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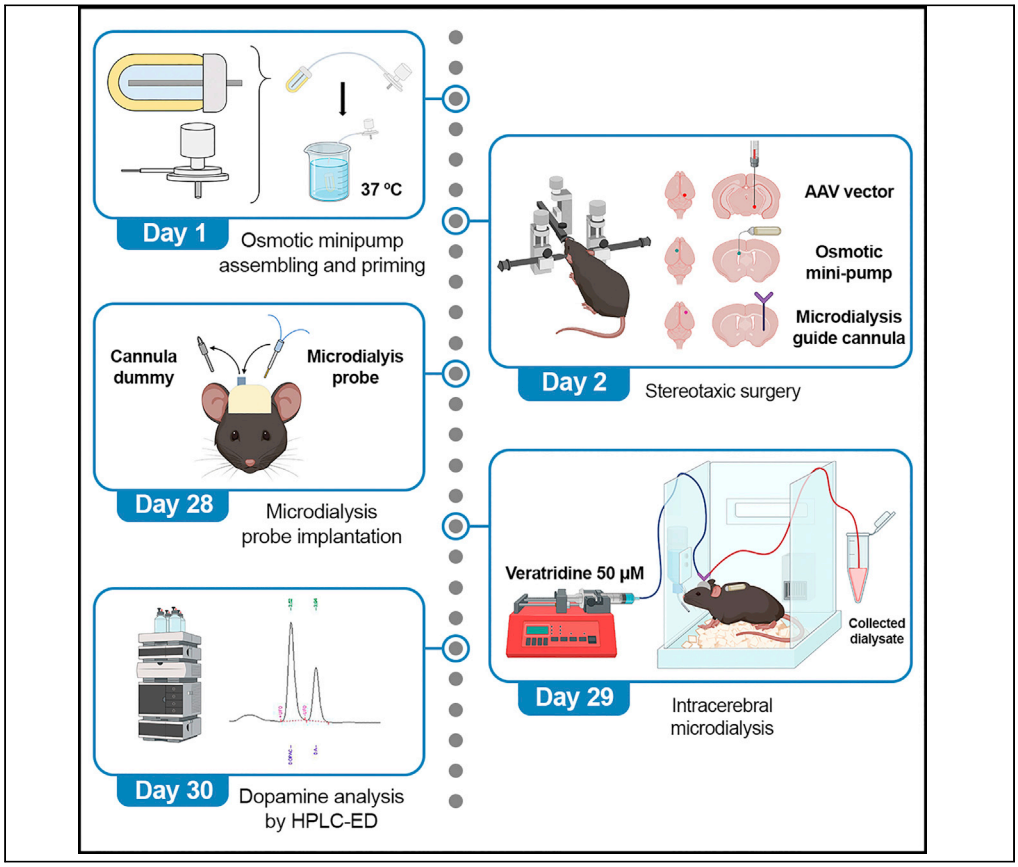
Intracerebral administration of a modified antisense oligonucleotide targeting the dopamine system in a mouse model of Parkinson's disease



Here, we present an optimized protocol for generating a mouse model overexpressing human α-synuclein in dopamine (DA) neurons driven by an adeno-associated viral (AAV) vector and for the examination of the benefit of an antisense oligonucleotide (ASO)-based therapy on DA neurotransmission under Parkinson's disease (PD)-like conditions. We describe AAV injection, followed by implantation of an osmotic minipump for ASO delivery and a guide cannula for microdialysis to measure DA release. This protocol can be used to evaluate oligonucleotide-based therapies for PD.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Optimization of ASO sequences for *in vivo* delivery to selected dopaminergic neurons

Protocol for implantation of brain devices and AAV infusion in several brain areas

Step-by-step guide for intracerebral microdialysis in freely moving mice

Protocol for assessing pharmacological agents on synaptic DA release

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Intracerebral administration of a modified antisense oligonucleotide targeting the dopamine system in a mouse model of Parkinson's disease

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SUMMARY

Here, we present an optimized protocol for generating a mouse model overexpressing human α-synuclein in dopamine (DA) neurons driven by an adeno-associated viral (AAV) vector and for the examination of the benefit of an antisense oligonucleotide (ASO)-based therapy on DA neurotransmission under Parkinson's disease (PD)-like conditions. We describe AAV injection, followed by implantation of an osmotic minipump for ASO delivery and a guide cannula for microdialysis to measure DA release. This protocol can be used to evaluate oligonucleotide-based therapies for PD.

For complete details on the use and execution of this protocol, please refer to Alarcón-Arís et al. (2020).

BEFORE YOU BEGIN

Synucleins are a family of proteins mainly expressed in neurons and are concentrated in synaptic terminals. Specifically, α-synuclein (α-Syn) is a neuropathological hallmark of Parkinson's disease (PD), thought to be an early mediator of functional deficits in the DA system, leading to subsequent degeneration of DA neurons. α-Syn is considered a hub protein for synaptic functional homeostasis since it interacts with many presynaptic proteins, such as monoamine transporters and synaptic vesicles-associated proteins. PD-like animal models are important for understanding the molecular/cellular mechanisms of the disease and may contribute to the development and validation of novel therapies. Here, we describe a protocol to generate a mouse model based on human α-Syn -(h)-α-Syn- overexpression in DA neurons using an adeno-associated viral vector (AAV). One of the advantages of this model is that it does not require extensive cross-breeding of transgenic rodents for α-Syn. Briefly, AAV-induced overexpression of (h)-α-Syn in DA neurons triggered progressive accumulation and aggregation of (h)-α-Syn protein in the DA system. AAV-injected mice displayed early axonal impairment in output DA regions, resulting in cognitive and motor deficits, which mimics some outcomes of PD pathogenesis (Alarcón-Arís et al., 2020).

Using this PD-like mouse model, we have established a delivery strategy of an indatraline-conjugated antisense oligonucleotide (ASO) effectively achieving a selective reduction of (h)-α-Syn expression/function in DA neurons. Despite the marked difficulties in delivering oligonucleotides

to the brain, there is a growing interest in developing therapeutic oligonucleotides for brain disorders. In contrast to prior approaches (Collier et al., 2016; Benskey et al., 2018), the present study takes advantage to establish an innovative strategy, in which the oligonucleotide was covalently bound to indatraline (ligand with high affinity for monoamine transporters) for its selective delivery to monoaminergic cells (Alarcón-Arís et al., 2018, 2020; Pavia-Collado et al., 2021). This strategy allows the possibility of a very precise cellular targeting of α-Syn mRNA, abundantly expressed in monoamine neurons. Indeed, reduced (h)-α-Syn synthesis/accumulation contributed to rescuing early deficits in DA neurotransmission as α-Syn modulates neuronal DA activity (Alarcón-Arís et al., 2020).

The protocol describes the specific steps for using an AAV5 construct overexpressing (h)-α-Syn under the chicken-β-actin promoter, intracerebroventricular administration of indatraline-conjugated ASO, and assessment of striatal DA neurotransmission in mice. However, we have also used this protocol with different serotypes of AAVs (AAV1/2, AAV10) and promotors (cytomegalovirus) to overexpress endogenous or human genes in DA or serotonin (5-HT) neurons (Pavia-Collado et al., 2022; Miquel-Rio et al., 2022). We have also designed different oligonucleotide sequences conjugated to sertraline (selective 5-HT transporter ligand) or reboxetine (selective norepinephrine-NE transporter ligand) targeting specific genes of monoamine neuron subtypes. Using these tools, we assessed the impact on 5-HT and NE neurotransmission in the prefrontal cortex and hippocampus (Bortolozzi et al., 2012, 2021; Ferrés-Coy et al., 2016; Alarcón-Arís et al., 2018; Fullana et al., 2019; Pavia-Collado et al., 2022).

Procedures performed on adult male C57Bl/6 mice (8–12 weeks), begin with stereotaxic injection of an AAV5 vector, followed by implantation of an osmotic mini-pump in the lateral ventricle and a guide cannula for a microdialysis probe in the striatum. The protocol requires experimenter training in surgical techniques and in vivo microdialysis procedures.

Institutional permissions

All procedures were carried out according to regulatory standard ethical guidelines (EU Directive, 2010/63 of September 22, 2010) and were approved by the local ethical committee on animal research (University of Barcelona). Any use or reproduction of this protocol will require the acquisition of permissions from the relevant institution.

AAV construct

Timing: 30 min

The AAV vector used consists of an AAV5 serotype construct encoding wild-type human α-Syn under the chicken-β-actin (CBA) promoter, which was acquired through the Michael J. Fox Foundation for Parkinson's Research. Currently, the viral vector (AAV2/5 serotype) encoding wild-type human α-Syn will be a new formulation to replace AAV5-CBA-α-Syn previously offered through the University of Iowa (Martinez et al., 2014; https://www.michaeljfox.org/research-tools-catalog).

1. Prepare the AAV working solution from stock, which should be stored at −80°C. The concentration of the solution depends on the experimental design; we suggest ~10^9 gc/μL. The infusion volume is 1 μL/mouse, but we suggest reducing it to 0.5 μL, if possible, to avoid the excessive AAV diffusion around the injection site.
   a. Thaw an aliquot of stock, keeping it on ice. We suggest using the whole aliquot to prepare working solutions, avoiding thaw-freeze cycles.
   b. Transfer the volume to RNase-free Eppendorf tubes, preparing as many aliquots as planned for the total days of surgery. If necessary, dilute the viral titer using the same solution in which AAV is stored or RNase-free PBS.
   c. Freeze again the working solution aliquots until the surgery day.

⚠ CRITICAL: All AAV manipulation must be performed in a biosafety cabinet, under sterile conditions, and with adequate personal protection to avoid AAV contact with the experimenter.

ASO solution

Timing: 3 h

The ASO used in this protocol corresponds to the 669–709 complementary sequence of the human α-Syn mRNA (GenBank: NM_000345.4), which was optimized to fully match the sequence inserted in the AAV5-CBA construct. In addition, a nonsense ASO with no homology to *Mus musculus* genome was used as a control group. Sequences are ASO [5′-CGCCTTCCACGGTTUUCU-3′] and non-sense ASO [5′-CCGTATCGTAAGCAGTAC-3], commercially available for synthesis. However, chemical modifications of ASO involving conjugation with selective inhibitors of monoamine transporters (e.g., indatraline) at the 5′-carboxy-C10 of the oligonucleotide through amide bond are under patent license (WO2011131693, WO-2014064257-A1, WO2014064258-A1).

2. Prepare the ASO working solution from stock, which must be kept at −30°C. We suggest purchasing lyophilized ASO stock and solubilizing it before the procedure. The final ASO concentration will depend on the experimental design and the solubility of the molecule. In our case, we will use a dose of 100 μg/day, which corresponds to a 37.88 μg/μL concentration. Also consider that 20% of the final volume corresponds to artificial cerebrospinal fluid (aCSF) 5×, in which ASO will be solubilized when administered.
   a. Calculate the necessary volume depending on the number of osmotic mini-pumps that will be used and the ASO desired dose. For the case of a dose of 100 μg/day in a 28-days Alzet ® 1004 pump (approximate capacity of 100 μL, 0.11 μL/h flux):

$$\text{ASO solution} = 100\ \mu g/day\ /\ 2.64\ \mu L/day = 37.88\ \mu g/\mu L$$

$$100\ \mu L\ (\text{pump volume}) + 5.61\ \mu L\ (1.5\ mm\ \text{catheter volume}) = 105.61\ \mu L\ /\ \text{mouse}$$

$$\text{Final volume / mouse} = 84.488\ \mu L\ \text{ASO}\ (80\%) + 21.122\ \mu L\ \text{aCSF}\ 5\times\ (20\%)$$

$$\text{ASO corrected concentration} = (105.61\ \mu L\ /\ 84.488\ \mu L) * 37.88\ \mu g/\mu L = 47.35\ \mu g/\mu L$$

   b. Put all necessary lyophilized ASO aliquots on ice and do a short spin to displace all content to the bottom of the tube.
   c. Add RNase-free water to each tube to get the necessary concentration.
   d. Tap the tubes gently for 5–10 s and do a short spin.
   e. Store the aliquots at 4°C for 2 h to allow resuspension of the molecules.
   f. Tap every tube again, do a short spin and transfer the volumes of all solubilized aliquots into a single RNase-free Eppendorf tube.
   g. Store the solution at −30°C.

⚠ CRITICAL: every material and equipment used in the ASO preparation must be kept in RNAse-free conditions, including gloves and personal equipment. If necessary, clean the instruments with RNaseZap ® or an equivalent RNase Decontamination solution.

Note: the prepared ASOs solution should not be frozen with the aCSF. The corresponding volume of aCSF 5× should be added on the same day as the priming of osmotic mini-pump.

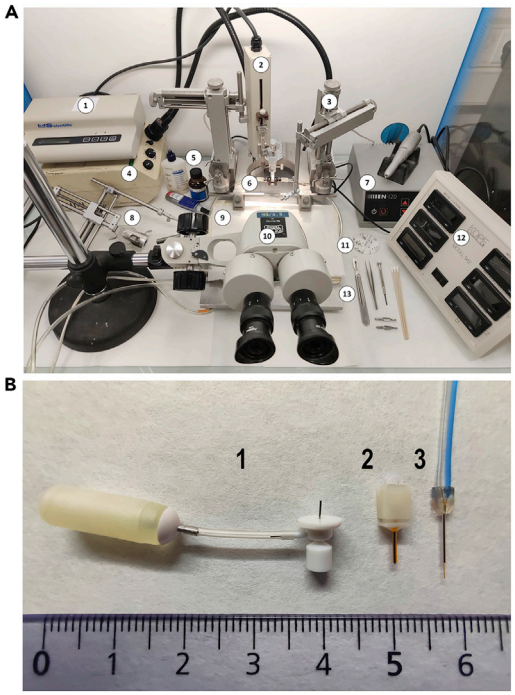

Figure 1. Stereotactic surgery materials

(A) Components of the stereotactic setup. 1: syringe pump system; 2: syringe pump with a 10-μL Hamilton syringe attached, equipped on one arm of the stereotactic frame; 3: stereotactic arm equipped with a microdialysis guide cannula clip; 4: arm light lamp; 5: dental cement powder (white bottle) and acrylic solvent (brown bottle), and cyanoacrylate adhesive (Loctite ®); 6: isoflurane vaporizing anesthetic system mask attached to the stereotactic teeth grip; 7: dental drill; 8: additional stereotaxic arm equipped with a mini osmotic pump cannula holder; 9: heating pad for the anesthetized mice; 10: surgical stereomicroscope; 11: 2-mm anchor screws; 12: digital display of the stereotaxic frame; 13: surgical tools (scalpel with sterile blade, needle-tipped forceps, screwdriver, cotton swabs, bulldog forceps).

(B) Size of brain implants in millimetric scale. 1: Alzet ® osmotic minipump; 2: microdialysis guide cannula with a dummy; 3: CMA7 microdialysis probe with a 2-mm membrane, to be inserted into the guide cannula the day before the microdialysis experiment.

Stereotaxic apparatus preparation

The stereotaxic system (Figure 1) should be prepared before starting the surgeries, including a syringe pump attached to one arm, a clip to hold the guide cannula attached to the other arm, an additional arm equipped with an osmotic mini-pump brain infusion cannula holder, a surgical stereomicroscope, an arm light, a dental drill, dental cement, and surgical tools. The syringe pump should be equipped with a sterilized Hamilton syringe (10 μL) and a 32 G needle.

The use of an isoflurane mask anesthetic system or an alternative long-lasting anesthesia is recommended, given the long duration of the surgical procedure. On the vaporizer system, also prepare the nose mask attached to the anterior teeth grip of the stereotaxic frame.

> *Note:* keep in mind that after AAV infusion, the arm attached with the syringe pump should be exchanged with the arm equipped with the holder of the osmotic mini-pump brain infusion cannula.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
AAV5-CBA-Alpha-Synuclein (Viral vector (AAV5 serotype) encoding wild-type human alpha-synuclein)	The Michael J. Fox Foundation For Parkinson's Research	N/A
AAV5-CBA-eGFP (Viral vector (AAV5 serotype) encoding eGFP)	The Michael J. Fox Foundation For Parkinson's Research	N/A
Chemicals, peptides, and recombinant proteins		
DMSO	Sigma-Aldrich	Cat#472301
Veratridine	Tocris Bioscience	Cat#2918
NaCl	Merck	Cat#106404
KCl	Merck	Cat#104936

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
$MgCl_2 \cdot 6H_2O$	Merck	Cat#105832
$CaCl_2 \cdot 2H_2O$	Merck	Cat#142002
Na_2HPO_4	Merck	Cat#106586
Disodium EDTA	Sigma-Aldrich	Cat#E5134
Phosphoric acid	Scharlau	Cat#AC10981000
MeOH HPLC grade	HiperSolv Chromanorm	Cat#83638
1-Octanesulfonic acid HPLC grade	Scharlau	Cat#AC17020025
Dopamine hydrochloride	Sigma-Aldrich	Cat#H8502
3,4-Dihydroxyphenylacetic acid	Sigma-Aldrich	Cat#850217
Experimental models: Organisms/strains		
Mouse: C57BL/6 (male, 8–12 weeks).	Charles River	Cat#632
Oligonucleotides		
Indatraline-conjugated antisense oligonucleotide targeting (h)-α-synuclein [5′-CGCCTTCCACGGTT UUCU-3′]	Axolabs GmbH	N/A
Indatraline-conjugated antisense non-sense oligonucleotide [5′-CCGTATCGTAAGCAGTAC-3′]	Axolabs GmbH	N/A
Software and algorithms		
TotalChrom software	PerkinElmer	Cat#N515-6021
MCID Core	Interfocus	mcid.co.uk/
Other		
RNaseZap	Ambion	Cat#AM9780
Nonstick, RNase-free Microfuge Tubes, 0.5 mL	Ambion	Cat#AM12350
Nuclease-Free Water	Ambion	Cat#AM9930
Barrier (Filter) Tips, 10 µL size	Ambion	Cat#AM12640
Micro-osmotic pump	Alzet Osmotic Pumps	Cat#1004
Brain Infusion Kit 3	Alzet Osmotic Pumps	Cat#0008851
CMA 7 guide cannula	Harvard Apparatus	Cat#CMAP000138
CMA 7 microdialysis probe	Harvard Apparatus	Cat#CMAP000083
Harvard Apparatus Single Animal Tabletop Isoflurane Anesthesia System with Small Induction Box	Harvard Apparatus	Cat#72-6468
Arm light lamp	Schott	Cat#KL1500 LCD
Dental Drill	Navfram	Cat#N-120
Heating Pad	DAGA	Cat#EP
Small Animal Stereotaxic Instrument with Digital Display Console	KOPF	Cat#942-C
Surgical stereomicroscope	BoliOptics	Cat#SZ02010421
10 µL Microliter Syringe	Hamilton	Cat#701RN
32 gauge, Small Hub RN Needle	Hamilton	Cat#7762-05
Nanoliter syringe pump	KDScientific	Cat#KDS 310 Plus
CMA 7 and 8 Probe Clip	Harvard Apparatus	Cat#P000136
Cannula Holder 1	Alzet Osmotic Pumps	Cat#0008860
Anchor Screws 2 mm	Microbiotech/se AB	Cat#4002002
Cyanoacrylate adhesive	Loctite	Cat#45404
TAB2000 Powder medium 50 g	Kerr	Cat#61772
TAB2000 Liquid Fast 50 mL	Kerr	Cat#61775
PHD ULTRA Syringe Pump	Harvard Apparatus	Cat#70-3007
22ga single channel plastic swivel	Instech	Cat#375/22PS
Counter-balanced lever arm for mice, 3.5 in	Instech	Cat#SMCLA
FEP Tubing (1.2 µL / 10 cm)	Microbiotech/se AB	Cat#4001005
FEP Tubing red (0.15 mm ± 0.05 mm inner diameter)	Microbiotech/se AB	Cat#4001006
Tubing Adapter	Microbiotech/se AB	Cat#4001036
Sterile Luer Slip 1 mL Syringes	BD Plastipak	Cat#303172
Sample Vials Polypropylene	Microbiotech/se AB	Cat#4001048
2.6 µm particle size (7.5 × 0.46 cm) C18 column	Phenomenex	Cat#00C-4462-E0
Isocratic HPLC Pump	Waters	Cat#515

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
HPLC Autosampler	Waters	Cat#717Plus
Electrochemical Detector	Waters	Cat#2465
Vacuum degasser	Waters	Cat#186001272

MATERIALS AND EQUIPMENT

Artificial cerebrospinal fluid (aCSF) for ASO solution preparation

Reagent	Final concentration	Amount
NaCl	625 mM	3.65 g
KCl	12.5 mM	93 mg
$CaCl_2 \cdot 2\ H_2O$	6.3 mM	92.5 mg
$MgCl_2 \cdot 6\ H_2O$	5.9 mM	120 mg
Glucose	25%	25 g
RNase-free water	n/a	Up to 100 mL
Total	n/a	100 mL

Note: pH must be adjusted to 5.5–6.0. It is suggested to aliquot the solution into 2 mL aliquots. All material should be prepared under RNase-free conditions. Final solution should be kept at −30°C until usage for a maximum of 6 months.

Artificial cerebrospinal fluid (aCSF) for intracerebral microdialysis

Reagent	Final concentration	Amount
NaCl	125 mM	7.31 g
KCl	2.5 mM	186 mg
$CaCl_2 \cdot 2\ H_2O$	2.52 mM	370 mg
$MgCl_2 \cdot 6\ H_2O$	1.18 mM	240 mg
Milli-Q water	n/a	Up to 1 L
Total	n/a	1 L

Note: pH must be adjusted to 5.5–6.0. Final solution can be stored at 4°C for a maximum of 4–5 months.

Mobile phase for HPLC

Reagent	Final concentration	Amount
$Na_2H_2PO_4 \cdot H_2O$	0.15 mM	48.3 mg
1-Octanesulfonic acid (sodium salt monohydrate)	0.9 mM	421.7 mg
Disodium EDTA	0.5 mM	336.2 mg
Methanol	10%	200 mL
Milli-Q water	n/a	Up to 2 L
Total	n/a	2 L

Note: pH must be adjusted to 2.8 with phosphoric acid before adding methanol. Final solution can be stored at room temperature (20°C–25°C) for a maximum of 1 month.

STEP-BY-STEP METHOD DETAILS

Part one: Osmotic mini-pump filling and priming

Timing: 24 h

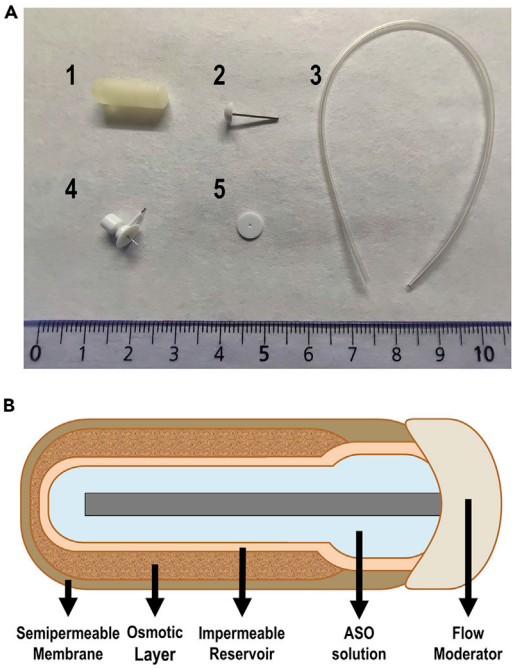

Figure 2. Components and internal structure of the Alzet osmotic mini-pump

(A) Components of the mini-pump in a millimetric scale. 1: 100 µL volume mini-pump body (1.5 × 0.6 cm); 2: Flow moderator (1.3 cm) composed of a styrene acrylonitrile flange and a 21-gauge stainless steel tube; 3: 3.7 µL/cm polyvinylchloride catheter tubing; 4: brain infusion cannula composed of a 30-gauge 3-mm stainless steel tube and a polycarbonate flange and pedestal; 5: polycarbonate spacer disc for height adjustment (0.5-mm height). (B) Internal structure of the mini-pump body, composed by a cellulose ester blend semipermeable outer membrane, a high osmolarity salt osmotic layer, and a thermoplastic hydrocarbon elastomer impermeable drug reservoir. Due to the high osmotic pressure difference between the osmotic layer and the tissue environment where it is implanted, the water fluxes into the pump through the semipermeable membrane. As the water enters the osmotic layer, it compresses the flexible reservoir, displacing the ASO solution from the pump at a 0.11 µL/h constant rate.

This section describes how to assemble the Alzet ® osmotic mini-pumps with the Brain infusion Kit 3 components, and how to prepare the pumps for their priming period before implantation. According to Alzet ® instructions, the pumps are composed of a semipermeable outer membrane, an intermediate osmotic layer, and a flexible reservoir for the solution (Figure 2). Due to the high osmotic pressure difference between the osmotic layer and the tissue environment where it is implanted, the water fluxes into the pump through the semipermeable membrane. As the water enters the osmotic layer, it compresses the flexible reservoir, displacing the ASO solution from the pump at a 0.11 µL/h constant rate in the case of 28-days long 100-µL capacity pumps. Also, the pumps have a start-up gradient until the pumps are ready to deliver the solution. To accomplish this period and start the infusion at the time of surgery, all pumps must be primed in vitro prior to implantation.

1. Prepare 100 mL of RNase-free aCSF.
2. Place the following items in a sterilized biosafety cabinet, taking care not to directly touch anything to maintain sterility: osmotic mini-pump, a flow moderator, a catheter, a brain infusion cannula, and a spacer disc from Alzet ® osmotic mini-pump in a biosafety cabinet. In addition, prepare a 1 mL sterile syringe, a scalpel blade, a millimetric ruler, and a rubber band.
3. Put the ASO working solution on ice and let it slowly thaw.
4. Add three drops of cyanoacrylate adhesive (e.g., Loctite ®) to the brain infusion cannula pedestal and insert into one spacer disc. Place the cannula in the cabinet and wait until the adhesive is fully dry.
5. Cut a catheter into 1.5 mm segments.
6. Push the flow moderator cap until it has a third of the tube in front of it.
7. Connect the catheter with the flow moderator and the brain infusion cannula and spread a drop of adhesive around each junction to prevent leakage. Wait until the adhesive is fully dry.
8. Attach the filling needle to the syringe and load it with ASO solution.

⚠ CRITICAL: do not start the loading of osmotic mini-pumps until all are ready to avoid keeping the ASO at room temperature.

9. Fill the flow moderator and catheter with ASO solution or vehicle (aCSF) until a drop of the liquid comes out of the brain infusion cannula.
10. Slowly fill the osmotic mini-pump with the ASO solution or vehicle (aCSF). Observe the pump shadow to control the level of the solution and stop filling when a drop comes out of the pump.

⚠ CRITICAL: avoid the formation of bubbles in the pump and catheter that will impede the flow of the solution.

11. Carefully insert the flow moderator into the pump.
12. Tie all filled osmotic mini-pumps with the rubber band and attach them into a 100 mL beaker. This will avoid any mixing of the ASO and the saline solutions.
13. Fill the beaker with 0.9% saline until all the bodies of the osmotic mini-pumps are submerged. Incubate the pumps at 37°C for 24 h to initiate ASO delivery.

Note: priming time could differ depending on the duration of the osmotic mini-pump. Refer to the corresponding Alzet ® instructions to adjust the incubation time.

Part two: Stereotaxic surgery

Timing: 90 min / mouse

This section details how to prepare mice and perform the stereotaxic surgery procedure. The entire process will consist of the injection of the AAV solution into mouse SNc/VTA, followed by the implantation of the osmotic mini-pump and the guide cannula for the microdialysis probe. The double implant will be fixed to the mouse skull with anchor micro-screws attached to the skull and dental cement to attach each piece.

Phase 1: Animal preparation

Timing: 5–10 min

14. Prepare the isoflurane vaporizing system with 0.8 L/min O_2 and open the induction box circuit with 3% isoflurane flow.
15. Anesthetize the mouse by placing it in the induction box.

Alternatives: mice can be anesthetized with intraperitoneal pentobarbital (25 mg/kg) or another long-lasting anesthetic solution.

16. Wait until the mouse is fully anesthetized. Inject buprenorphine 0.1 mg/kg subcutaneously to alleviate pain,
17. Place the mouse on the heating pad to avoid dropping body temperature during the surgery, shave the head hair and sanitize the head skin with 70% ethanol.
18. Fix the mouse head in the stereotaxic system by inserting the ear bars into the mouse's ear canals.

⚠ CRITICAL: ensure that the mouse is fully fixed before starting the surgery to avoid it from moving while in the frame. Check that the graduation marks of both ear bars coincide and that both eyes are parallel to the bars. If the animal is properly positioned, a gentle movement of the nose should not alter the position nor loosen the head.

19. Hook the front teeth into the adapter clamp and adjust the position of the adapter away from the mouse until you feel resistance.

20. Exchange the flux circuit towards the anesthesia vaporizing mask and reduce the isoflurane concentration to a maintenance dose (1.5%).
21. Gently approximate the anesthesia vaporizing mask to the mouse nose and check its sedation state by loss of pedal reflex.

Note: if the mouse wakes up before this step, place it back in the induction box.

Phase 2: Skull exposure and drilling

Timing: 15 min

22. Once mouse is fixed and fully anesthetized, apply an ocular lubricant to the eyes to prevent dryness.
23. Make an anterior-posterior incision in the head skin using a sterile scalpel, using sufficient pressure to cut through the skin but not into the skull.

Note: the incision should be large enough to access the skull until the occipital bone is visible.

24. Pinch both sides of the skin with bulldog clamps and open them to obtain a wider view of the skull.
25. Prior to starting the AAV administration procedure, very carefully insert the hemostatic forceps or a blunt surgical scissor into the mouse body and slightly open and close the jaws to create a subcutaneous pocket next to the scapula, where the osmotic mini-pump will be placed.

Note: the osmotic mini-pump should be placed parallel to the brain and the spinal cord. The pocket should be at least 1 cm longer than the pump, but not too large as the pump could move to the side of the animal.

26. Gently remove the connective tissue with the scalpel and clean the skull surface with a sterile cotton swab. Wait a few minutes to ensure drying of the skull and to see the cranial sutures.
27. Level the mouse head to obtain a right coordinate reference:
    a. Move the syringe pump attached to the Hamilton syringe to place the needle tip to the bregma and annotate the dorsoventral coordinate.
    b. Repeat the process at the lambda point.
    c. Reposition the dial of the anterior stereotaxic clamp to adjust the z-coordinate until the dorsoventral coordinate is equal at the bregma and lambda points.
28. Move the needle tip to the bregma and write down the anteroposterior and mediolateral coordinates.
29. Find the coordinates of the target areas based on the atlas of the mouse brain (Franklin and Paxinos, 2008; Allen Institute for Brain Science, 2011), using bregma as the zero point (Figures 3C–3F):
    a. SNc/VTA: –2.9 mm posterior; –1.3 mm lateral to bregma; –4.25 mm ventral from skull.
    b. Lateral ventricle: –0.34 mm posterior; +1.0 mm lateral to bregma; –2.2 mm ventral from skull.
    c. Striatum: +0.5 mm anterior; –2.4 mm lateral to bregma; –2.8 mm ventral from skull.

Lift the syringe at each point and mark the target sites with a fine waterproof marker, which will serve as puncture sites.

Note: depending on the mouse strain, body weight and age, coordinates may change and often need to be adjusted. We recommend administering several dye injections in the target brain areas and confirming the accuracy of the stereotaxis coordinates before starting the AAV administration.

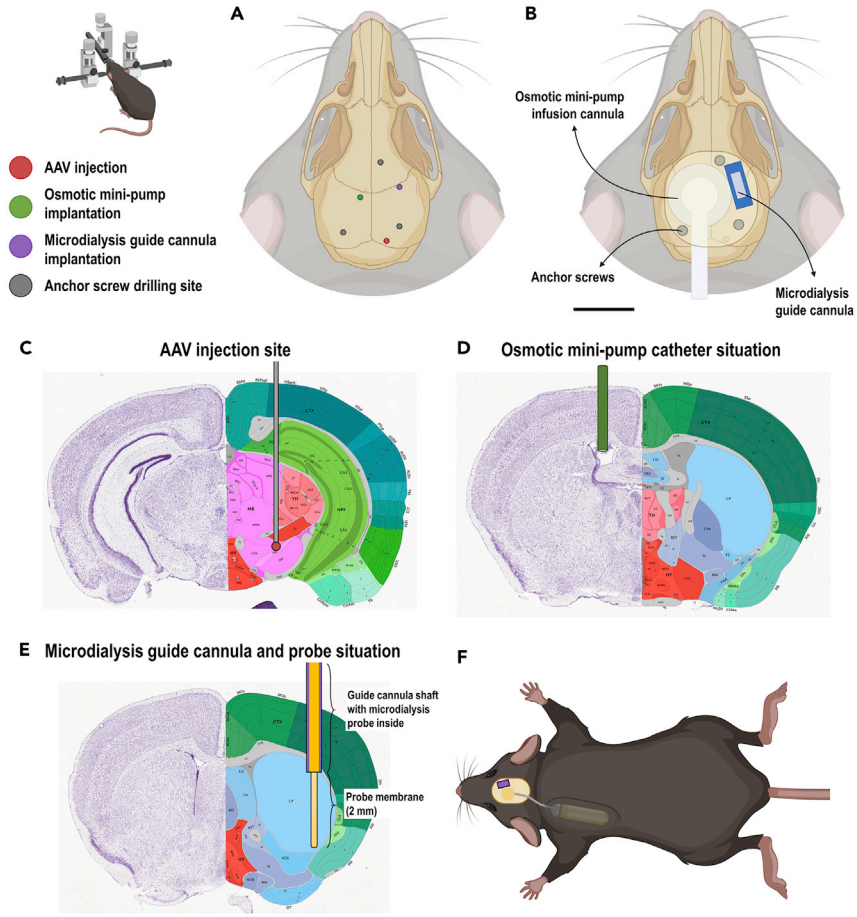

Figure 3. Injection and implantation coordinates in the stereotaxic surgery procedure

(A) Perforation sites in the mouse skull (steps 32 and 33), indicating the site of AAV injection in SNc/VTA (red), the site of implantation of the osmotic minipump brain infusion cannula in the ventricle side (green) and cannula microdialysis guide in striatum (purple). The three additional gray dots correspond to the suggested locations for the anchor screws.

(B) Site of the implants and anchor screws after surgery. After the application of the dental cement, only the upper part of the guide cannula will be visible outside the implant structure, while the brain infusion cannula and the anchor screws must be completely covered by the cement. Scale bar: 5 mm.

(C–E) Nissl (left) and anatomical annotations (right) from the Allen Mouse Brain Atlas and Allen Reference Atlas – Mouse Brain (Allen Institute for Brain Science, 2004, 2011), respectively showing the specific site for AAV injection into SNc/VTA (C, step 39), the specific site of the osmotic minipump catheter implanted in the lateral ventricle (D, step 52), and the microdialysis guide cannula implant site in the striatum (E, step 59). The neurotransmitter exchange membrane is 2 mm.

(F) Representative diagram showing the location of the cranial implants and the osmotic minipump inside the scapular subcutaneous pocket, connected by the minipump catheter.

Note: considering unilateral infusion surgery, in order to have enough space for both the guide cannula and the osmotic mini-pump, make sure to place the AAV infusion and guide cannula sites in the same hemisphere and the osmotic mini-pump brain infusion cannula on the opposite side.

30. Lift the syringe on the vertical axis and move the tip to a security position.
31. Mark three additional points where three anchor screws will be implanted, equidistant from the osmotic mini-pump point.

⚠ CRITICAL: to allow each implant to take place in the mouse skull, ensure that the screws are at least 1.5 mm away from the osmotic mini-pump brain infusion cannula, and with

sufficient distance from the guide cannula site. To ensure maximum fixation, make sure that the three screws are located in different skull bones.

32. Drill three small shallow holes (for SNc/VTA, lateral ventricle and striatum, respectively) by using the dental drill at the marked sites on the skull, applying gentle downward pressure while drilling (Figure 3A).

⚠ CRITICAL: at target sites of interest, stop immediately once the drill stoke has passed through the skull, so as not to puncture the brain and avoid tissue damage. Some bleeding may occur if the dura or parenchyma are damaged; in that case, use a cotton swab to clean the blood.

33. Partially drill the three anchor screw sites, without drilling completely through the skull, by placing the dental drill more parallel to the skull. This will allow a better fixation of the screw and prevent the implants from loosening.

Phase 3: AAV injection

Timing: 20–25 min

34. Take an aliquot of AAV working solution and place it on ice to prevent severe thawing.
35. Bring the needle back to the bregma point and recalculate the coordinates for AAV injection.
36. Bring the needle to the calculated position and lower it into the hole, until the needle tip is just at the level of the skull. Use this point as the zero for the depth coordinate.

Note: if the needle tip does not penetrate through the hole, drill again to be sure there are no debris of the skull or dura mater.

37. Lift the syringe and clean the needle of the microinjection syringe with RNase-free water. Check for clogging. Withdraw automatically 1 μL of the AAV working solution.
38. Lower the syringe as slowly as possible to reach the SNc/VTA.
39. Inject 1 μL of AAV solution at a rate of 0.15 μL/min. After completing the infusion, wait an additional 10 min to ensure complete infusion of AAV solution.

Note: to avoid ocular damage to the mouse while infusing, turn off the arm light in the meantime.

40. Confirm that the syringe volume has been delivered. Draw the syringe as slowly as possible to avoid backflow of solution along the needle track.
41. Lift the syringe and rinse the needle tip with RNase-free water. If there is blood residue, use sterile cotton swabs to remove it and re-rinse the needle.
42. Remove the syringe from the syringe pump and place it in a safe, sterile box.
43. Remove the syringe pump arm from the stereotaxic frame and place it in a safe place. Replace it with the arm equipped with the osmotic mini-pump brain infusion cannula holder.

Phase 4: Osmotic mini-pump implantation

Timing: 20–25 min

44. Uncap the primed osmotic mini-pumps and place them in a Petri dish filled with sterile saline solution (0.9%).

45. Hold an anchor screw with needle-pointed forceps and place it over one of the screw marks. Using a micro-screwdriver, apply gentle pressure towards the skull while turning it. The anchor screw should enter firmly into the skull.
46. Insert the screw until there is less than 1 mm between its head and the skull surface. Check the screw fixation by touching it carefully on one side.

⚠ CRITICAL: do not insert the screw completely. This will allow dental cement to flow between the skull surface and the screw head, which will provide a stronger implant fixation. If the screw hole is too wide, the screw will feel loose when touched, or will fall directly into the hole without screw-driving it in; in that case, the implants risk moving or falling out during the post-operatory time, so drill another narrower hole for the screw and discard the large one.

47. Repeat steps 45 and 46 for the remaining two anchor screws.

Note: the distance between the screw holes and the osmotic mini-pump brain infusion cannula should be large enough for implantation (Figure 3B), but it is recommended to fix the anchor screws pointing obliquely away from the center of the skull and not completely vertical.

48. Place a primed osmotic mini-pump into the cannula holder by the removable tab and adjust the fixation, with the pump pointing toward the mouse body.
49. Place the brain infusion cannula over the drilled hole and very carefully insert the pump into the subcutaneous pocket of the mouse body, leaving the catheter and the cannula out.
50. Lower the cannula until the needle is just level with the skull. Use this as the dorsoventral coordinate zero point.
51. Lift the cannula and carefully add several drops of cyanoacrylate adhesive around the hole.

Note: make sure that the adhesive does not cover the hole or adhere to the tip of the cannula needle to prevent clogging. It is recommended to use a 10 μL micropipette tip to apply the adhesive more precisely.

52. Insert the brain infusion cannula carefully and slowly until the appropriate depth is reached.

Note: If necessary, apply more adhesive around the contact area between the cannula pedestal and the skull.

53. Hold the cannula in position for 5 min until the cyanoacrylate is completely dry and the cannula is fixed.

Note: make sure that the adhesive is completely dry before proceeding. The following steps will require dental cement which may interfere with the cyanoacrylate if it is still wet.

54. Loosen the cannula holder grip and lift it up.
55. Heat a scalpel for several seconds and carefully cut the cannula holder. The plastic composition will melt in contact with the scalpel, so make the cut in one movement to avoid the cooling of metal and plastic.
56. If necessary, close the caudal part of the skin incision with grips or absorbable sutures, avoiding contact with the pump catheter.

Phase 5: Microdialysis guide cannula implantation and post-operative care

Timing: 20–25 min

57. Attach a guide cannula to the clip equipped on the other stereotaxic arm.
58. Place the cannula over the last drilled hole and lower it until the cannula tip is just in contact with the skull. Use this as the zero point of the dorsoventral coordinate.
59. Carefully and slowly insert the cannula until determined depth is reached.

Note: keep in mind that, after replacing the dummy, the probe will exceed the depth of the cannula. Therefore, the implantation depth of the guide cannula should be calculated by subtracting the length of the probe membrane (See part three: microdialysis probe implantation) and the desired depth of the probe. In our case, the dorsoventral coordinate for implanting the guide cannula was -2.8 mm, whereas for the microdialysis probe it was -4.8 mm, indicating that the dialysis membrane for neurotransmitter exchange is 2-mm long.

60. Mix the dental cement powder with the acrylic solvent in a Petri dish or an equivalent resistant container.
61. When the mixture is partially fluid, carefully apply it to the skull, making sure that it makes contact with all implants and the screws are still partially visible.
62. Allow the cement to dry for 10 min, checking by touching the cement with a needle.

Note: avoid letting the mixture leak into the subcutaneous pocket. If necessary, touch the closed skin to make it contact with the caudal region of the skull. The fluidity of the mixture will allow the cement to flow below the screw heads.

63. When the cement is dry, release the cannula clip and lift the stereotaxic arm.
64. Release the skin from the bulldog clamps.
65. Remix the dental cement components until a slightly less fluid mixture than in step 61 is obtained.
66. Apply a second layer of cement over the implants until all components are covered, except for the guide cannula, which should be covered until the lower half of the cannula body.

⚠ CRITICAL: All skin around the implants should be in contact with the cement to prevent the reopening of the incision. If necessary, repeat from step 65.

67. Allow the cement to dry completely.
68. Gently remove the ear bars from the stereotaxic frame, remove the anesthetic mask, and take the mouse from the instruments.
69. If suturing was necessary in the caudal part of the incision, apply appropriate antiseptic treatment over it.
70. Individualize the mouse in a new clean cage and keep it on a heating pad.
71. Monitor the mouse until it wakes up and moves on its own.

Note: cement implantation and subcutaneous insertion of the pump may generate pain in mice, so once the animal has recovered, provide buprenorphine or other analgesic treatment as indicated by the institutional guidelines. The mini-pump implant may make it difficult for mice to freely access water and food, so provide them at the bottom of the cage along with environmental enrichment.

Note: the recovery time after surgery should not be less than 72 h before proceeding to the next part of the protocol.

Part three: Microdialysis probe implantation

Timing: 30 min

This section describes the preparation of the microdialysis probes and their implantation in the mice cannulas the day before the microdialysis experiment. The probes should be rinsed before use and then exchanged by the dummy, which avoids clogging around the guide cannula. In this example, CMA7 6 kDa microdialysis probes will be used.

72. Immerse the tube adaptors in 70% ethanol for 15 min.
73. Fill one syringe with aCSF and one with deionized water and mount them in a microdialysis pump.
74. Prepare the microdialysis probe.
    a. Mount the probe in a holder clip and place it in a vial filled with aCSF.
    b. Attach a tubing adapter and FEP tubing to the blue tubing of the microdialysis probe and connect it to the syringe cannula.
    c. Flush the probe with ethanol at 10 μL/min flow to wash out the air inside the probe. If bubbles remain, gently tap the clip holding the probe.
    d. Adjust the pump to the required perfusion flow rate (See part four: microdialysis) and check for leaks.
    e. Connect the FEP tubing to the water syringe and flush the probe again.

⚠ CRITICAL: do not touch the probe membrane during the process. If it receives any hit and/or leaks are detected, discard the probe.

Note: while preparing all the necessary probes, place the ready probes in a vial filled with deionized water.

75. Prepare the isoflurane vaporizing system with 0.8 L/min O_2 and open the induction box circuit with a 3% isoflurane flow.
76. Attach a primed probe to the aCSF syringe on the microdialysis pump and set it at a rate of 10 μL/min. This flow pressure will allow the membrane to stand straighter and prevent bending.
77. Anesthetize the implanted mouse by placing it in the induction box. Wait until the mouse is fully anesthetized.
78. Place the mouse on a firm surface and carefully hold its head.
79. Remove the dummy from the cannula guide and carefully insert the microdialysis probe until the main body of the probe is completely inside the cannula body.
80. Place the mouse in the microdialysis cage and allow it to wake up from anesthesia. After implantation of the microdialysis probes, the mice should be allowed to rest overnight (12–16 h).

Note: if the mouse recovers from anesthesia before probe insertion, place the mouse back in the induction box.

Note: the microdialysis cage can be a regular standard mice cage and should be large enough for the mouse to freely move. It must have attached a support for the balance arm (See Figure 4). In this case, we use a 20 × 20 × 20 cm methacrylate box open at the top.

Part four: Microdialysis

Timing: 6–8 h

This section details how to perform the in vivo microdialysis experiment on freely moving mice. During the experiments, a physiological solution is perfused at a constant flow rate by a microdialysis pump through the inner tube of the probe to its distal end, reaching the exposed semipermeable membrane located in the brain area of study. Low molecular weight substances (less than 6 kDa),

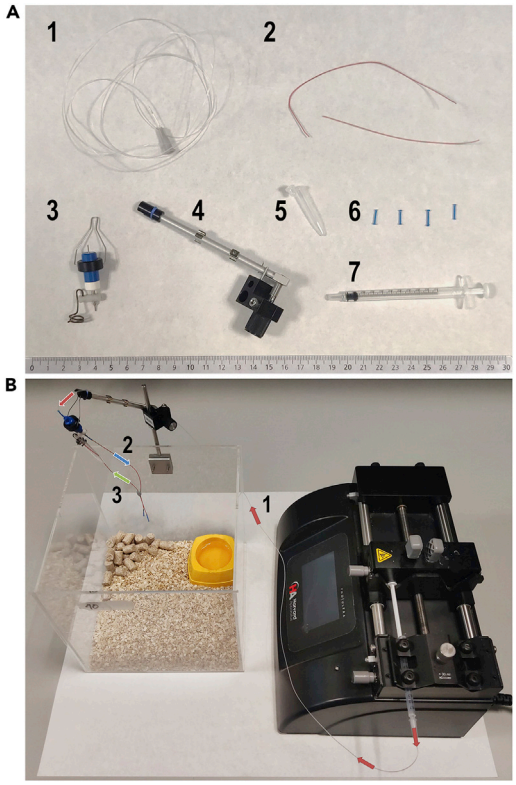

Figure 4. Components and setup of the microdialysis system

(A) Components of the microdialysis set-up system in a millimetric scale. 1: transparent fluorinated ethylene propylene (FEP) tubing with the syringe connector attached; 2: red FEP tubing; 3: 22-gauge single channel plastic swivel with an attachment for the collecting vial; 4: multi-axis counter-balanced lever arm; 5: 300-µL polypropylene collecting vial; 6: blue tubing adapters for connecting FEP tubing; 7: 1-mL sterile syringe of 4.70 mm inner diameter. (B) Assembled microdialysis set-up with a 20 × 20 × 20 cm methacrylate box for mice placement and a syringe pump. Arrows indicate the direction of the perfusate flow: 1, from the syringe to the swivel (red arrows); 2, from the swivel to the implanted microdialysis probe (blue arrow); 3, from the microdialysis probe to the collecting vial attached to the swivel (green arrow).

such as neurotransmitters, diffuse from the outside to the inside of the probe according to a concentration gradient.

81. Prepare the solutions for perfusion.
    a. Prepare artificial cerebrospinal fluid (aCSF) containing 1% DMSO as basal perfusate.
    b. Prepare the veratridine working solution:
        i. To prepare the stock solution, dissolve the veratridine powder in DMSO at 5 mM. For safe storage, the stock solution should be kept at −20°C.
        ii. Dilute the stock solution in aCSF to obtain 50 µM veratridine perfusate. This perfusate will have the same concentration of DMSO as the basal perfusate.

Note: ensure that both basal perfusate and veratridine-containing perfusate have the same final concentration of DMSO (1%).

    c. Prepare the perfusate collection vials by adding 5 µL of concentrated perchloric acid ($HClO_4$ 10 mM) in each vial, which prevents oxidation of DA in the dialysate.

Note: the collecting vials should be filled with $HClO_4$ up to a maximum of one day before starting the microdialysis experiment to avoid evaporation.

82. Set up the microdialysis room and system (Figure 4B):
    a. To allow for proper adaptation of the mice, turn on the room light 1 h before the start of the experiment.
    b. Prepare the system to allow the animals to move freely. Attach the balance arm to the cage and mount the swivel on it. The swivel arm has a holder for holding the plastic vials.
83. Purge the tubing system and connect the probes to the pump.
    a. Fill the sterile syringes with aCSF + 1% DMSO and tap them gently to remove all air bubbles. Place them in the pump.
    b. Connect the inflow line from the syringe to the swivel using the FEP tubing and tubing adaptors.
    c. To ensure good perfusate flow, set the pump to a rate of 100 μL/min and perfuse aCSF + 1% DMSO through the system before connecting it to the dialysis probes, to be sure that there is no air in the entire system.
    d. Change the flow rate to 10 μL/min and attach the FEP tubing to the probe inlet tubing. Wait until the perfusate flows freely out of the probe outlet tubing.
    e. Connect the outlet tubing to the probe outlet and place the end of the tubing into the vial to collect perfusate fractions.

⚠ CRITICAL: check that the inlet and outlet tubing is protected against kinking, as well as to prevent it from being chewed by mice. Before connecting the entire dialysis circuit, make sure that the perfusate comes out normally and the probes are not obstructed.

    f. Change the flow rate to 1.5 μL/min. Check that the volume of dialysate collected is as expected.

Note: the flow rate of 1.5 μL/min is adjusted for a syringe of 1 mL capacity and 4.70 mm inner diameter. In case of using another type of syringe (different volume and/or internal diameter), the infusion rate should be corrected in order to provide the adequate volume of dialysate for subsequent analysis in HPLC system (in our study it is 30 μL/20 min).

84. Stabilize the mice in the established conditions for 1 h before starting the collection of the dialysate fractions, to ensure an adequate exchange of neurotransmitters across the membrane.
85. Collect the perfusate samples in vials containing perchloric acid in 20 min fractions. Keep the collected vials at −20°C. The six initial samples obtained are used to establish basal DA concentrations.

Note: depending on the experimental design, the number of fractions and the collecting time can be modified.

86. Change the perfusate solution. This step begins when the sixth sample has been collected.
    a. Prepare new syringes filled with veratridine 50 μM solution.
    b. Place a discard vial to the vial holder, disconnect the inlet tubing from the swivel, and place the new syringes in the pump.
    c. Set the pump at a rate of 100 μL/min and perfuse veratridine solution through the system until there is no air in the tubing system.

Note: The required time perfusing at 100 μL/min depends on the dead volume of the tubing, which is determined by the length of the tubes.

    d. Change the flow rate to 1.5 μL/min and connect the inlet tubing to the swivel. Wait 5 min to ensure that no aCSF solution remains in the tubing.
    e. Place a new vial in the holder and collect the dialysate sample after infusing veratridine for 20 min.

87. After collecting the dialysate corresponding to the veratridine treatment, repeat step 85 using syringes filled with aCSF to collect eight more basal samples.

Pause point: if necessary, perfusate samples can be stored at −20°C for 24 h.

88. After collecting the last fraction, disconnect the dialysis system and flush sequentially with de-ionized water, 70% ethanol, and air.
89. At the end of the microdialysis experiment, euthanize the animals and remove their brains for verification of the implantation of the microdialysis probes.

Part five: Analysis by high-performance liquid chromatography

Timing: 1–2 days

This section describes how to measure the DA and DOPAC levels in the collected dialysate fractions by reversed-phase high-performance liquid chromatography (HPLC) coupled to electrochemical detection (EC). The procedure described here uses HPLC equipment consisting of a Waters 515 isocratic pump, a Waters 717plus HPLC auto-sampler, a 2.6 μm particle size (7.5 × 0.46 cm) C18 column, a Waters 2465 electrochemical detector (+0.7 V), and a Waters IN-Line AF degasser. The equipment is connected to an interface that transmits the collected signal to a computer, where it is converted into a chromatographic profile using Total-Chrome software.

90. Prepare 2 L of mobile phase for the HPLC system.
91. Allow the mobile phase to flow during 14–16 h through the chromatographic circuit. The mobile phase flows through the degasser to purge any air bubble or solubilized air.
92. Connect the mobile phase inlet lines to the HPLC/EC system and set the flow rate at 0.8 mL/min with an internal column pressure of 2000–2200 psi. Set the oxidation-reduction potential of the electrode to +0.7 V.

Note: using these conditions, the chromatographic retention time of DA and 3,4-dihydroxyphenylacetic acid (DOPAC) is 2.5–2.8 min and 3.0–3.2 min, respectively. The detection limit for DA is approximately 3 fmol.

93. Prepare the standard solutions of DA and DOPAC at different concentrations. This will require a serial dilution in order to obtain an adequate calibration curve for determining the DA and DOPAC levels in perfusates (Figure 5).
    a. Prepare 100 mL of aCSF with $HClO_4$ 10 mM for the serial dilutions.
    b. Weight 1.13 mg of DA hydrochloride and dissolve it in 9.5 mL methanol + 500 μL $HClO_4$ 10 mM [solution 1.1].

    Note: the actual DA and DOPAC weight must be corrected according to manufacturer's commercial formulation of the compound and/or purity. In this case, the subtraction of the hydrochloride molecular weight will correspond to a 0.81× correction factor for DA, and the 98% purity of DOPAC will correspond to a 0.98× correction factor.

    c. Dilute serially solution 1.1 until reaching the working solution adequate concentration:
        i. Add 20 μL of solution 1.1 into 1 mL aCSF+$HClO_4$ [solution 1.2].
        ii. Add 20 μL of solution 1.2 into 1 mL aCSF+$HClO_4$ [solution 1.3].
        iii. Add 20 μL of solution 1.3 into 1 mL aCSF+$HClO_4$ [DA working solution, W.S.].
    d. Weight 2.25 mg of 3,4-dihydroxyphenylacetic acid (DOPAC) and dissolve it in 9.5 mL methanol + 500 μL $HClO_4$ 10 mM [solution 2.1].
    e. Dilute serially solution 2.1 until reaching the working solution adequate concentration:
        i. Add 20 μL of solution 2.1 into 1 mL aCSF+$HClO_4$ [solution 2.2].

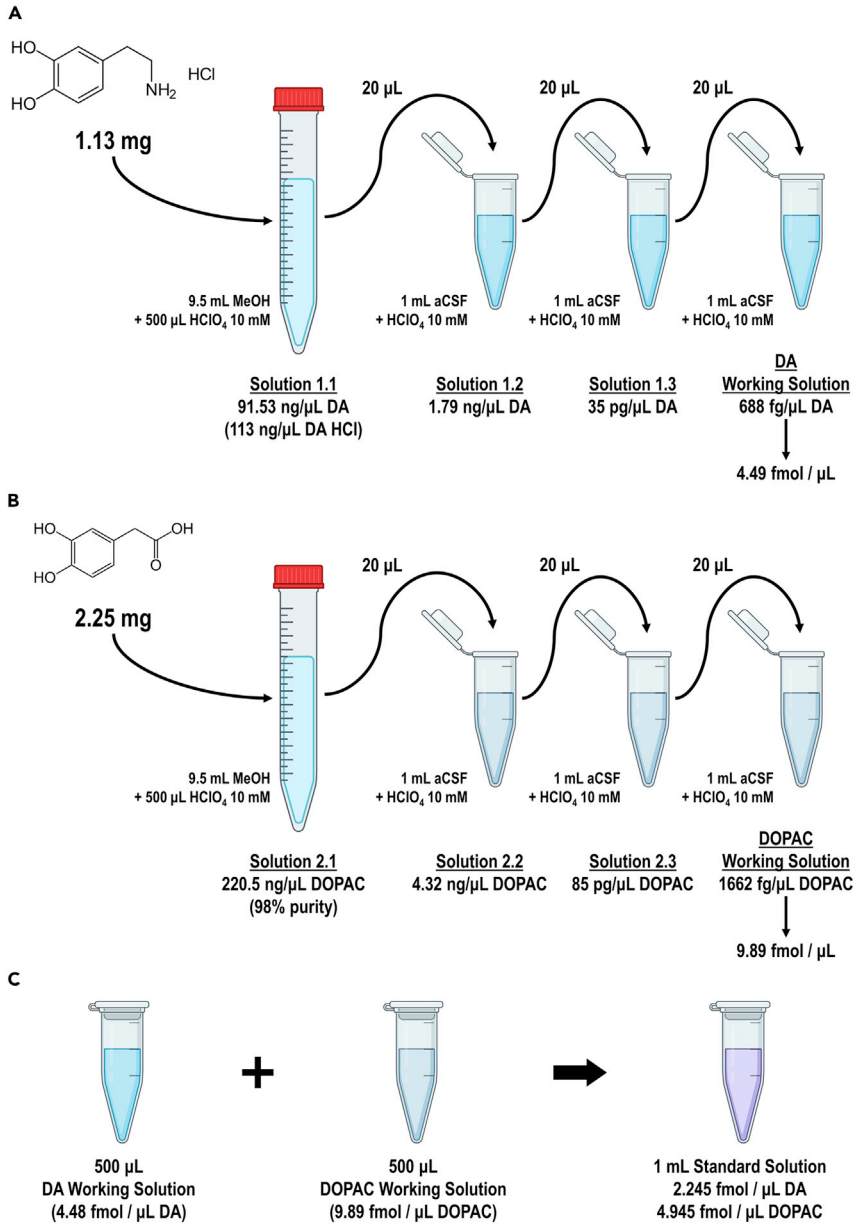

Figure 5. Preparation of the DA and DOPAC standard solution for the HPLC-ED calibration curve

(A) Serial dilution of DA hydrochloride in aCSF + $HClO_4$ 10 mM to obtain a 4.49 fmol/µL working solution.

(B) Serial dilution of DOPAC in aCSF + $HClO_4$ 10 mM to obtain a 9.89 fmol/µL working solution.

(C) Mix of DA and DOPAC working solutions to obtain the standard solution, which will be injected into the HPLC-ED system for the generation of the calibration curve.

ii. Add 20 µL of solution 2.2 into 1 mL aCSF+$HClO_4$ [solution 2.3].

iii. Add 20 µL of solution 2.3 into 1 mL aCSF+$HClO_4$ [DOPAC working solution, W.S.].

f. Mix 500 µL of DA and 500 µL of DOPAC working solutions for getting the standard solution. The final standard solution will consist of a mix of DA (2.245 fmol/µL) and DOPAC (4.945 fmol/µL).

94. Inject the following volumes of the standard solution to determine the calibration curve for the linear regression analysis:

a. 2 µL: 4.49 fmol DA, 9.89 fmol DOPAC.

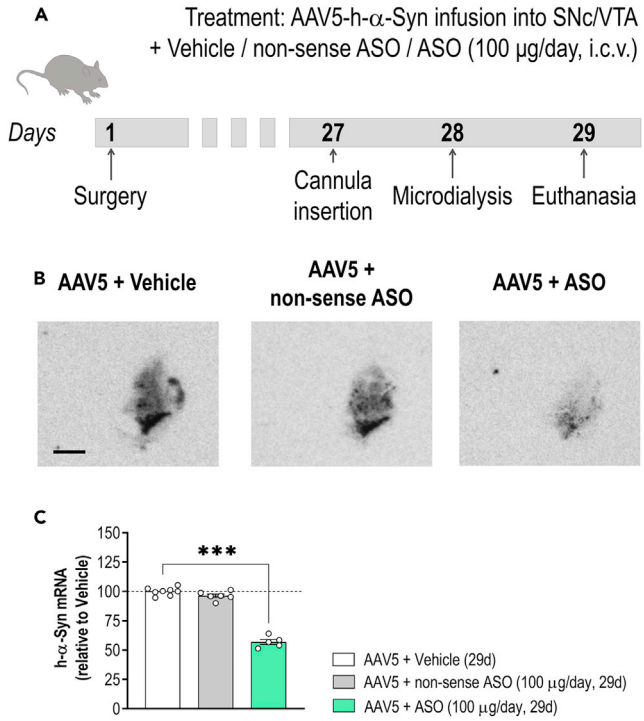

Figure 6. Intracerebroventricular conjugated ASO therapy prevents (h)-α-Syn accumulation in an AAV-driven overexpression mouse model

(A) Treatment timeline. Mice were unilaterally injected with 1 µL AAV5 into SNc/VTA and also received: i) vehicle, ii) non-sense ASO (100 µg/day) or iii) ASO (100 µg/day) into the lateral ventricle for 28 days using osmotic mini-pumps. Mice were sacrificed 24 h later.

(B) Coronal brain sections showing (h)-α-Syn mRNA expression in SNc/VTA of AAV5-injected mice treated with vehicle, non-sense ASO or ASO assessed by *in situ* hybridization procedures. Images of the sections autoradiograms were obtained with a computer-assisted image analyzer (MCID Core); for further information on quantification and analysis we refer to Alarcón-Arís et al. (2020). Scale bar: 1 mm.

(C) Decreased (h)-α-Syn mRNA expression in AAV5-injected mice treated with ASO compared to control groups (data are represented as mean ± SEM; n = 5–7 mice/group; *** $p < 0.001$ versus AAV5 + vehicle group; one-way ANOVA and Tukey's multiple comparisons test).

b. 5 µL: 11.23 fmol DA, 24.725 fmol DOPAC.
c. 10 µL: 22.45 fmol DA, 49.45 fmol DOPAC.
d. 20 µL: 44.90 fmol DA, 98.90 fmol DOPAC.

95. Inject 20 µL of each collected perfusate sample. Also interleave 20 µL samples of standard solution (i.e., corresponding to 44.92 DA fmol and 98.86 DOPAC fmol) every 15–20 perfusate samples, in order to check the electrochemical detection quality.
96. Perform a linear regression analysis with the calibration curve and determine the actual DA concentration of the perfusate samples.

EXPECTED OUTCOMES

To validate a novel oligonucleotide-based α-Syn-targeted therapy and investigate its effect on nigrostriatal DA neurotransmission, we developed a murine model of overexpression by injecting an AAV5-(h)-α-Syn construct into the SNc/VTA. At the same time, we implanted an osmotic mini-pump containing an indatraline-conjugated antisense oligonucleotide (ASO) to be continuously administered into the cerebrospinal fluid for 28 days (Figure 6A). Conjugation with indatraline facilitated the delivery and internalization of ASO selectively into monoamine neurons, including DA neurons that express dopamine transporter (DAT) (Javitch et al., 1985; Alarcón-Arís et al., 2018), for which indatraline has a high in vitro affinity and in vivo occupancy (Lengyel et al., 2008).

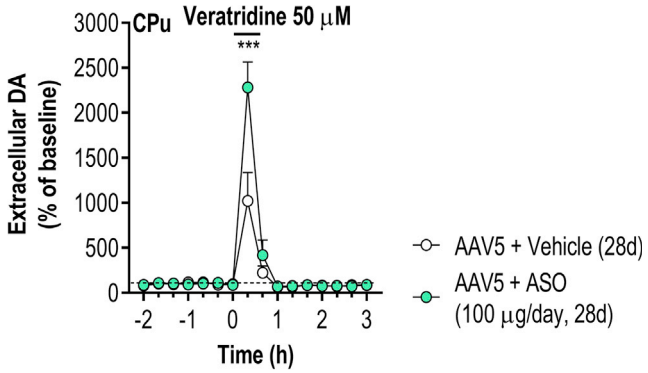

Figure 7. Intracerebroventricular conjugated ASO therapy recovers (h)-α-Syn-induced DA neurotransmission deficits

Microdialysis approach using veratridine (50 μM, depolarizing agent inhibiting sodium channel inactivation) confirmed an improvement of DA neurotransmission in the CPu of AAV5 mice treated with ASO (100 μg/day) compared to vehicle-treated mice (data are represented as mean ± SEM; $n = 4–6$ mice/group; ***$p < 0.001$; two-way ANOVA and Sidak's multiple comparisons test).

Therefore, ASO reduces the synthesis and accumulation of (h)-α-Syn by close to 40% only in this neuronal subset, early vulnerable to PD (Figures 6B and 6C). Importantly, this strategy does not induce degeneration of DA neurons, as previously reported using a viral vector delivering a short hairpin RNA (shRNA) targeting α-Syn, reducing α-Syn expression to over 80% (McCormack et al., 2010; Collier et al., 2016). Taken together, these data argue for the need to maintain a threshold for α-Syn knockdown since this protein is essential for neuronal homeostasis and neurotransmission, and its elimination would dramatically affect brain function.

Reduced levels of (h)-α-Syn and its phosphorylated form in the DA system induced by ASO lead to enhanced nigrostriatal DA neurotransmission compared to mice overexpressing (h)-α-Syn treated with vehicle (Alarcón-Arís et al., 2020; Corrigendum 2021) (Figure 7). The changes in DA release were observed after pharmacological stimulation using a depolarizing agent veratridine, but not under basal conditions, suggesting that nigrostriatal terminals display a standard tonic DA activity that is independent of α-Syn transcription levels in both mouse models. Supporting this hypothesis, several reports indicated that α-Syn-null mice exhibited a normal tonic DA activity in the nigrostriatal pathway after electrical stimulation with single pulses; however, they showed increased DA release with paired stimuli that elevated Ca^{2+} levels (Abeliovich et al., 2000). Since, α-Syn plays a key role in endocytosis/exocytosis processes, for instance, in the assembly of the SNARE complex, an essential event in synaptic vesicle docking and membrane fusion (Burré et al., 2010; Nemani et al., 2010), this is important to address α-Syn-dependent changes in DA neurotransmission under basal and stimulated (electrically or pharmacologically) conditions.

QUANTIFICATION AND STATISTICAL ANALYSIS

Determine dialysate concentrations of DA by comparing peak heights with those of known concentrations of standards. Perform a linear regression analysis of the standards and use the resulting linear regression equation to determine actual concentrations in samples.

For the group statistical comparison, the mean DA concentration of the six initial basal fractions in each mouse (step 85) is used as a normalization value. The DA concentration in each 20-min perfusate fraction should be expressed as a percentage relative to this value. All values are expressed as the mean ± standard error of the mean (SEM). Statistical comparisons and differences among groups may be analyzed by a 2-way analysis of variance (ANOVA, group versus time). If

ANOVA shows significant differences, pairwise comparisons between means should be subjected to Sidak's multiple comparisons test.

LIMITATIONS

Osmotic mini-pumps provide a long-term and continuous way of administration of a wide variety of molecules. However, the nature of the substance administered is limited by the biocompatibility of its solvent with the pump. In addition, the small volume of the mini-pump determines the maximum concentration of the molecule, given the possibility of precipitation within the pump.

The combination of AAV injection to generate the PD-like model and intracerebral treatment with ASO also limits the duration of the experiment. One of the aims of our study was to prevent the AAV-induced accumulation of (h)-α-Syn in the DA system and to analyze whether this improved DA neurotransmission at early stages, before the loss of nigral DA neurons as we found at 8 weeks post-AAV infusion (Alarcón-Arís et al., 2020). Since the available minipumps to infuse oligonucleotide molecules are of 28 days maximum duration, we decided to initiate ASO treatment together with AAV5 injection to overexpress (h)-α-Syn and evaluate the effects at the end of ASO treatment.

The small surface of the mouse skull also limits the possibility of studying DA release in several brain areas in a parallel fashion. In fact, the size of the pedestal of the osmotic minipump brain infusion cannula implanted in the lateral ventricle (Figure 3B) makes it impossible to implant a microdialysis cannula in nearby brain regions. It should be taken into account that the strong fixation of the different implants and anchor screws as well as during the process of brain extraction some superficial cortical regions may be damaged. This should be taken into consideration for further histological analysis.

In addition, it should also be taken into account that after the intracerebral implantation of the different devices, the mice require individualization in their home cages, which could limit the performance of several behavioral tests due to the lack of social interaction.

Once the microdialysis probe is implanted into the guide cannula, the microdialysis experiment should not exceed two days due to reduced effectiveness of molecular exchange through the membrane of the probe as a consequence of the precipitation of solutes in the probe.

Finally, the limitations of the HPLC-ED DA separation and detection system should be taken into account when designing the experimental procedure. It is important to consider the possible interactions between the neurotransmitter to be evaluated and the infused molecules, the separation on the chromatographic column, and the oxidation-reduction potential. For instance, ascorbic acid and uric acid have oxidation-reduction potentials close to those of DA and therefore their chromatographic profile is similar to that of DA, being considered an interference in various types of biological samples. (Wu et al., 2020).

TROUBLESHOOTING

Problem 1

Failure mounting the mouse in the stereotaxic frame (steps 18 and 19).

Potential solution

Make sure that the mouse is fully anesthetized. The ear bars should be inserted and fixed into the mouse ear canals, while the front teeth inserted into the anterior clamp.

Problem 2

Failure of AAV injection (steps 34–41).

Potential solution

Always work in RNase-free conditions when handling AAV solutions and quickly withdraw the working solution into the infusion syringe to avoid exposure for too long at room temperature.

Make sure that the mouse head is well fixed and leveled in the stereotaxic frame and ensure that the bregma and lambda are visible.

Make sure that the drilled hole is free of debris that may disturb the vertical movement of the injection needle, and that the needle is not obstructed before inserting it into the brain.

Problem 3

Failure of ASO delivery (steps 2–13 and 44–55).

Potential solution

Work in RNase-free conditions when preparing the ASO working solution and the preparation and priming of the osmotic mini-pump.

To avoid bubble formation, completely fill the osmotic mini-pump, flow moderator and the catheter, and carefully connect each part. Also, check the amount of solution remaining inside the pump at the end of the experiment.

In surgery, make sure that the cannula needle is not clogged and that the size of the subcutaneous pocket is sufficient to place the pump.

Ensure that there is enough cyanoacrylate adhesive to fix the cannula and the skull surface is sufficiently clean and dry to allow complete adhesion.

Ensure that the adhesive is completely dry and that the cannula is fully attached to the skull before continuing with the protocol.

Problem 4

Limited space for the implantation of the mini-pump cannula and the microdialysis guide cannula (steps 58 and 59).

Potential solution

Ensure that the anchor screws point outward from the skull and are not completely vertical.

Make sure that the osmotic mini-pump cannula and guide cannula are positioned in opposite hemispheres.

Problem 5

Post-operatory recovery time is too long (steps 70 and 71).

Potential solution

Check that the mice are anesthetized with a maintenance dose of isoflurane (1.5%) once fixed in the stereotaxic frame.

Administer appropriate analgesic to relieve pain during recovery and provide soft food and water on the bottom of the cage.

Problem 6

Recovered dialysate volumes are lower than expected (steps 85–87).

Potential solution

Ensure that there are no air bubbles in the syringes and tubing system. A transient increase in flow rate (no more than 10 μL/min) may be applied to remove bubbles or minor obstructions.

Check that mice have not chewed through the tubing system and/or that their probes are disconnected from the system. If so, replace the damaged section and reconnect the probe.

Problem 7

The DA concentration in dialysates is below the detection limit (3 fmol) (step 96).

Potential solution

Check the surgery coordinates for the guide cannula implantation in the striatum. Consider examining the exact cannula site by histological study.

Make sure that the collection vials contain perchloric acid to prevent oxidation of the DA in solution.

Inject dialysates into the HPLC system as soon as possible (at most 48 h after collection) and after a single freeze-thaw cycle.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Analia Bortolozzi (analia.bortolozzi@iibb.csic.es).

Materials availability

Recombinant (h)-α-Syn-expressing AAV5 viral vector was produced and tittered by the UNC Vector Core and gently provided by the Michael J. Fox Foundation (https://www.michaeljfox.org/research-tools-catalog). Indatraline-conjugated antisense oligonucleotides were produced by Axolabs GmbH and are patented by miCure Therapeutics Ltd. (WO2011131693, WO-2014064257-A1, WO2014064258-A1). The rest of the materials used in this protocol are commercially available.

Data and code availability

This protocol did not generate datasets nor code.

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AUTHOR CONTRIBUTIONS

A.B. conceptualized and supervised the research and acquired funding. R.P.-C., L.M.-R., and L.C. performed the investigation and formal analysis. R.P.-C. and L.M.-R. wrote the original draft of the manuscript. A.B. and R.P.-C. reviewed and edited the manuscript.

DECLARATION OF INTERESTS

A.B. is an inventor of the issued patents WO2011131693, WO-2014064257-A1, and WO2014064258-A1 for ligand-conjugated siRNA and ASO molecules and the targeting approach for monoamine systems related to this work. The rest of the authors declare no competing interests.

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