged neurons for further analysis.

Note: It is important to keep a record of the OT neurons before, during, and after social interaction, because the signal can be noisy during social interaction due to large motion noise. Therefore, the OT neurons identified previously can serve as a template for spike sorting.

Alternative: temporary implantation of opto-electrodes and acute recording of oxytocin neurons in anesthetized rats

Timing: 2–3 h

Acute recordings in anesthetized rats allows for the monitoring of neural activity from opto-tagged OT neurons while mimicking social somatosensory stimuli. This mimicry can be achieved by applying external stimuli, such as 'airpuffs', to the rat as described in Tang et al., 2020. To record neural activity of OT neurons with temporary opto-electrodes in anesthetized rats, follow the same procedure as described in steps 1–13 and 14–21 for chronic implantation with the following changes:

Note: Opto-tetrodes are not suitable for acute recordings because they bend easily during repeated use and are difficult to clean between implantations.

29. Attach a temporary opto-electrode (Neuronexus) and it's connectors to the stereotaxic arm. Then immediately connect the preamplifier and optic fiber patch cord to the probe as it is not feasible to connect them after the implantation.
30. Implant only two screws on the rat's skull for grounding and referencing purposes. No anchor screws are needed because the probe will be removed after recording.
31. Connect the probe's ground and reference pins with the wires attached to the ground and reference screws, respectively.

Note: It is recommended to solder micro connectors to the grounding wires on screws and probes before implantation to facilitate the process. Otherwise you will need to solder the wires together during implantation.

32. Periodically apply sterilized PBS solution on the rat's skull to keep the brain moist during the whole implantation and recording procedure. Monitor the rat's breathing rate during the whole process.

⚠ CRITICAL: Electrophysiological recordings of anesthetized rats that are head-fixed to the stereotaxic device require an electrically isolated environment (ideally a faraday cage over the whole setup) and proper grounding (Figure 2E), as any open-end electronic devices nearby can generate electrical noise detectable by the preamplifier. In our experience, the digital meter of the stereotaxic frame, the heating plate, and the laser generators (Figure 2G) were the major sources of electrical noise. It is important to ground everything to the faraday cage and check the basal noise level of the signal (<25 μV after applying band-pass filter) before implanting and recording. If neural recordings display high amounts of electrical noise and/or a low signal-to-noise ratio, please refer to 'problem 3' for potential solutions.

33. If opto-tagged neurons were not found after the first implantation, retract the opto-electrode (200 μm/min for the first 5 min, then 2 mm/min until fully retracted), wash it with PBS, and redo the same procedure. We are typically able to record from each site 2–3 times, after which the brain tissue becomes too damaged for further recording. If action potentials (spikes) still cannot be detected after repositioning the opto-electrode in the target region, please refer to 'problem 5' for potential solutions.
34. Perform an acute recording of opto-tagged OT neurons while delivering external sensory stimulation, such as 'airpuffs', to the rat (Figure 2F).
35. After recording, carefully retract the opto-electrode and unplug the connectors. Wash the probe following the protocol described below. Gold-plating is advised after repeated usage, make sure the impedance of each channel is 0.3–0.5 MΩ.
36. Sacrifice the rat after recording, perfuse it with PFA (4%) and extract the brain to verify the implantation site.

Note: Silicon Probe cleaning and care protocol:

After withdrawing the probe from the brain tissue at the end of an experiment, immediately rinse it with a gentle stream of distilled water. Soak the probe (only the silicon shank; avoid soaking the epoxy or PCB) in a protein-dissolving detergent (e.g., enzyme cleaner AN000689 from Thomas Recording, 1 g/50 mL of distilled water) or diluted surgical instrument detergent (e.g., EmPower from Metrex, 1:128 in 20°C–40°C water). This process may take 0.5–2 h depending on the residual tissue. After the protein dissolving procedure, isopropyl alcohol (e.g., 70% IPA) can be used for sterilization, but not for more than 15 s. Using isopropyl alcohol without firstly dissolving the residual tissue from the probe could cause protein to stick to the electrode sites. Do not use ultrasonic cleaners and do not autoclave probes as this may cause damage.

Steps 37–46 describe how to image the oxytocin neurons population with fiber photometry *in vivo*.

Optically assisted implantation of optic fiber for photometry recording

Timing: 30–45 min

Fiber photometry allows for the capture of calcium-dependent fluorescent activity in a cell-type specific neuronal population, such as the OT neurons in the PVN, using an optic fiber. This technique relies on the expression of a genetically encoded calcium indicator (GECI) that changes its fluorescence as a function of intracellular calcium ion concentration. Among these sensors, the most commonly used are proteins from the GCaMP family (Chen et al., 2013), which are able to rapidly emit a fluorescent signal (λ = 473 nm excitation peak wavelength, λ = 515 nm emission peak

wavelength) in response to changes in intracellular free calcium concentration. These fluorescent signals are commonly used as a proxy for neural activity. Three to four weeks after the injection of a viral vector designed to express GCaMP6s in OT neurons (rAAV-OTp-GCaMP6s) in the PVN, an optic fiber is implanted above the PVN where it collects the fluorescent signal emitted by the OT neural ensemble (Grund et al., 2019; Tang et al., 2020).

Note: The implantation procedure can be optimized by recording the signal from the optic fiber during surgery. Once the optic fiber tip is approaching the target region where GCaMP6s is expressed, it is expected to detect an increase of the fluorescence collected in the green channel (515 nm).

37. Follow the initial steps (1 to 5 in the 'before you begin' section) for surgery preparation, as described in the section for viral injection, to obtain a clean and exposed skull (Figure 3A1).
    a. Perform the craniotomy (circular shape, 1 mm diameter) above the target region using a high-speed drill.
    b. Be careful when drilling, especially when approaching blood vessels toward the end. It is important to avoid contaminating the optic fiber tip with blood as it might reduce the fiber's optical transparency.
    c. If excessive bleeding occurs, please refer to 'problem 1' for potential solutions.

Note: Clean the craniotomy region with 2% H_2O_2 and let it dry for 1 min, make sure there is no blood or exudates. If blood contacts the fiber tip, retract the tip, clean it with ethanol (70%), and repeat the procedure.

38. Connect the fiber tip to the fiber photometry recording setup with an optic patch cord (mono fiber optics patch cord, Doric Lenses) and visualize the output fluorescence. Then connect the implantable fiber cannula (Thorlabs, CFMXD10, CFMC54L10) to the fiber patch cord with quick release interconnectors (Thorlabs ADAF2, ADAL3) or mating sleeves.

Note: We recommend using a 400 μm diameter, 0.5 NA implantable fiber cannula that can capture the fluorescence emitted from a larger brain volume and thereby increase the signal-to-noise ratio up to 4 times greater than a fiber cannula with a 200 μm core diameter.

39. Attach the optic fiber and patch cord to the stereotaxic holder, making sure the optic fiber is perfectly aligned to the vertical bars on the stereotaxic frame in all directions. Then slowly (10 mm/s) insert the fiber until it is ~100 μm above the target region (for PVN, DV −7.9 mm) to maximize the fluorescence signal collected from the target cell population (Qin et al., 2019).
40. While inserting the optic fiber into the brain tissue, the experimenter should see a visible increase in the fluorescent signal when approaching the region where the calcium sensor is expressed. Fine tune the final depth of the fiber in such a way as to maximize the fluorescent signal.

Note: If the optic fiber was successfully implanted above the GCaMP6s sensor expression site, it should be detected as an increase of the background fluorescent signal, as well as transient calcium signals due to spontaneous activity of the cells.

⚠ CRITICAL: The optic fiber cannula used for imaging (400 μm diameter) is larger than the opto-probe (100–200 μm), thus the insertion process needs to be very slow in order to avoid tissue damage. It is recommended to withdraw 0.3 mm for every 1 mm of insertion to allow the surrounding brain tissue to adapt. Inserting the cannula too rapidly may cause the brain tissue to be pushed down, leading to the dislocation of the target site.

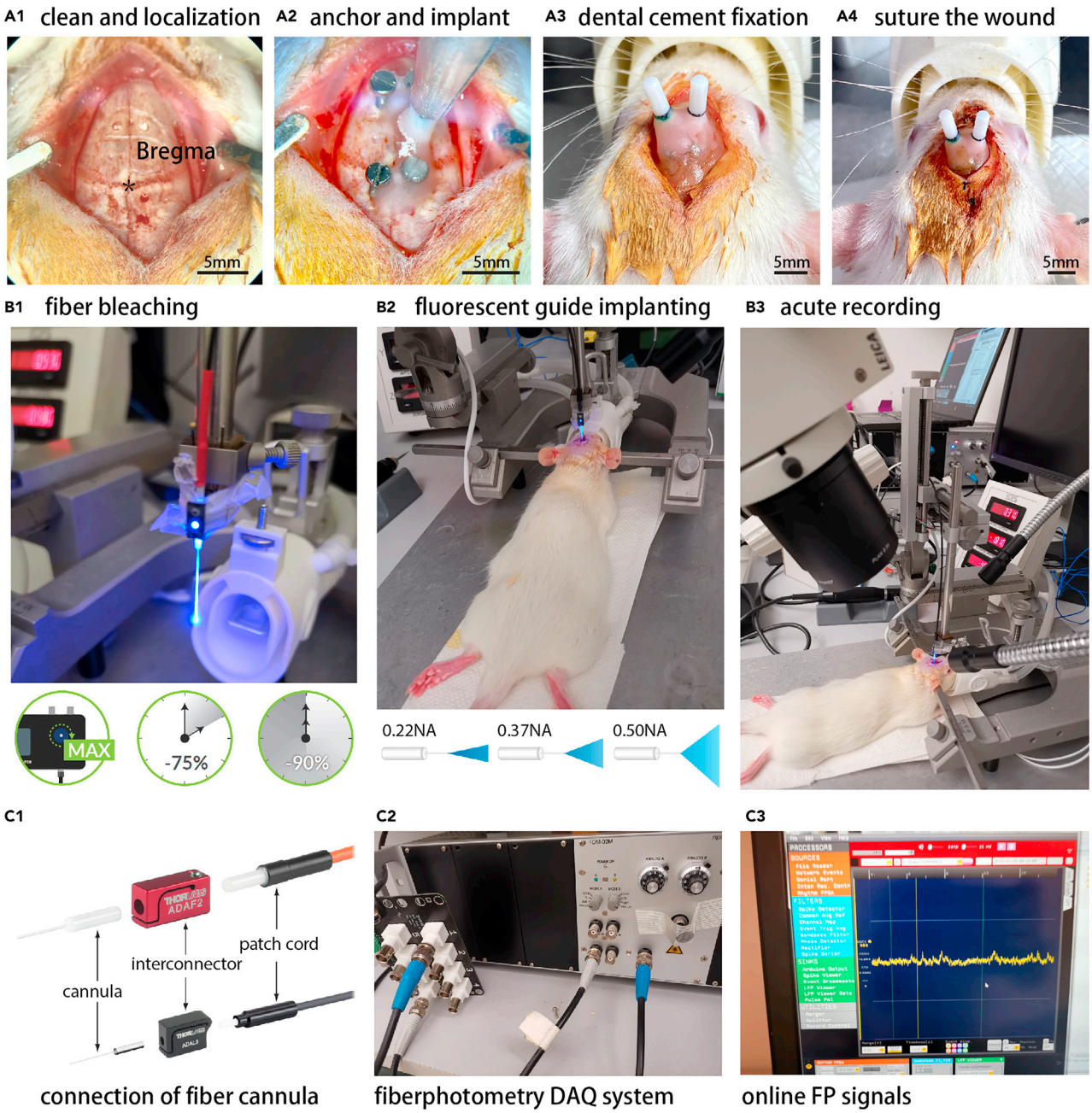

Figure 3. Implantation of fiber optic cannulas for fiber photometry imaging of oxytocin neuronal populations

(A1) Picture of a clean, exposed skull prepared for the implantation of the cannulas. (A2) Implantation of anchor screws and optic fiber cannulas. (A3) UV glue and dental cement are used to secure the implanted cannulas while leaving the fiber connectors (ferrule) free from dental cement. (A4) Surgical sutures are used to close the wound after the dental cement has hardened. Scale bar, 5 mm.

(B1) Setup for fiber photometry imaging and photobleaching of the fiber optic patch cord. (B2) Picture of an anesthetized rat implanted with an optic fiber connected to the fiber patch cord for photometry imaging. (B3) Optic fiber cannula mounted on the stereotaxic arm and connected to the fiber photometry LED.

(C1) Schema illustrating connections of an optic fiber cannula with the fiber patch cord using an interconnector (a mating sleeve). (C2) Fiber photometry data acquisition (DAQ) system. (C3) Real-time visualization of calcium signal acquired with fiber photometry imaging.

41. After reaching the desired depth, secure the optic fiber on the rat's skull using dental cement and four anchor screws (Figure 3A2), as described in steps 3 and 8. Wait until the dental cement has solidified.
42. Release the optic fiber then carefully lift up the arm of the adaptor (Figure 3A3).
43. Suture the wound closed and place the rat in a heated recovery cage (Figure 3A4).

Note: We do not recommend using a protective cap for the optic fiber tip because, in our experience, the rat will always remove the cap within a few days. Furthermore, if the cap is attached too tightly there is a risk of breaking the fiber implants each time the cap is removed. Alternatively, simply clean the fiber tip with 70% ethanol and a cotton swab before each recording.

44. In the 3 days following the surgery, check the animal's health status daily and administer analgesics as described for the surgical procedure in the viral delivery section.

Photometry recording of a neural population in vivo

Timing: 1–3 h

Note: Allow at least 1 week of recovery from the surgery before starting the imaging session. Usually, the signal to noise ratio of fiber photometry will increase after recovery from bleeding and inflammation caused by implantation surgery. Choose a fiber patch cord with low autofluorescence (LAF, Doric Lenses). Photo-bleaching is advised 1 h prior to the recording session to minimize auto-fluorescent noise (for details refer to the protocol below). The auto-fluorescent noise is mainly generated from the external layers of the fiber patch cord. If using a CMOS camera-based fiber photometry system, this issue can be softened by selecting a region of interest only in the center of the fiber core.

Note: Patch Cord Photobleaching protocol (derived from Doric Lenses, (https://www.doriclenses.com/downloads/ApplicationNotes/LAF_Application_Note_V1.0.0.pdf).

Due to their chemical composition optical fibers possess an innate fluorescence (autofluorescence), which may interfere with photometry measurements. Autofluorescence can be drastically reduced through photobleaching, which involves exposing the fiber to light of a high intensity in order to bleach the fluorophores responsible for autofluorescence. Photobleaching is necessary before any photometry experiment and has to be performed while the system is disattached from experimental subjects (Figure 3B1).

The procedure includes the following steps: Connect one end of the patch cord to the optical fiber output of a blue LED light source, typically the one used for the experiment. Place the opposite end of the patch cord in a beam dump or black box. The disconnected end of the patch cord will emit intense light during the photobleaching process; it is important that it is isolated to avoid harm or damage. Set the output current of the light source to its maximum value. Leave the light on for 1–2 h. An illumination for 1–2 h should decrease the autofluorescence by 75 % (typically) of its initial value. Further photobleaching (12+ hours) can reduce the natural autofluorescence by 90% (typically) of its initial value. Repeat the process for each patch cord should there be multiple channels. Photobleaching provides a temporary reduction of autofluorescence. Repeat this procedure before each experiment to ensure a minimal level of autofluorescence. As autofluorescence recovery is very slow, a simple 1 h exposure can be sufficient if the patch cord was used in the last few days. If motion noise or artifacts are present during fiber photometry in vivo imaging, please refer to 'problem 7' for potential solutions.

45. Attach the low-auto fluorescent patch cable to the cannula tip on the rat's head and record the baseline calcium activity (Figures 3B2 and 3B3).

Note: Reduce the amount of light present in the recording room to a minimum in order to avoid interference with the collected fluorescent signal. With the Thorlabs interconnector, the fiber cannula is sheltered from the environmental light (Figure 3C1). If using other connectors (mating sleeves), it is advised to put a black cover on it (e.g. heat-shrink tubing) to block the environmental light. It is also important to make sure the DAQ system itself is grounded because electrical noise can contaminate the voltage-converted fluorescent analog signal (output of the fiber photometry recording system, Figure 3C2). If neural recordings display high amounts of electrical noise and/or a low signal-to-noise ratio, please refer to 'problem 3' for potential solutions.

46. Visualize the collected fluorescent signals (both 405 nm isosbestic control channel and 473 nm GCaMP6s calcium signal channel) from the optic fiber in the DAQ software.
    a. To visualize the change in fluorescent signal online, apply a band-pass filter (low-cut 0.1 Hz and high-cut 100 Hz), and a 3kHz sampling rate to reduce data size due to oversampling, which is sufficient considering the slow calcium dynamics of GCaMP6s (Figure 3C3).
    b. If no fluorescent signal or only a very weak signal is detected during imaging, please refer to 'problem 8' for potential solutions.

⚠ CRITICAL: Due to GCaMP6s sensor fluorescence bleaching, the optical signal will decrease over time, thus it is necessary to adjust the final output of the LED power to minimize the bleaching effect. We recommend using an output power of 50–100 μW/mm^2 (from the fiber tip) with a 20 Hz stimulation rate. We also do not recommend recording for more than 3 hours continuously (this time may vary for different fluorescence sensors).

Note: Photometry imaging can be employed in animals that are awake and freely moving or while they are anesthetized. In the case of recording exclusively in anesthetized animals (acute recording Figures 3B2 and 3B3), no anchor screws are needed and imaging can be done immediately after implantation of the fiber cannula in the GCaMP6s-expressing region (refer to the 'airpuffs' protocol with fiber photometry imaging in Tang et al., 2020). After recording, the optic fiber can be retracted from the brain, washed with ethanol (70 %) and reused more than once.

Histological verification of recording locations

Timing: 3 days

The recording location of opto-electrodes and optic fibers are verified *post-hoc* with immuno-histological techniques. If the expression of genes of interest delivered by viral vectors is absent or not sufficient, please refer to 'problem 9' for potential solutions.

47. After the experiment, euthanize the rat using a single intra peritoneal injection of a ketamine (400 mg/kg)/xylazine (80 mg/kg) solution.
48. Perfuse the brain transcardially with phosphate-buffered saline (PBS) followed by a 4% paraformaldehyde (PFA) solution.
49. Decapitate the rat, dissect out the brain and post-fix it in 4% PFA at 4°C with gentle agitation for 12 h.
50. Cut the brain coronally and slice it into 50-μm thick sections with a vibratome (VT1000S, Leica).
51. Collect the slices containing the brain region of interest (e.g., PVN) in a 12-well plate and perform immunohistochemistry on the free-floating sections.

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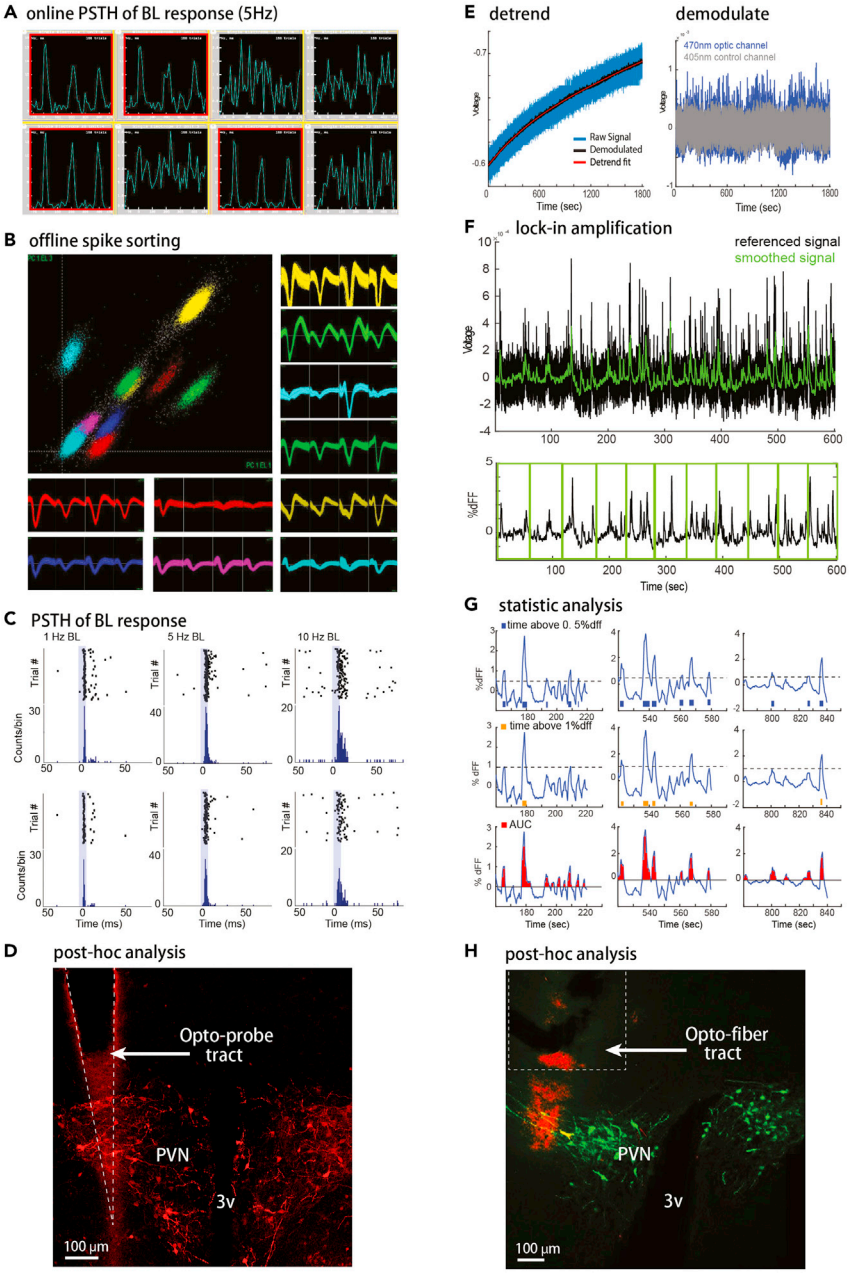

Figure 4. Data analysis pipeline for electrophysiology and photometry imaging of oxytocin neurons

(A) Example of "online" PSTHs aligned to blue light stimulations (5 Hz, 10 ms, 473 nm, 10 mW/mm^2) of eight recorded single-units. Four of them (marked with a red border) show a clear increase of spiking activity following light pulses with a very short (<10 ms) delay and are classified as ChR2-expressing OT neurons.

(B) Illustration of principal component analysis (PCA) used to separate clusters of spikes into sorted single units using Offline Sorter (Plexon) software. 10 well-separated clusters each represent a signal unit sorted with unique waveforms all recorded from one tetrode in the PVN.

(C) Examples of PSTHs aligned to blue light stimulations (10 ms, 473 nm, 10 mW/mm^2) of two optically tagged OT neurons at different stimulation frequencies (1 Hz, 5 Hz, 10 Hz). In all cases, light pulses evoked action potentials within a short latency of 2–10 ms from stimulus onset.

(D) Opto-electrode (dashed outline) above the PVN expressing ChR2 in OT neurons after infection of the nucleus by the OTp-ChR2-mCherry AAV. Immunostaining for mCherry shown in red. 3v - third ventricle, PVN - paraventricular nucleus. Scale bar, 100 µm.

Figure 4. *Continued*

(E) Analysis pipeline for photometry imaging data processing. Left panel shows a representative example of a raw signal trace. A low-pass filtered signal and polynomial fit are used to remove the slow attenuation of the signal due to photo-bleaching of fluorophores. Right panel shows a comparison of detrended and demodulated calcium signal (470 nm optic channel) and calcium independent signal (405 nm control channel).

(F) Top panel shows the lock-in amplification and smoothing of the processed signal with a Savitzky-Golay filter. Bottom panel shows a transformation of the voltage signal to dF/F_0 values and the separation of the data into 60 s (optional) time bins for downstream analysis.

(G) Estimation of neural activity from calcium transient signals using threshold crossing (0.5 or 1% of dF/F_0) and integration of the area under the curve (AUC) above the baseline level.

(H) Optofiber tract (dashed outline) above the PVN and OT neurons expressing GCaMP6s after infection of the nucleus by the OTp-GCaMP6s AAV. Immunostaining for GFP shown in green. 3v - third ventricle, PVN - paraventricular nucleus. Scale bar, 100 μm.

52. Blocking step: incubate the sections in blocking buffer (1×PBS + 1% Triton + 5% normal goat serum (NGS)) for 2 h at room temperature with gentle agitation. Use 1 mL of blocking buffer per every 4 slices.
53. Primary step: incubate the sections in primary solution (1×PBS + 1% Triton + primary antibodies) for 24–48 h at 4°C (cold room) with gentle agitation.
54. First washing step: wash the sections in 1×PBS (4 washes, 10 min each) with gentle agitation.
55. Secondary step: incubate the sections in secondary solution (1×PBS + 1% Triton + fluorescent secondary antibodies) for 2 h at room temperature, in the dark with gentle agitation.
56. Second washing step: wash the sections in 1×PBS (4 washes, 10 min each) with gentle agitation.
57. Mounting step: mount the sections on Superfrost Plus (Thermo scientific) microscope glass slides (4 sections per slide), let them dry completely, then cover with glass coverslips (24 × 50 mm, Roth).
58. Acquire high-quality images of immunoimmune-stained sections (Figures 4D and 4H) with a confocal laser-scanning microscope (e.g., Leica TCS SP5).

EXPECTED OUTCOMES

Here, we illustrated two techniques to monitor the activity of OT neurons *in vivo*: electrophysiological recordings from individual opto-tagged neurons, and fiber photometry imaging of cell-type specific neuronal population activity. Proper implantation of the recording opto-electrodes should allow for 3–5 days of productive recording after optogenetic tagging, as well as recording of other neighboring neurons and local field potentials, while the animal is performing behavioral tasks. Between recording sessions, it is recommended to repeat the opto-tagging procedure to ensure that the recorded units are stable over time.

The relationship between OT neural activity and social behavior is still not fully understood. We recently demonstrated that social interactions between conspecific female rats elicit a synchronous increase of OT neuron firing activity together with an intensification of the bursting activity of the cells. These physiological responses of individual OT cells were mostly driven by the physical contacts which naturally occur during social behavior between the two rats. This observation was made possible by closely monitoring animal movements during social interactions with a high-speed, high-resolution video camera and then matching the timing of specific behaviors with the neural activity of opto-tagged neurons.

Single-unit recordings of OT neurons in socially interacting rats revealed synchronous activation of individual cells, however the number of neurons that can be recorded at the same time from the same animal is limited. To probe the activity of the whole OT cell ensemble, we employed photometry recording of GCaMP6s-expressing OT neurons with an optic fiber. This technique allows us to monitor in real time the calcium-dependent activity of the neuronal population expressing the calcium indicator within a certain volume of tissue which, in this case, is enough to cover the majority of OT cells residing in the PVN. The data collected with fiber photometry imaging gave us a

comprehensive overview of the overall activity of PVN OT neurons in response to non-noxious somatosensory stimulation in real-time.

QUANTIFICATION AND STATISTICAL ANALYSIS

Spike sorting of neural data

After collecting neural signals with extracellular recording, single units can be isolated offline for classification and analysis. This step is necessary to obtain high-quality data from single units. We use Offline Sorter (Plexon) to sort spikes recorded from individual neurons (Figure 4B).

Note: The manual sorting method described below is recommended for opto-tetrode based recording, but not for Neuronexus opto-probes, in which case we recommend Phy-sorter (https://phy.readthedocs.io/en/latest/), a software designed for high-density channel spike sorting.

1. Load the recorded neural data into the Offline Sorter program. OpenEphys or Intan formatted data needs to be converted into plx format first; this can be done with MATLAB tools published by OpenEphys (https://github.com/open-ephys/analysis-tools/tree/master/ephys2plx).
2. Set the data into 'tetrodes mode' for simultaneous detection of spikes across four channels. Apply a high-pass, 250 Hz butterworth filter and set a threshold (3 times standard deviation of the noise level) for detection of action potentials independently in each of the four channels.
3. Exclude the high amplitude noise in each channel as well as the noise shared across all channels (offline sorter built-in function, use with caution). Align the waveforms such that there is only 100 ms between the valley points (global minimum between sort start and sort end).
4. Visualization of waveform features and clustering:
   a. Visualize the spike clusters in 3D feature projections, the most commonly-used features are principal component analysis (PCA), peak-valley slice and timesteps.
   b. Adjust the features and view angles from 3D clusters, until clear clusters are found.
   c. Manually draw a circle at the center of each potential cluster then apply template sorting with 70% tolerance. This step helps to keep the sorting quality equal in all channels.
5. Invalidate the noise in all units manually (sometimes noise can be similar to a waveform, but it can be distinguished with the timestep feature) and remove the waveforms that have inter-spike intervals (ISI) shorter than 1500 ms (Offline Sorter will show a line between two points in the 3D clusters). Check that the sorted units have a clear refractory period by visualizing the autocorrelation histogram for each unit.

   Note: OT neurons usually have long absolute refractory periods (>2 ms). Other neuronal cell types, such as fast-spiking interneurons, sometimes have shorter absolute refractory periods (<1 ms). Adjust this ISI parameter in Offline Sorter according to the recorded cell-types.

6. When analyzing multiple recording sessions from the same cells, it is recommended to keep the sorted units in the same order across multiple recorded files.
7. Save the sorted neural data by exporting it to a new plx file. Convert the data format in order to visualize the results in a data analysis software such as Neuroexplorer.

   Note: for a complete guideline on spike sorting consult the Offline Sorter user manual: https://plexon.com/wp-content/uploads/2018/02/Offline-Sorter-v4.4-User-Guide-a.pdf.

Analysis of local field potentials

Local field potentials (LFPs) can be studied by analyzing the same raw data acquired during extracellular recording.

8. Load the recorded neural data into MATLAB and downsample it to 3 kHz.
9. Filter the data with a low-pass (200 Hz) butterworth filter to remove high frequency noise.

10. Examine the power spectrum density (PSD) of the LFP signal by applying a multitaper PSD estimate ('pmtm' function in MATLAB with a frequency range of 1–200 Hz).
11. Extract the oscillatory activity of specific frequency bands and calculate the point-by-point phase with Hilbert's transformation ('hilbert' function in MATLAB).
12. Correlate the activity of single units (action potentials) with the phase of LFP oscillations in different frequency bands to detect phase-locking of neuron activity with specific phases of the oscillation cycle.
13. Use Rayleigh's test for circular uniformity to assess the significance of the correlation between action potential timing and cycle phase.

Analysis of fiber photometry data

14. Load the recorded raw voltage signal data into MATLAB and downsample it to 300 Hz
15. Filter the data with a low-pass (10 Hz) butterworth filter to remove high frequency noise.
16. Apply the same step on the reference channel (if a 405 nm control signal was recorded simultaneously), then subtract it from the calcium dependent (473 nm) signal (Figure 4D).
17. Visualize the collected signal and remove possible non-neural artifacts (very fast, abrupt, and symmetric transients) due to micro-movements of the optic fiber cannula (motion noise).
18. Fluorophores are subject to photo-bleaching when excited with continuous light over time, which means that the average signal decreases over time as the emitted fluorescence slowly attenuates. To correct this effect, apply a non-linear polynomial fit (MATLAB 'polyfit' function) to the whole recording and subtract it from the raw data to obtain a stationary baseline (Figure 4E).
19. Smooth the signal with a Savitzky-Golay filter (MATLAB 'smooth' function, option 'sgolay').
20. Convert the signal F to DF/F by applying $DF/F(t) = (F(t) - F_0)/F_0$, where t is time and F_0 is the average value of F calculated over a 600 s window at the beginning of the recording session (Figure 4F).
21. Divide the data into 1 min time bins and calculate the area under the curve (AUC) of the signal using the MATLAB 'trapz' function to estimate the amount of calcium transients (Figure 4G).
22. Use ratios of AUCs between different experimental conditions to quantify the activity of the recorded neural population.

LIMITATIONS

This protocol is applicable only to cell types for which viral vectors with specific transgene expression or transgenic animals are available. The two recording methods described in this protocol (electrophysiology with opto-tagging and fiber photometry imaging) have opposing strengths and limitations. For example, extracellular recordings from opto-electrodes have both high temporal (30 kHz sampling rate to capture extracellular spikes in sub-milliseconds scale) and single cell resolution with opto-tagging, but only a limited number of units can be recorded in each animal due to the probe channel density, implantation precision, and viral expression efficiency; on the contrary, calcium imaging with fiber photometry using a large fiber core and NA allows for monitoring the activity of large neuronal populations that have been specifically labeled using viral vectors. However, the temporal resolution is limited by the dynamics of the fluorophore and single cell resolution is lost as well. Moreover, recording from opto-tagged cells may not be feasible if the neuronal population of interest is sparsely distributed in a large volume due to the physical distance from the recording electrodes. In the future, it may become more feasible to integrate the two techniques into one approach, which would ameliorate many of these limitations and provide an even greater level of detail regarding the function of specific cell-types at both the single-cell and population level.

TROUBLESHOOTING

In this section we summarize the potential problems which can occur during the surgical procedures or during the recording and highlight some possible solutions.

Problem 1

Excessive bleeding during the surgery after removing the dura (step 3).

Potential solution

When bleeding happens, repeatedly wash the brain surface with sterilized 0.9% saline solution for 1–2 min and apply a hemostatic gauze to stop the bleeding. Keep the skull moist with saline throughout the surgery.

Problem 2

Large photo-electrical artifacts from the laser stimulation are detected on the recording electrodes (step 4).

Potential solution

Move the optic fiber tip further away from the electrode recording sites. For the opto-probes, contact the manufacturer to customize the fiber tip and recording site distances to suit your specific needs. Check for leakage of light in the optic fiber shank as it may also result in artifacts. For opto-tetrodes, position the tetrode tips 300–500 μm from the tip of the optic fiber when assembling the microdrive. Decrease the laser power and check the interconnector light leakage, sometimes adding a shelter around the optic interconnector (mating sleeve) may prevent opto-electrical noise. The photo-electrical artifacts are present only at the beginning and the end of laser pulse stimulation (e.g., at 0 ms and 10 ms when delivering a 10 ms laser pulse), thus it may be possible to exclude them from the recorded data during the offline spike sorting process.

Problem 3

Neural recordings display high amounts of electrical noise and/or a low signal-to-noise ratio (step 10).

Potential solution

There are several potential causes for electrical noise and/or a low signal-to-noise ratio in electrophysiology recordings. Check the environmental electrical noise before implantation. The neural recording system should be separated from other electrical devices and all of them should be grounded. Ideally, the whole setup should be installed on an electrically isolated table (faraday cage). Computers, screens, power supplies, and the DAQ system should be grounded as well. Furthermore, make sure that the grounding electrode is properly connected to the ground on the skull screw during implantation. Use dental cement to fix the wire around the ground and reference screw to prevent movement of the wire while the rat is moving. In acute recording, lowering the electrode very slowly will increase the likelihood of obtaining high-quality recordings. In chronic recordings, wait a few days after surgery and propel the electrodes 100–200 μm each day until obtaining an optimal signal.

Problem 4

The implanted head-stage has become detached or broken (step 12).

Potential solution

This is often caused by poor implantation of the anchor screws, a bad dental cement seal between the skull, or using excessive force while connecting the wires (see Major Step 1–1). Thoroughly cleaning the skull before applying dental cement or increasing the number of anchor screws on the skull may help.

Problem 5

No action potentials (spikes) are detected with the recording electrodes in the target region (PVN) (step 19).

Potential solution

Make sure that all connections (e.g., preamplifier, recording cable) are still properly in place and not loose. Monitor the presence of spikes during the whole implantation procedure: while inserting into the brain tissue, spikes should be visible when the electrodes penetrate into regions with high neuronal density (i.e., hippocampus and thalamus). If the latter occurs, but no spikes are visible at PVN depth, it is likely that the electrode was implanted in the third ventricle. In which case, slowly retract the opto-electrode from the brain, wash the shank with enzymatic detergent and PBS, carefully removing any tissue attached to the probe or fiber tip. Next, correct the stereotaxic coordinate (try moving the position of the probe 100 μm anterior or posterior) and re-implant following the step-by-step procedure. The absence of action potentials might also be caused by a generally low level of neural activity due to the depth of anesthesia; this also depends on the type of anesthesia used and the region that is recorded from.

Problem 6

No neurons respond to the blue laser stimuli or have a long delay and/or jittered response (step 19).

Potential solution

This can happen for different reasons. Firstly, the opto-electrode must be implanted correctly at the ChR2 expression site. Secondly, the optic fiber aligned with the electrodes must be close enough (200–500 μm) to stimulate the ChR2 neurons with light pulses. Finally, a delayed signal may be indirectly caused by OT receptor expressing neurons in the PVN that are responding to OT released from the OT expressing neurons that were stimulated by the blue light (also see problem 9).

Problem 7

Motion noise or artifacts are present during *in vivo* fiber photometry imaging (step 45).

Potential solution

Make sure that the connection between the optic fiber and the fiber patch cord is properly aligned. Pre-bleach the fiber patch cord before recording (as described in steps 45–46). Using a longer fiber patch cord can help reduce the cable tension when recording in freely moving animals.

Problem 8

No fluorescent signal or very weak signal was detected with fiber photometry imaging (step 46).

Potential solution

This typically occurs when the optic fiber is too far from the GCaMP6s expression site. The maximum signal is usually achieved when the fiber tip is ~100–200 μm above the fluorescent region. Carefully verify the implant location each time after finishing a recording (steps 47–58). Another possible reason is that the fiber tip is dirty or damaged (either the implanted fiber cannula or the fiber patch cord); apply ethanol on the fiber tip and clean it carefully, polish both sides of the fiber patch cord to increase the light coupling efficiency (also see problem 2).

Problem 9

Absent or insufficient expression of transgenes delivered by viral vectors (step 47–58).

Potential solution

There are several potential reasons for absent or low expression of the genes of interest. Make sure that the viral titer is high enough with rt-qPCR ($>10^{10}$ genomic copies/mL). Check that the injection site was targeted correctly with histology. Wait at least 3–4 weeks after viral injection before performing the experiment. For theOT promoter, 2% salts in the drinking water can promote the virus expression.

⚠ CRITICAL: Do not use Lenti- or Rabies- virus in any *in vivo* opto-tagging approach as neurons will die shortly after transfection. We recommend using only rAAV for this technique.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Valery Grinevich (Valery.Grinevich@zi-mannheim.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

MATLAB code used for the analysis of fiber photometry data is available in the Supplementary Software section of the published article (Tang et al., 2020).

This study did not generate new datasets.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.T., D.B., A.L. and V.G.; investigation Y.T., D.B., and A.L.; writing – original draft D.B., and A.L.; writing – review & editing, all authors; visualization Y.T. and S.K.; funding acquisition, V.G.; supervision, A.C. and V.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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