YMTHE, Volume *26*

Supplemental Information

Selective α-Synuclein Knockdown in Monoamine Neurons by Intranasal Oligonucleotide Delivery: Potential Therapy for Parkinson's Disease

Diana Alarcón-Arís, Ariadna Recasens, Mireia Galofré, Iria Carballo-Carbajal, Nicolás Zacchi, Esther Ruiz-Bronchal, Rubén Pavia-Collado, Rosario Chica, Albert Ferrés-Coy, Marina Santos, Raquel Revilla, Andrés Montefeltro, Isabel Fariñas, Francesc Artigas, Miquel Vila, and Analia Bortolozzi

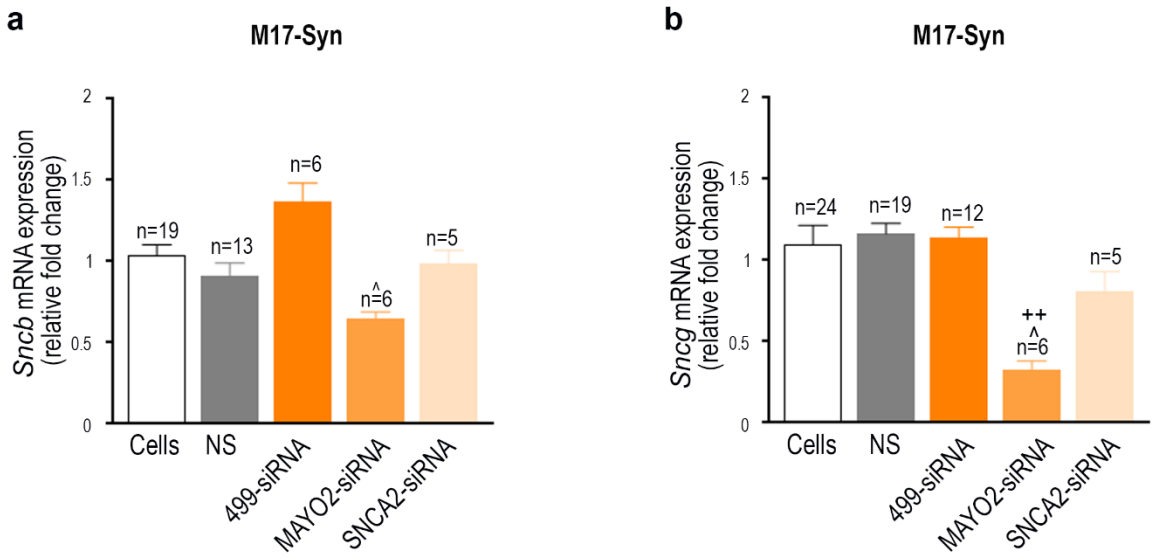

Figure S1. 499-siRNA does not suppress β- nor γ-synuclein expression in M17 cells overexpressing human α-synuclein (M17-Syn)

(a-b) RT-qPCR quantification of *Sncb* **(a)** and *Sncg* **(b)** expression in M17-Syn 24h after transfection with 499-siRNA, MAYO2-siRNA and SNCA2-siRNA. Cells transfected with nonsense siRNA (NS) were used as control. Target gene expression was normalized to two different housekeeping genes: GAPDHD and RPLPO. In all graphs, histograms represent average ± SEM. ^P<0.05 compared with cells + lipofectamine; ++P<0.01 compared with NS-siRNA (One-way ANOVA followed by Tukey's *post-hoc* test). M17-Syn, M17 human neuroblastoma cells overexpressing α-synuclein. 499-siRNA, MAYO2-siRNA or SNCA2-siRNA are siRNA sequences designed to target regions of the *Snca* mRNA encoding α-synuclein protein (Table S1 shows siRNA sequences).

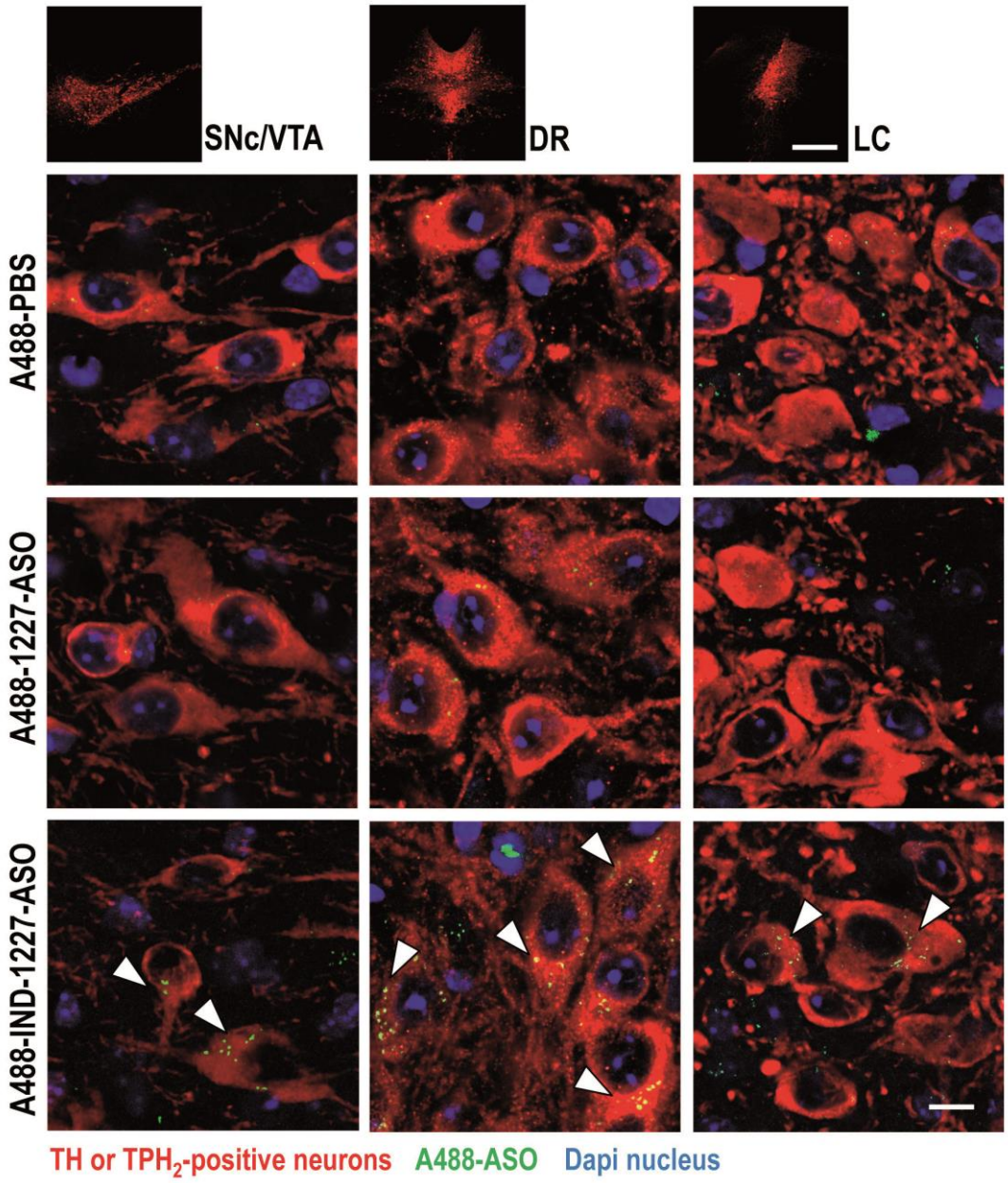

Figure S2. Preferential accumulation of indatraline-conjugated ASO molecules in monoamine neurons after intranasal administration

Mice were intranasally administered with alexa488 PBS (A488-PBS), alexa488-labeled nonsense 1227-ASO (A488-1227-ASO) or alexa488-labeled indatraline-conjugated 1227-ASO (A488-IND-1227-ASO) at 30 µg/day for 4 days and were sacrificed 6h after last administration (n=3 mice/group, new mouse cohort different from those used in the Figure 2). Confocal images showing co-localization of A488-IND-1227-ASO (yellow) with TH-positive neurons (red) in SNc/VTA and LC or TPH_2-positive neurons (red) in DR identified with white arrowheads. Cell nuclei were stained with Dapi (blue). Scarce or null co-localization of A488-1227-ASO with TH-positive or TPH_2-positive neurons was detected. Scale bars: low=200 µm, high=10 µm. SNc/VTA, substantia nigra compacta/ventral tegmental area; DR, dorsal raphe nucleus; LC, locus coeruleus.

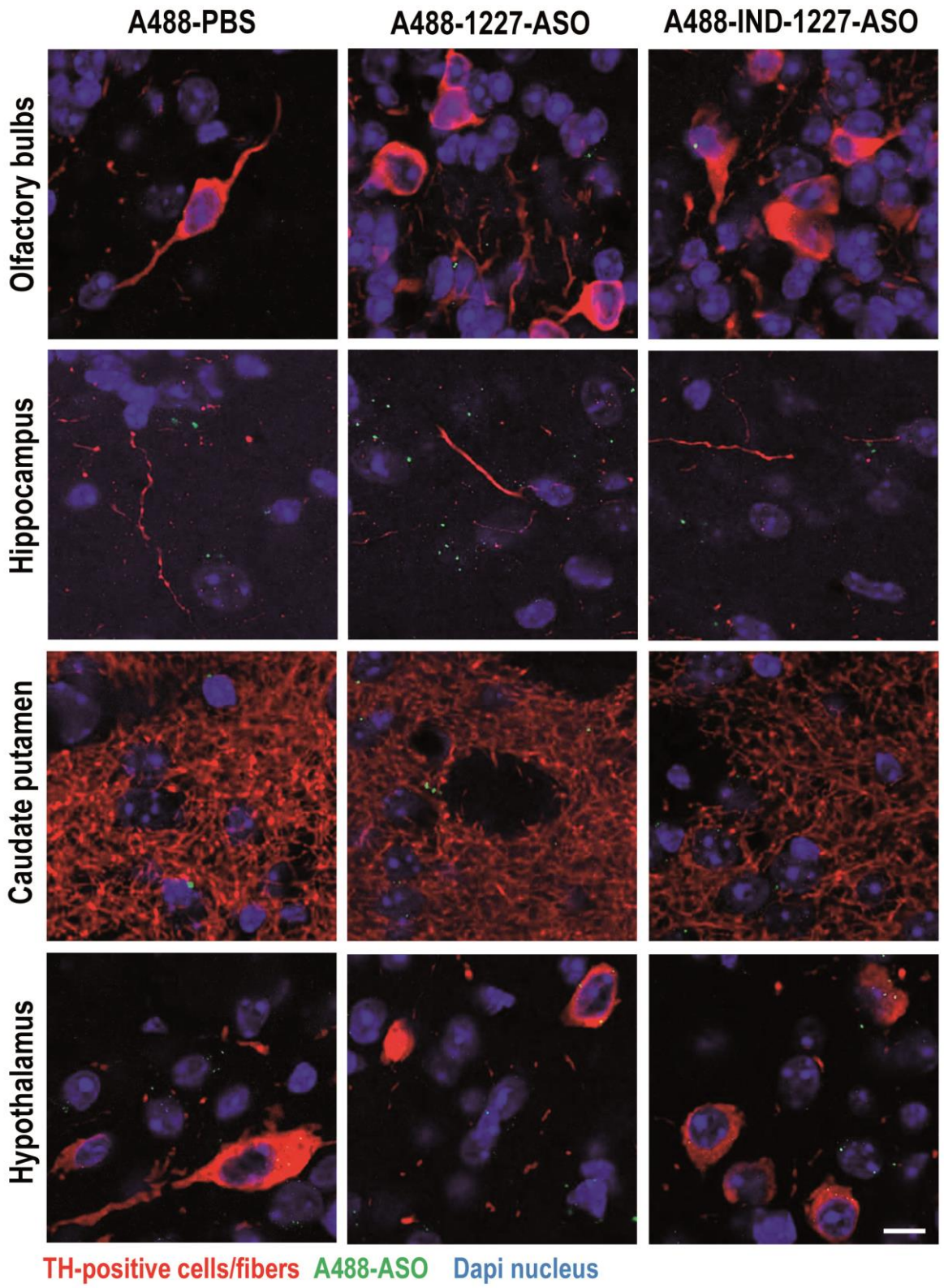

Figure S3. Absence of indatraline-conjugated nonsense-ASO (IND-1227-ASO) molecules in olfactory bulbs, hippocampus, caudate putamen and hypothalamus of mice

Laser confocal images show cell nuclei stained with Dapi (blue) in the different brain areas of mice treated intranasally with Alexa488 PBS (A488-PBS), alexa488-labeled 1227-ASO (A488-1227-ASO) or alexa488-labeled indatraline-conjugated 1227-ASO (A488-IND-1227-ASO) at 30 µg/day during 4 days (n=3 mice/group, the same mice as in Figure 3). Scale bars: 50 µm.

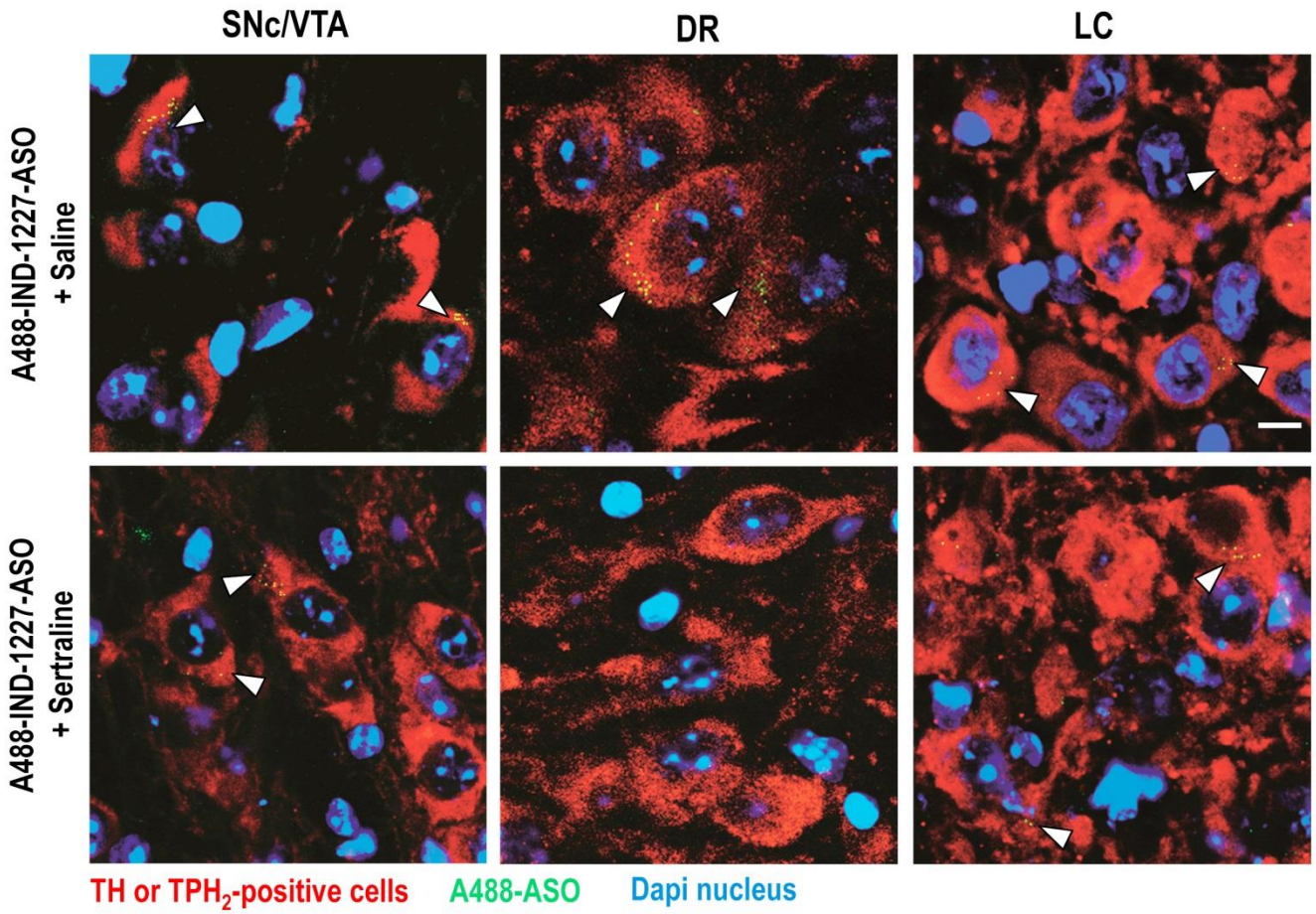

Figure S4. Effect of sertraline SERT inhibitor on *in vivo* cellular uptake of indatraline-conjugated nonsense-ASO (IND-1227-ASO) into 5-HT neurons

Mice were pretreated with: a) sertraline 20 mg/kg, i.p. (selective SERT inhibitor) or b) saline solution, i.p. for 3 h before the i.n. administration of IND-1227-ASO at 30 µg/day for 4 days. Mice were sacrificed 6h after last administration of IND-1227-ASO (n=3 mice/group). Representative confocal images showing the co-localization of A488-IND-1227-ASO (yellow) with TH-positive neurons (red) in SNc/VTA and LC or TPH_2-positive neurons (red) in DR identified with white arrowheads (Top panel, images are the same as in Figure 2a). Representative confocal images showing co-localization of A488-IND-1227-ASO (yellow) with TH-positive neurons (red) in SNc/VTA and LC, but not with TPH_2-positive neurons (red) in DR (Down panel). Cell nuclei were stained with Dapi (blue). As seem, the accumulation of IND-1227-ASO in TPH_2-positive 5-HT neurons was completely blocked by sertraline indicating that SERT is a requirement to uptake of oligonucleotide. Scale bars: 10 µm. SNc/VTA, substantia nigra compacta/ventral tegmental area; DR, dorsal raphe nucleus; LC, locus coeruleus; SERT, serotonin transporter; 5-HT, serotonin.

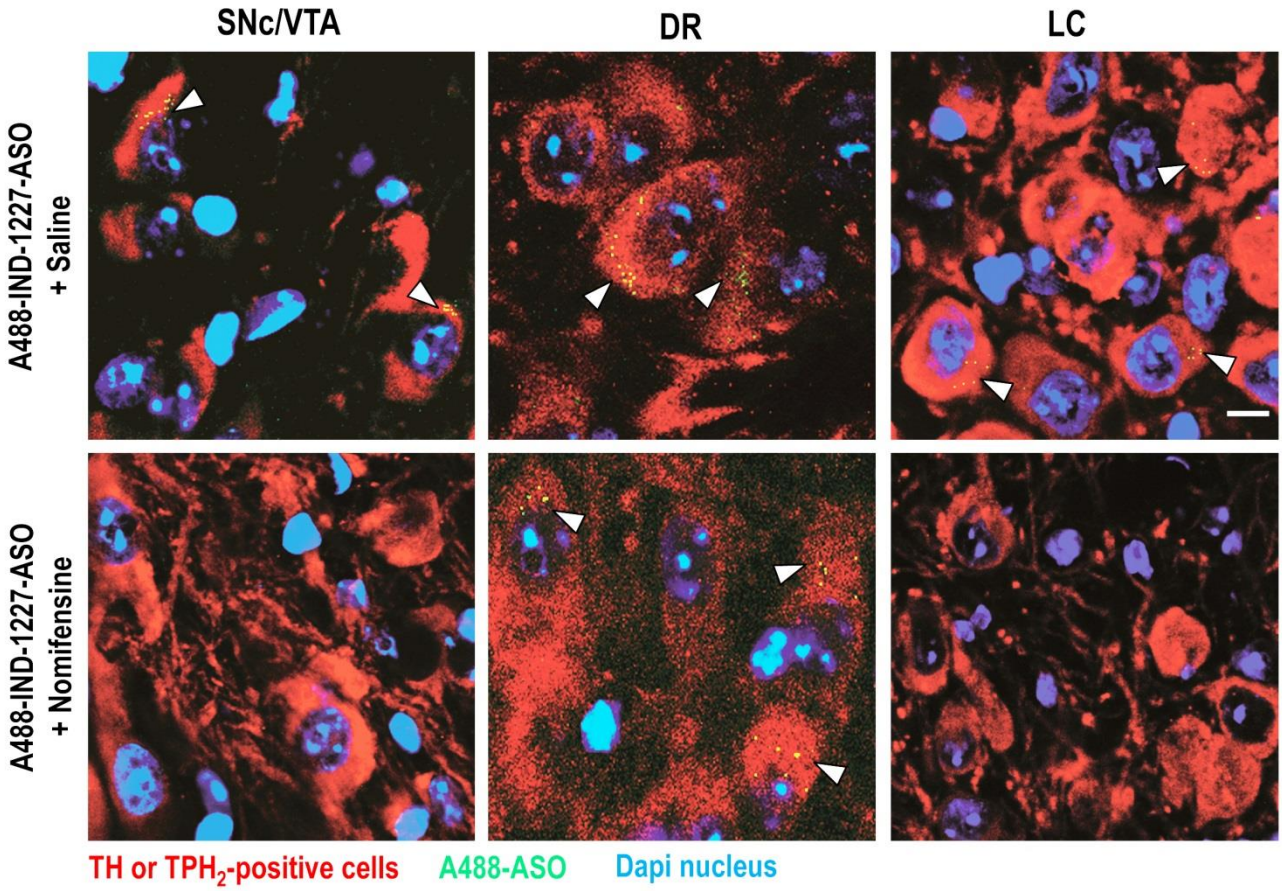

Figure S5. Effect of nomifensine DAT/NET inhibitor on *in vivo* cellular uptake of indatraline-conjugated nonsense-ASO (IND-1227-ASO) into DA and NE neurons

Mice were pretreated with: a) nomifensine 20 mg/kg, i.p. (DAT/NET inhibitor) or b) saline solution, i.p. for 3 h before the i.n. administration of IND-1227-ASO at 30 µg/day for 4 days. Mice were sacrificed 6h after last administration of IND-1227-ASO (n=3 mice/group). Representative confocal images showing the co-localization of A488-IND-1227-ASO (yellow) with TH-positive neurons (red) in SNc/VTA and LC or TPH_2-positive neurons (red) in DR identified with white arrowheads (Top panel, images are the same as in Figure 2a). Representative confocal images showing co-localization of A488-IND-1227-ASO (yellow) with TPH_2-positive neurons (red) in DR, but not with TH-positive neurons (red) in SNc/VTA and LC (Down panel). Cell nuclei were stained with Dapi (blue). As seem, the accumulation of IND-1227-ASO in TH-positive DA or NE neurons was completely blocked by nomifensine indicating that DAT and NET are a requirement to uptake of oligonucleotide. Scale bars: 10 µm. SNc/VTA, substantia nigra compacta/ventral tegmental area; DR, dorsal raphe nucleus; LC, locus coeruleus; DAT, dopamine transporter; NET, norepinephrine transporter; DA, dopamine; NE, norepinephrine.

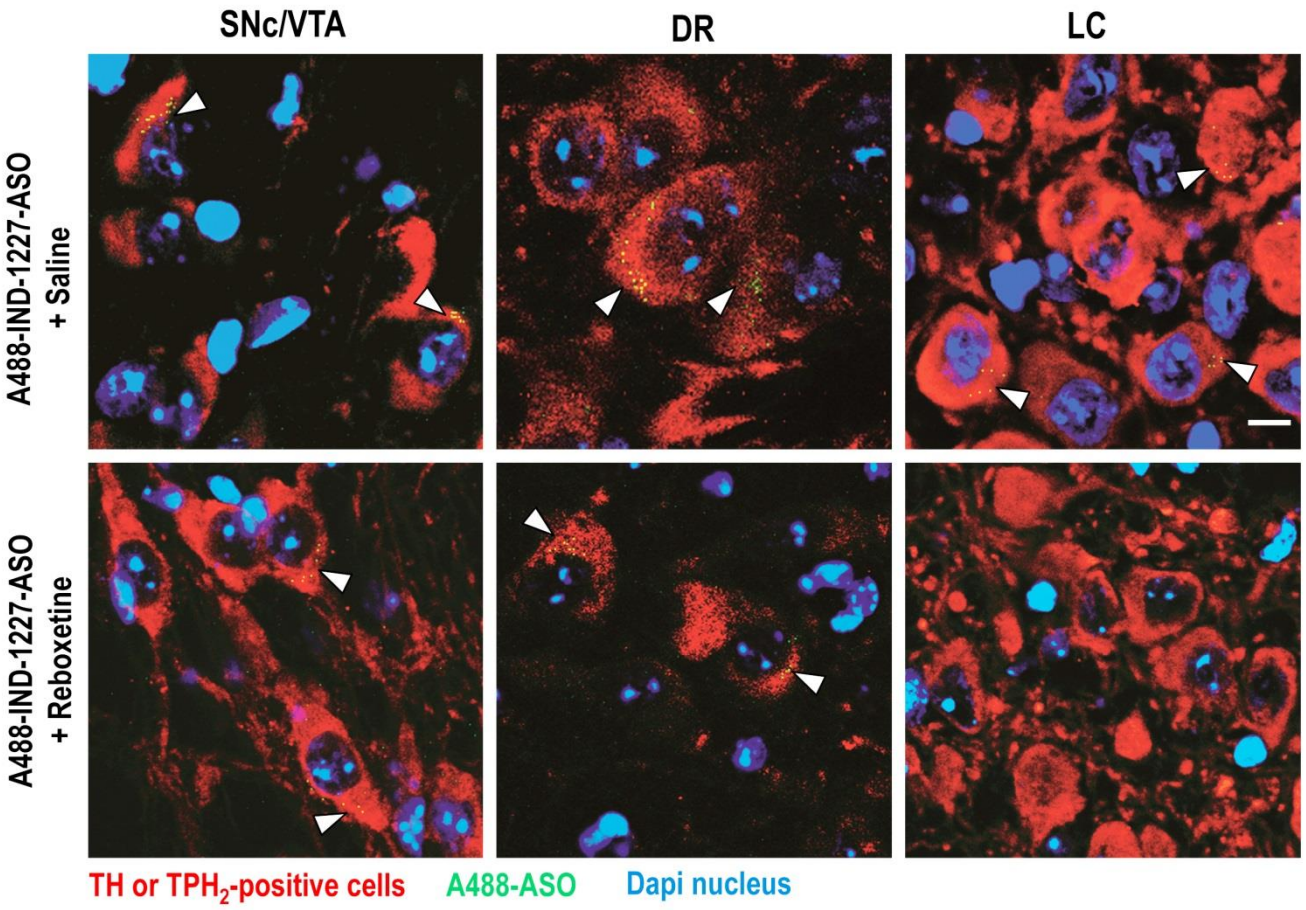

Figure S6. Effect of reboxetine NET inhibitor on *in vivo* cellular uptake of indatraline-conjugated nonsense-ASO (IND-1227-ASO) into NE neurons

Mice were pretreated with: a) reboxetine 10 mg/kg, i.p. (DAT/NET inhibitor) or b) saline solution, i.p. for 3 h before the i.n. administration of IND-1227-ASO at 30 µg/day for 4 days. Mice were sacrificed 6h after last administration of IND-1227-ASO (n=3 mice/group). Representative confocal images showing the co-localization of A488-IND-1227-ASO (yellow) with TH-positive neurons (red) in SNc/VTA and LC or TPH_2-positive neurons (red) in DR identified with white arrowheads (Top panel, images are the same as in Figure 2a). Representative confocal images showing co-localization of A488-IND-1227-ASO (yellow) with TH-positive neurons in SNc/VTA or TPH_2-positive neurons (red) in DR, but not with TH-positive neurons (red) in LC (Down panel). Cell nuclei were stained with Dapi (blue). As seem, the accumulation of IND-1227-ASO in TH-positive NE neurons was completely blocked by reboxetine confirming that NET is a requirement to uptake of oligonucleotide. Scale bars: 10 µm. SNc/VTA, substantia nigra compacta/ventral tegmental area; DR, dorsal raphe nucleus; LC, locus coeruleus; NET, norepinephrine transporter; NE norepinephrine.

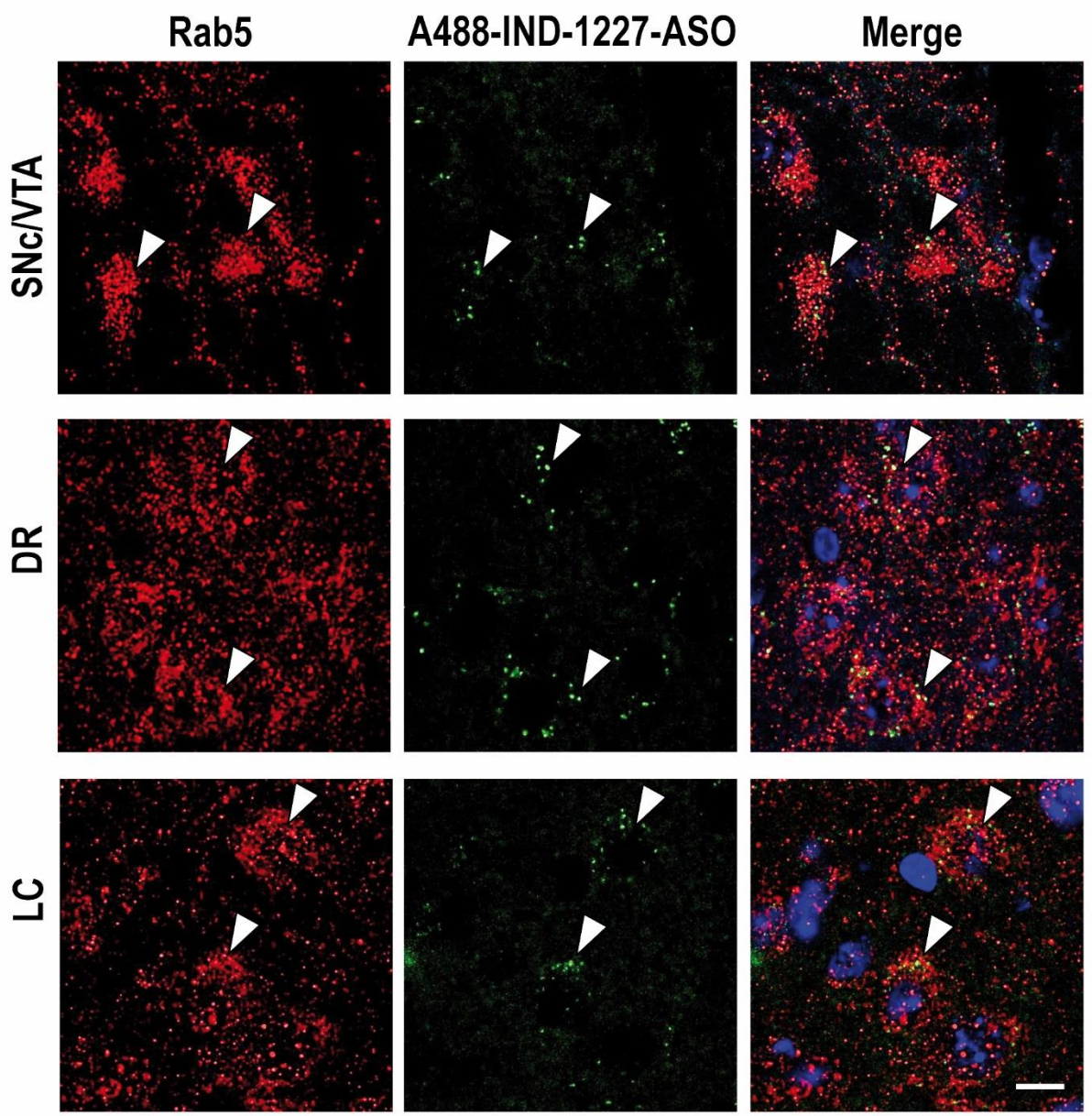

Figure S7. Co-localization of alexa488-labeled indatraline-conjugated nonsense-ASO (A488-IND-1227-ASO) with early endosome marker Rab5

Confocal images showing the co-localization (yellow) of A488-IND-1227-ASO (green) with Rab5 (red) in SNc/VTA, DR or LC neurons. Scale bar: 10 μm. Vesicles are marked with white arrowheads. DR, dorsal raphe nucleus; LC, locus coeruleus; SNc/VTA, substantia nigra compacta/ventral tegmental area.

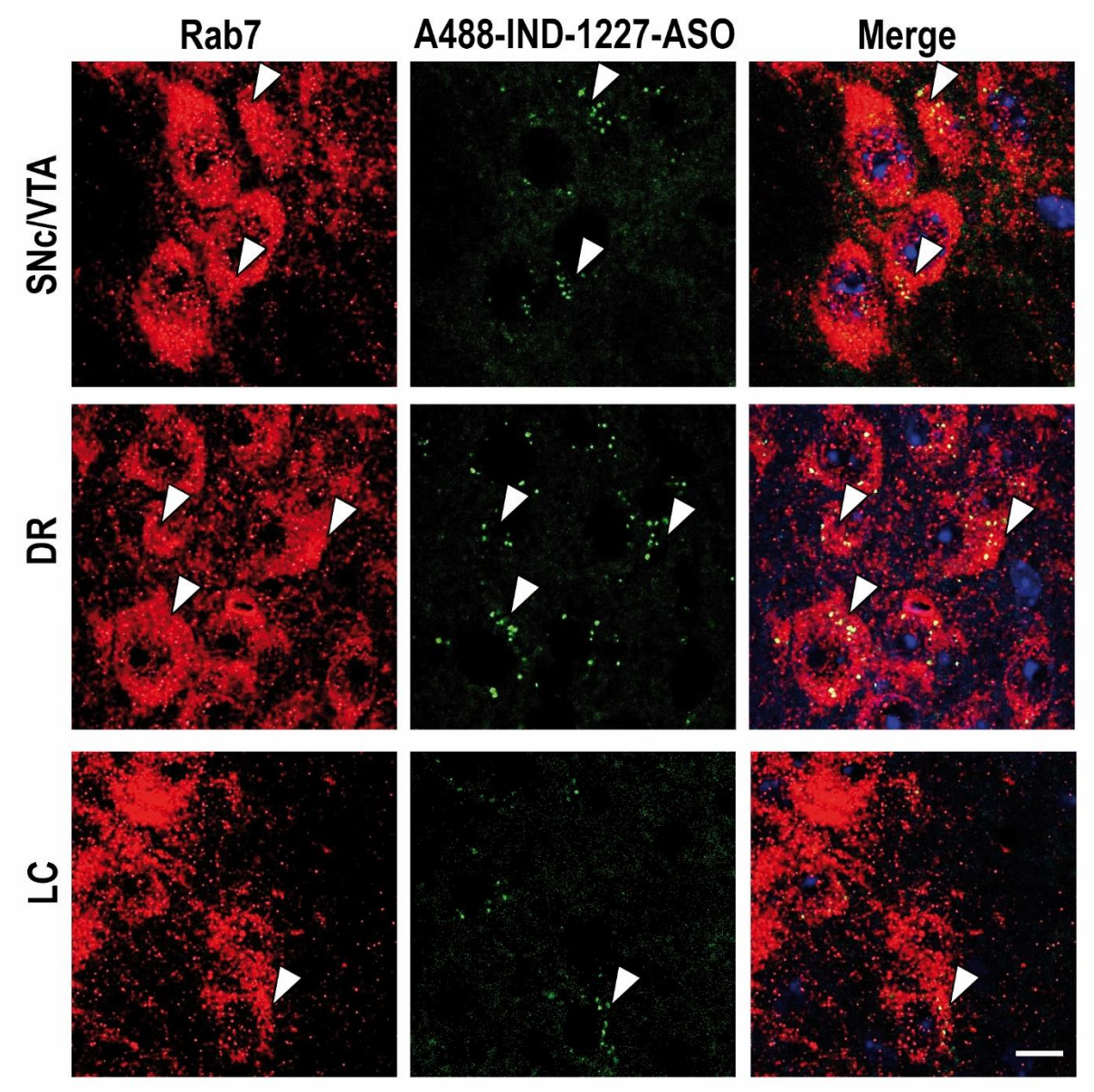

Figure S8. Co-localization of alexa488-labeled indatraline-conjugated nonsense-ASO (A488-IND-1227-ASO) with late endosome marker Rab7

Confocal images showing the co-localization (yellow) of A488-IND-1227-ASO (green) with Rab7 (red) in SNc/VTA, DR or LC neurons. Scale bar: 10 μm. Vesicles are marked with white arrowheads. DR, dorsal raphe nucleus; LC, locus coeruleus; SNc/VTA, substantia nigra compacta/ventral tegmental area.

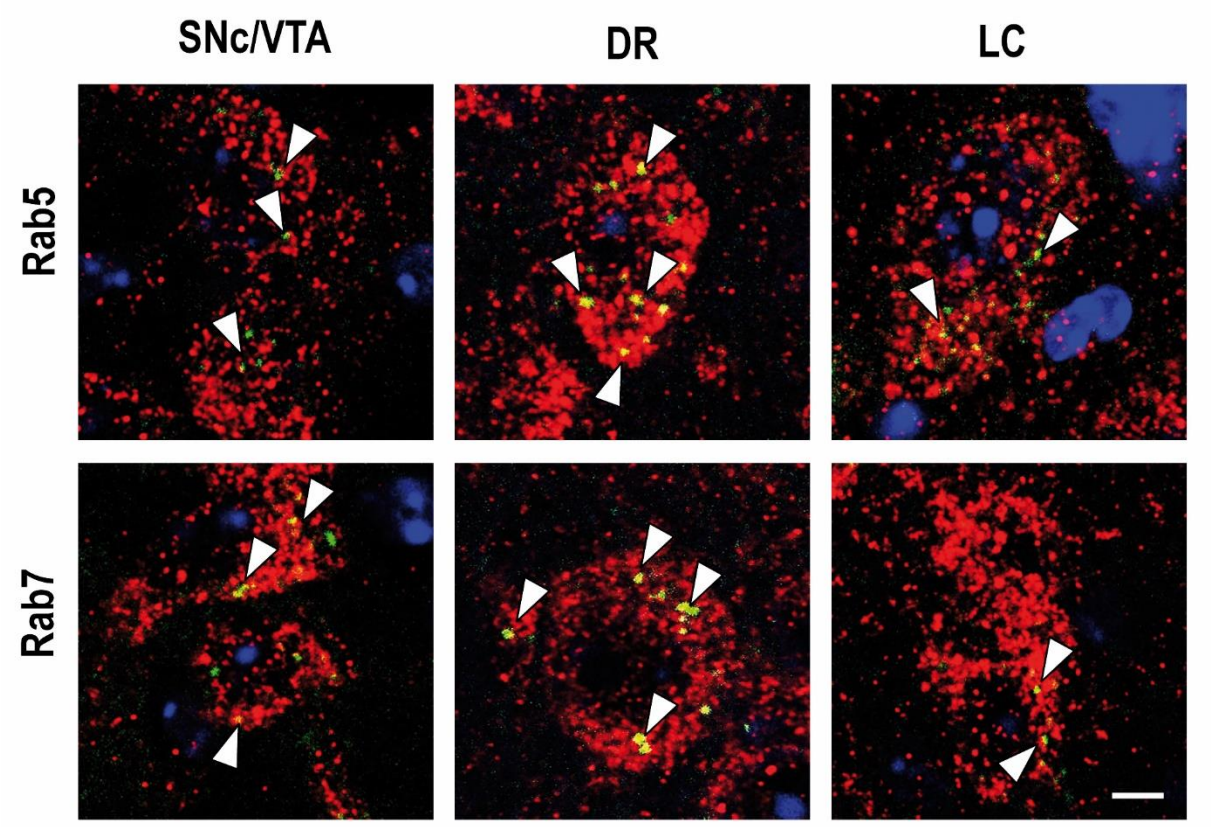

Figure S9. Co-localization of alexa488-labeled indatraline-conjugated nonsense-ASO (A488-IND-1227-ASO) with Rab5 or Rab7

Confocal images showing the co-localization (yellow) of A488-IND-1227-ASO (green) with Rab 5 or Rab7 (red) in SNc/VTA, DR or LC neurons. Enlarged images were obtained from brain sections of mice treated with A488-IND-1227-ASO at 30 µg/day for 4 days (the same mice as in Supp Figures S3 and S4) . Scale bar: 10 µm. Vesicles are marked with white arrowheads. DR, dorsal raphe nucleus; LC, locus coeruleus; SNc/VTA, substantia nigra compacta/ventral tegmental area.

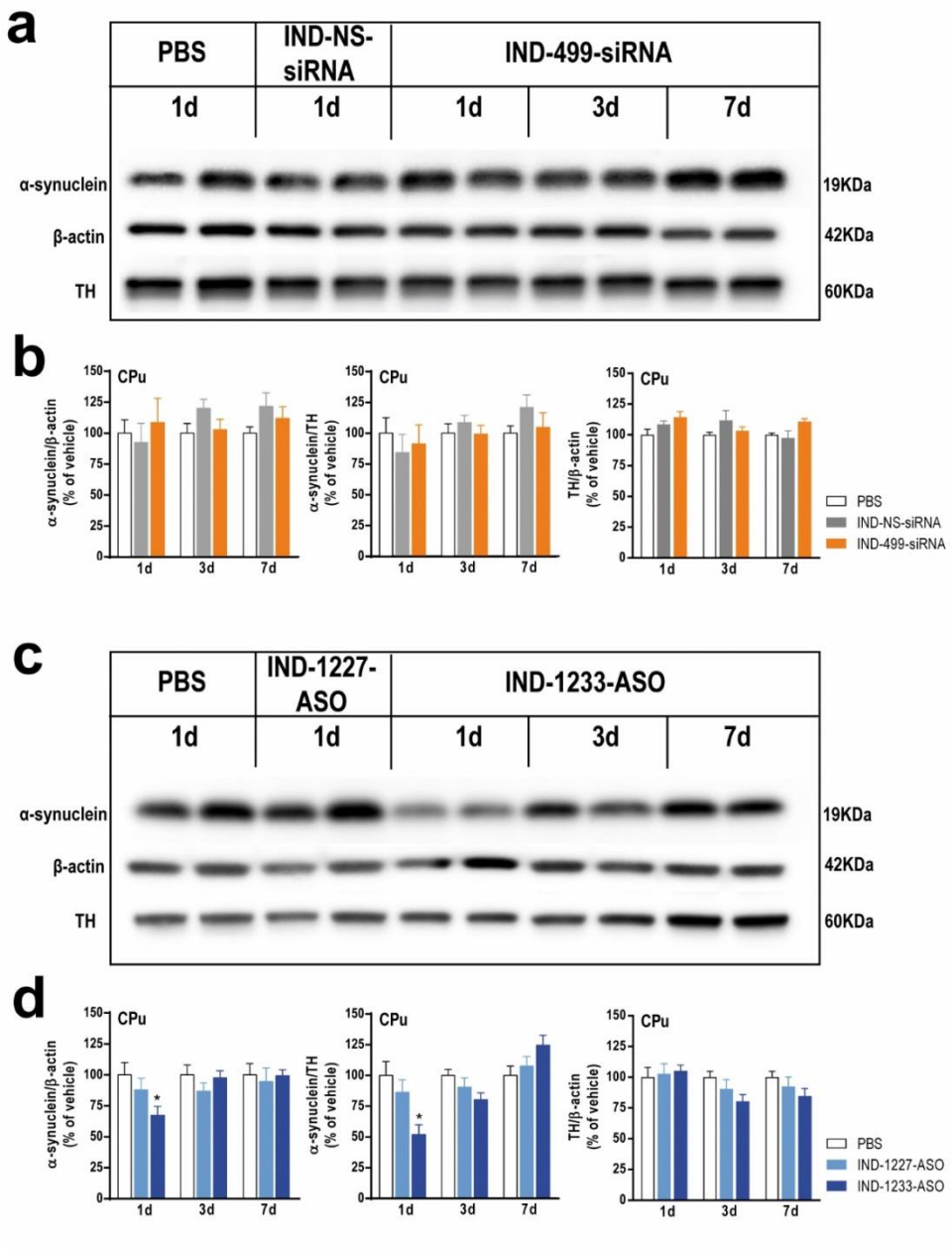

Figure S10. α-Synuclein protein levels in caudate putamen after intranasal treatment with indatraline-conjugated oligonucleotides

Mice were intranasally administered with PBS, indatraline-conjugated nonsense siRNA (IND-NS-siRNA), indatraline-conjugated 499-siRNA (IND-499-siRNA), indatraline-conjugated nonsense ASO (IND-1227-ASO) or indatraline-conjugated 1233-ASO (IND-1233-ASO) at 30 µg/day during 4 days and were sacrificed at 1, 3 or 7 days post-administration (1d, 3d or 7d, respectively; n=8-10 mice/group). **(a)** Image of immunoblot of α-synuclein, β-actin and tyrosine hydroxylase (TH) in caudate putamen (CPu) of mice treated with siRNA. **(b)** Bar graphs showing α-synuclein protein levels in CPu normalized against β-actin or TH and TH levels normalized against β-actin. IND-499-siRNA did not produce any alteration in the striatal α-synuclein protein level. **(c)** Image of immunoblot of α-synuclein, β-actin and TH in caudate putamen (CPu) of mice treated with ASO molecules. **(d)** Bar graphs showing α-synuclein protein levels in CPu normalized against β-actin or TH and TH levels normalized against β-actin. Unlike IND-499-siRNA, IND-1233-ASO reduced α-synuclein protein level in CPu 24h after last administration, and then the levels were recovered 3 days later. **P*<0.05 versus control groups (Two-way ANOVA followed by Tukey's *post-hoc* test). Values are mean ± SEM.

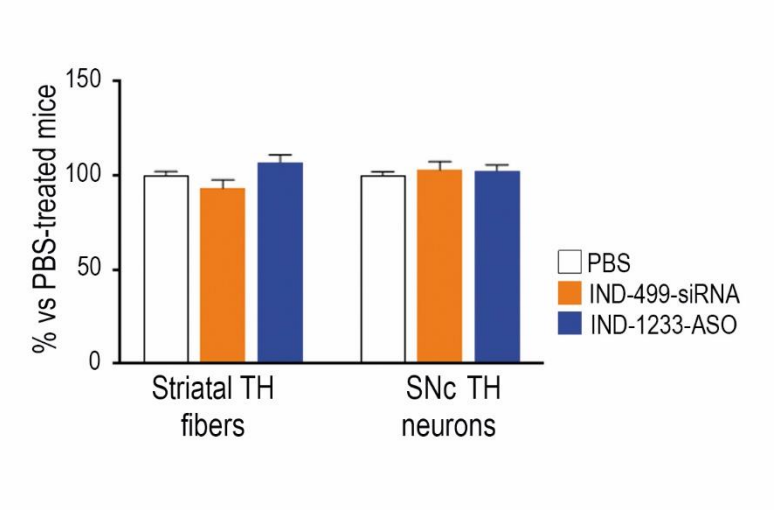

Figure S11. Absence of dopaminergic neurotoxicity after intranasal administration of indatraline-conjugated 499-siRNA (IND-499-siRNA) or indatraline-conjugated 1233-ASO (IND-1233-ASO)
Mice received intranasally PBS, IND-499-siRNA or IND-1233-ASO at 30 µg/day for 4 days and were sacrificied 7 days post-administration. Bar graphs show no differences in the number of SNc TH-positive neurons (right) or density of TH-positive striatal terminals (left), (n=5 mice/group). Values are mean ± SEM.

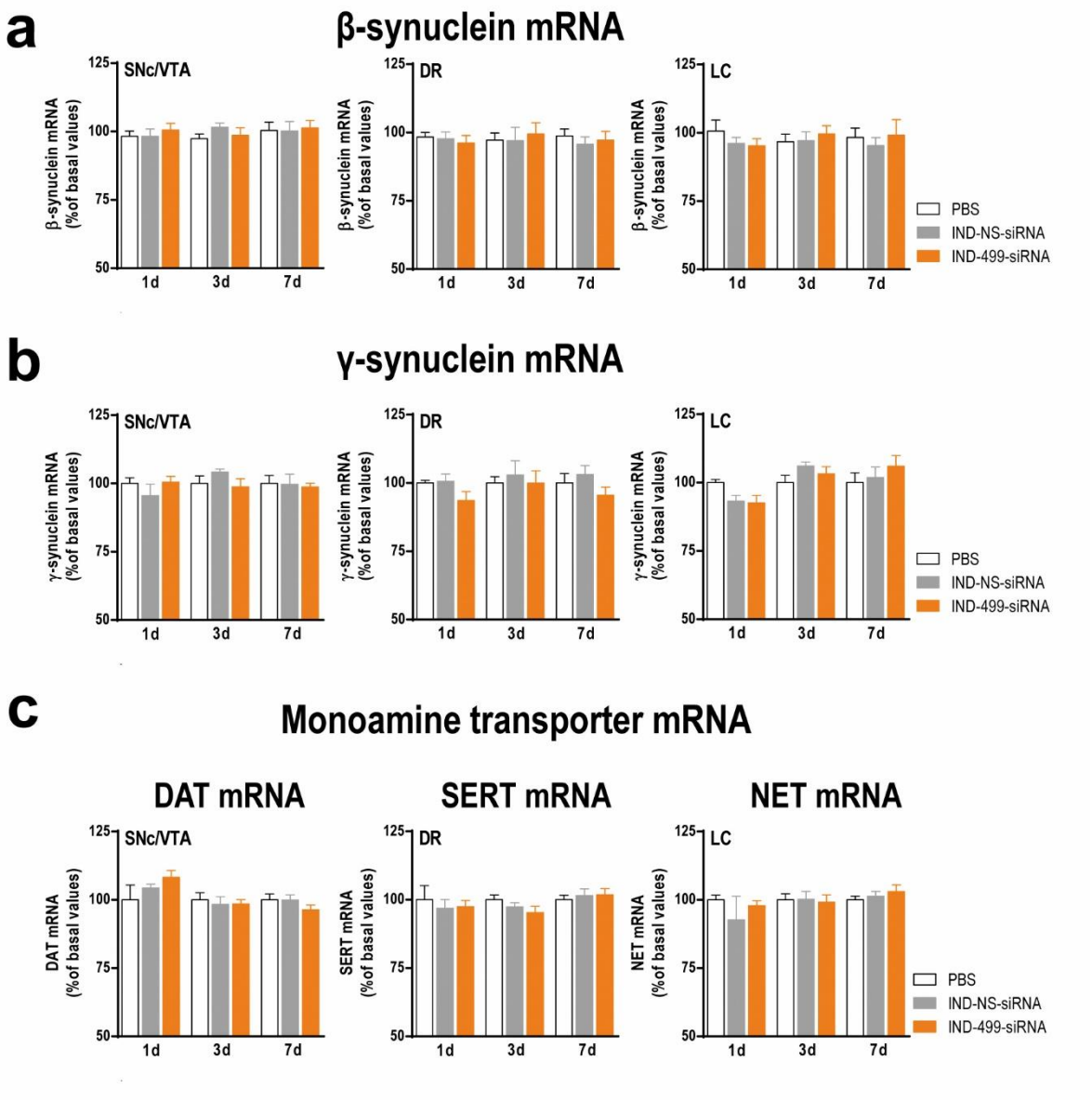

Figure S12. Selective suppresion of α-synuclein expression after intranasal administration of indatraline-conjugated 499-siRNA (IND-499-siRNA)

Mice received intranasally PBS, indatraline-conjugated nonsense siRNA (IND-NS-siRNA) or IND-499-siRNA at 30 μg/day during 4 days and were killed 1, 3 and 7 days post-administration (1d, 3d or 7d, respectively; n=4-5 mice/group). **(a-c)** Quantitative analysis of autoradiogrms (ROD) showed no differences in β-synuclein mRNA **(a)**, γ-synculein mRNA **(b)** and DAT, SERT and NET mRNA **(c)** densities in SNc/VTA, DR and LC assessed by in situ hibridization (ISH). Values are mean ± SEM and are normalized based on mice receiving PBS as 100%.

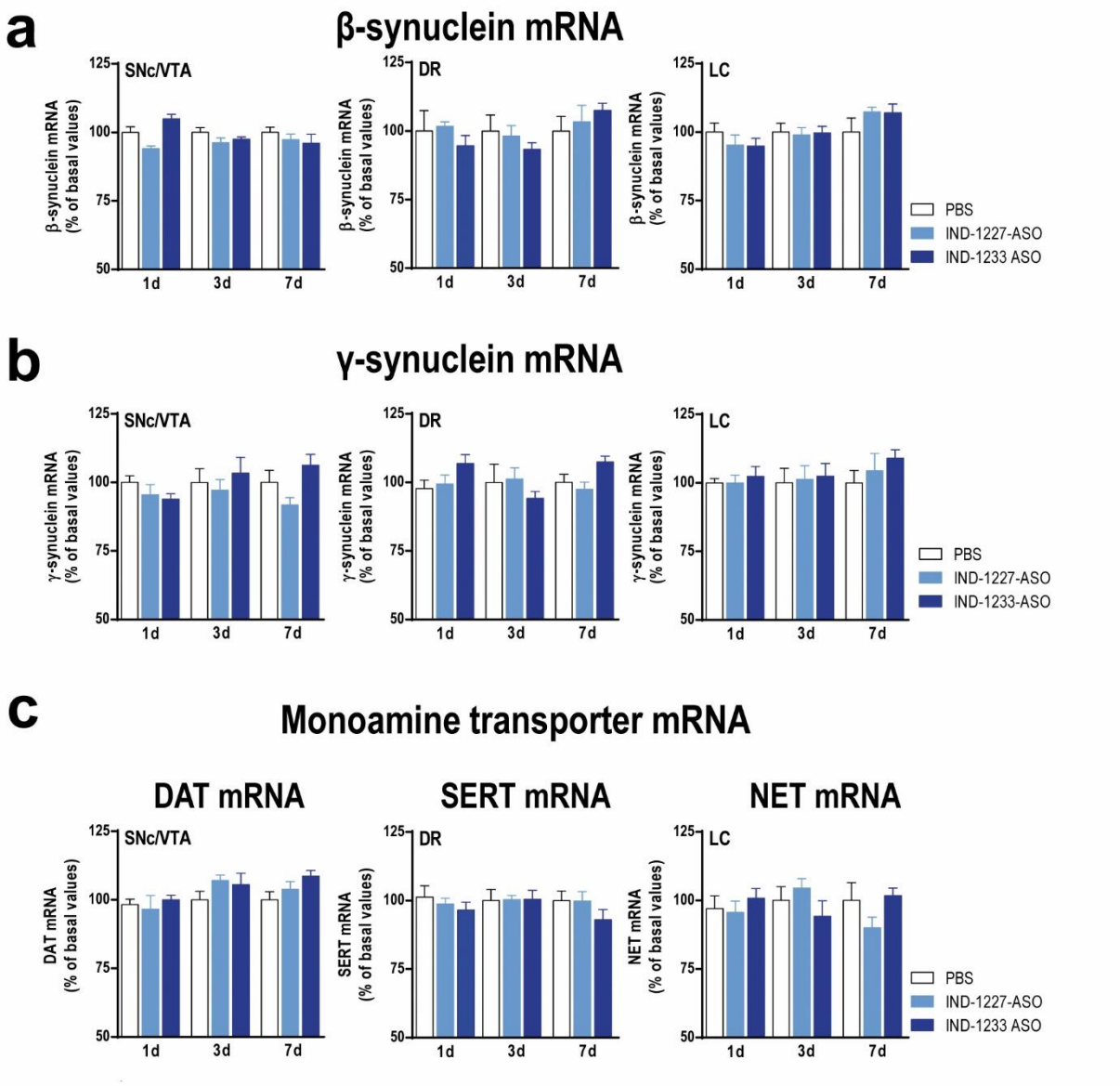

Figure S13. Selective suppresion of α-synuclein expression after intranasal administration of indatraline-conjugated 1233-ASO (IND-1233-ASO)

Mice received intranasally PBS, indatraline-conjugated nonsense ASO (IND-1227-ASO) or IND-1233-ASO at 30 μg/day during 4 days and were killed 1, 3 and 7 days post-administration (1d, 3d or 7d, respectively; n=4-5 mice/group). **(a-c)** Quantitative analysis of autoradiogrms (ROD) showed no differences in β-synuclein mRNA **(a)**, γ-synculein mRNA **(b)** and DAT, SERT and NET mRNA **(c)** densities in SNc/VTA, DR and LC assessed by in situ hibridization (ISH). Values are mean ± SEM and are normalized based on mice receiving PBS as 100%.

Table S1. Sequences of siRNA and ASO molecules

siRNAs/ASOs	forward	reverse
Nonsense		
NS-siRNA	aguacugcuuacgauacggTT	ccguaucguaagcaguacuTT
1227-ASO	*not applicable*	ccgtATCGTAAGCAgtac
α-Synuclein		
499-siRNA	agaagacaguggagggagcTT	gcucccuccacugucuucuTT
MAYO2-siRNA	aacaguggcugagaagaccaaTT	uuggucuucucagccacuguuTT
SNC2-siRNA	ggaguccucuauguaggguTT	accuacauagaggacuccTT
1233-ASO	*not applicable*	cuccCTCCACTGTCuucu

ASOs are 18mer single stranded DNA molecules with four 2'-O-methyl RNA bases at both ends. 1233-ASO: 5'-cuccdCdTdCdCdAdCdTdGdTdCuucu-3' and 1227-ASO: 5'-ccgtdAdTdCdGdTdAdAdGdCdAgtac-3' with lower case letters = 2'-OMethyl and "d" = 2'-deoxy nucleotides.

Table S2. Baseline DA and 5-HT dialysate concentrations in the CPu and mPFC of mice

Groups	Experimental Conditions	CPu		mPFC	
		DA	5-HT	DA	5-HT
PBS	aCSF[(a)]	15.3±1.9 (n=25)	3.8±0.5 (n=18)	3.1±0.4 (n=17)	7.5±1.3 (n=9)
IND-1227-ASO	aCSF[(a)]	13.3±1.5 (n=18)	n.e	n.e	n.e
IND-1233-ASO	aCSF[(a)]	13.4±1.5 (n=18)	2.7±0.4 (n=15)	2.1±0.3 (n=16)	6.8±0.7 (n=8)
PBS	aCSF+Nom/Cit[(a,c)]	49.4±9.6 (n=9)	10.1±1.1 (n=12)	28.4±4.6 (n=5)	13.7±1.7 (n=10)
IND-1227-ASO	aCSF+Nom/Cit[(a,c)]	39.2±9.4 (n=7)	n.e	n.e	n.e
IND-1233-ASO	aCSF+Nom/Cit[(a,c)]	52.8±21.3 (n=6)	11.3±1.2 (n=13)	27.7±2.6 (n=5)	15.3±2.1 (n=9)
PBS	aCSF+Nom/Cit[(b,d)]	34.7±5.6 (n=9)	17.0±4.5 (n=5)	13.5±2.1 (n=6)	18.8±1.3 (n=5)
IND-1227-ASO	aCSF+Nom/Cit[(b,d)]	45.3±5.50 (n=9)	n.e	n.e	n.e
IND-1233-ASO	aCSF+Nom/Cit[(b,d)]	41.1±10.9 (n=9)	22.9±7.1 (n=6)	11.8±1.9 (n=6)	15.9±1.1 (n=5)
TG-	aCSF[(a)]	13.4±1.1 (n=16)	n.e	n.e	n.e
TG+	aCSF[(a)]	15.2±1.6 (n=14)		n.e	n.e
TG-	aCSF+Nom/Cit[(a,c)]	35.9±11.3 (n=7)	n.e	n.e	n.e
TG+	aCSF+Nom/Cit[(a,c)]	33.6±7.1 (n=8)	n.e	n.e	n.e
TG-	aCSF+Nom/Cit[(b,d)]	50.2±10.4 (n=3)	n.e	n.e	n.e
TG+	aCSF+Nom/Cit[(b,d)]	52.3±9.1 (n=5)	n.e	n.e	n.e

Extracellular DA and 5-HT levels are expressed as fmol/20-min fraction[(a)] or fmol/10-min fraction[(b)]. In the experiments involving the evaluation of effects of quinpirole, CP93129 or 8-OH-DPAT on extracellular DA or 5-HT levels, nomifensine (Nom) or citalopram (Cit) was added in the aCSF at 10 or 1μM[(c)], respectively. In addition, Nom or Cit at 50μM[(d)] was added in the aCSF to evaluate tetrabenazine effects[46,48,79,80]. Data are means ± SEM of the number of mice shown in parentheses. n.e., not examined. TG+, transgenic mice expressing human wild-type α-synuclein cDNA under the control of TH promotor; TG-, control mice under the same background.

Supplemental Methods

Conjugated siRNA and ASO synthesis

The synthesis and purification of unmodified and indatraline-conjugated molecules of ASO targeting α-synuclein (1233-ASO, GenBank accession AH008229.3), nonsense-ASO (1227-ASO) and siRNAs (499-siRNA, GenBank accession NM_001042451.2; MAYO-siRNA, GenBank accession XM_012774027.2 and SNCA2-siRNA, GenBank accession NM_001042451.2) were performed by nLife Therapeutics S.L. (Granada, Spain). ASO molecules consisted of an antisense GapMer of 18 nucleotides with a central block of DNA flanked by 2-O methyl RNA bases to protect the internal DNA from nuclease degradation and improve the binding to the target sequence.

In brief, ASO and siRNA synthesis was performed using ultra mild-protected phosphoramidites (Glen Research, Sterling, VA, USA) and H-8 DNA/RNA Automatic synthesizer (K&A Laborgeraete GbR, Schaafheim, Germany). Indatraline (hydrochloride, trans-racemate) was conjugated to 5´-carboxy-C10 modified oligonucleotide through an amide bond. This condensation was carried out under organic conditions (DIPEA / DMF, rt, 24h). Conjugated oligonucleotides were purified by high performance liquid chromatography using a RP-C18 column (4.6x150 mm, 5 µm) under a linear gradient condition of acetonitrile. The molecular weights of the oligonucleotide strands and the conjugate were confirmed by MALDI-TOF mass spectrometry (Ultraflex, Bruker Daltonics). The concentration of the conjugates was calculated on the basis of absorbance at 260nm wavelength. Complementary strands were annealed in an isotonic RNA-annealing buffer (100mM potassium acetate, 30mM HEPES pH 7.4, 2mM magnesium acetate) at 90ºC by 1min, centrifuged for 15s and incubated 1h at 37ºC. Duplex RNA formation was confirmed using 20% polyacrylamide gel electrophoresis (PAGE, 30mA, 60min) and visualized by silver staining (DNA Silver stain kit, GE Healthcare, Piscataway, NJ). Sequences are shown in **Supplemental Table S1**.

***In situ* hybridization**

Frozen tissue sections were first brought to room temperature, fixed for 20min at 4ºC in 4% paraformaldehyde in phosphate-buffered saline (1xPBS: 8mM Na_2HPO_4, 1.4mM KH_2PO_4, 136mM NaCl, and 2.6mM KCl), washed for 5min in 3xPBS at room temperature, twice for 5min each in 1xPBS, and incubated for 2min at 21ºC in a solution of predigested pronase (Calbiochem, San Diego, CA) at a final concentration of 24 U/mL in 50mM Tris-HCl, pH 7.5, and 5mM EDTA. The enzymatic activity was stopped by immersion for 30s in 2 mg/ml glycine in 1xPBS. Tissues were finally rinsed in 1xPBS and dehydrated through a graded series of ethanol. For hybridization, the radioactively labeled probes were diluted in a solution containing 50% formamide, 4x standard saline citrate, 1x Denhardt's solution, 10% dextran sulfate, 1% sarkosyl, 20mM phosphate buffer, pH 7.0, 250 µg/ml yeast tRNA, and 500 µg/ml salmon sperm DNA. The final concentrations of radioactive probes in the hybridization buffer were in the same range (~1.5 nM). Tissue sections were covered with hybridization solution containing the labeled probes, overlaid with Nescofilm coverslips (Bando Chemical Ind., Kobe, Japan), and incubated overnight at 42ºC in humid boxes. Sections were then washed 4 times (45min each) in a buffer containing 0.6M NaCl and 10mM Tris-HCl (pH 7.5) at 60ºC. Hybridized sections were exposed to Biomax-MR film (Kodak, Sigma-Aldrich, Madrid, Spain) for 1-4 weeks with intensifying screens. For specificity control, adjacent sections were incubated with an excess (50x) of unlabeled probes. The cytoarchitecture of different mouse brain areas was analyzed in an adjacent series of cresyl-violet stained frozen sections.

Confocal fluorescence microscopy

Brain sections were rinsed with PBS/Triton 0.2%, incubated with 10% normal serum from secondary antibody host and treated with primary antibodies: sheep anti-TPH_2 (1:2500; ref.: AB1541, Merck Millipore), rabbit anti-TH (1:1250; ref.: AB112, Abcam), rabbit anti-Rab5 (1:500; ref.: ab18211, Abcam) or mouse anti-Rab7 (1:2000; ref.: R8779-200UL, Sigma-Aldrich). Sections were then incubated at 4ºC overnight, rinsed and treated with respective secondary Alexa555-conjugated antibodies (1:500, A-20000, Life Technologies) for 120min. After subsequent washes, the sections were dehydrated and mounted in the anti-fading agent Prolong Gold with DAPI (Life Technologies). DAPI, Alexa488 and Alexa555 images were acquired sequentially using 405, 488 and 561 laser lines, AOBS (Acoustic Optical Beam Splitter) as beam splitter and emission detection ranges 415- 480, 500-550 and 571-625 nm, respectively and, the confocal pinhole set at 1 Airy units. Images were acquired at 400Hz in a 1024 x 1024 pixel format. Images were composed using NIH ImageJ 1.47n software.

Treatments

To assess the involvement of monoamine transporters (DAT, SERT and NET) as a gate for the accumulation of indatraline-conjugated ASOs in the monoaminergic neurons, different cohorts of mice were pretreated with: a) sertraline 20 mg/kg, i.p. (selective SERT inhibitor), b) nomifensine 20 mg/kg, i.p. (DAT/NET inhibitor) or c) reboxetine 10 mg/kg/day, i.p. (selective NET inhibitor) for 3 h before the i.n. administration of IND-1227-ASO at 30 µg/day for 4 days. Control group received saline solution, i.p. in the same conditions. Mice were sacrificed

6h after last administration of IND-1227-ASO and their brain were removed and processed by immunofluorescence.