Targeted Interneuron Depletion in the Dorsal Striatum Produces Autism-Like Behavioral Abnormalities in Male but not Female Mice

Supplemental Information

SUPPLEMENTARY METHODS

Animals

All experiments were approved and overseen by the Yale University IACUC, consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were housed in a temperature (23°C) and humidity-controlled vivarium, 2-5 per cage, on a 12h light/dark cycle. Food (standard laboratory chow) and water were provided *ad libitum*.

To target FSIs we used Pv-cre transgenic mice, which have been previously described (1). This is a distinct line from that used in our previous study of striatal FSIs (2); this PV-cre transgenic line appears to express in a larger fraction of striatal PV-positive striatal interneurons. To target CINs we used Chat tm1(cre)Lowl transgenic mice (www.jax.org:006410), as in our previous work (3). Mice of these two lines were interbred to produce PV-cre homozygous/ChAT-cre hemizygous breeders; these mice were bred with wild-type C57Bl/6 mice to produce PV-cre hemizygous/ChAT-cre double-hemizygous and PV-cre single-hemizygous mice for experimental use. To produce ChAT-cre single-hemizygous mice for control experiments, ChAT-cre homozygotes were bred with C57Bl/6 mice. Control mice were littermates in all experiments.

Viruses, Surgical Procedures and Viral Infusions, and Interneuron Depletion

To achieve targeted interneuronal depletion specifically in the dorsal striatum we infused a virus expressing the simian diphtheria toxin receptor (sDTR) into the dorsal striatum of transgenic mice, as previously described (2, 3). We used the AAVrh10 EF-DIO-sDTR-FLAG (A46) vector previously used to target FSIs (2); this expresses sDTR at higher levels and is more effective in depleting FSIs than the originally described A06 construct (3). Control animals received the control AAVrh10-EF- mutDIO-sDTR-FLAG (C46) construct, which is identical to A46

except for multiple point mutations in the 3' loxP sites of the DIO cassette and thus is not subject to activation by cre-mediated recombination (2). Viruses were produced by the Salk Institute Vector Core (http://www.salk.edu/science/core-facilities/gene-transfer-targeting-and-therapeutics-core/) at a titer of 10^{13} viral genomes/ml.

Both male and female mice were used. Mice were 2 months old at the time of surgery. Viruses were infused into the central dorsal striatum stereotaxically under ketamine/xylazine anesthesia as described previously (3), targeting coordinates AP +0.5mm, ML ±1.5mm, DV -2.7 mm (4). 0.5 µl virus was infused on each side at a flow rate of 0.1 µl/min using a 1 µl microsyringe (Hamilton) attached to a micropump (Ultramicropump II, WPI). Typical viral spreads are shown in **Figure 1A-C**. After infusion was completed on each side the syringe was left in place for an additional 5 min to minimize regurgitation of virus. After surgery and recovery from anesthesia, mice were returned to the vivarium and allowed to recover for 2 weeks to allow for robust viral expression.

In mice infused with the A46 virus, cre recombinase in cre-expressing cells (FSIs and/or CINs) inverts the DIO cassette, leading to expression of DTR and rendering these cells susceptible to ablation after systemic DT. Neighboring cre-negative cells are infected by virus but cannot invert the DIO cassette and thus express GFP rather than DTR; they are resistant to ablation after systemic DT. The C46 virus cannot be activated even in cre-expressing cells, and thus all infected cells express GFP rather than DTR; all cells are resistant to ablation after systemic DT.

Two weeks after surgery, all mice received two injections of diphtheria toxin (DT, 15 or 30 µg/kg IP, one injection/day on 2 consecutive days). Behavioral analysis began 1 week after DT injection and continued for ~3 weeks, after which mice were euthanized and their brains examined.

Drugs

Diphtheria toxin (DT, Calbiochem #322326) was dissolved in sterile saline and administered IP. Oxytocin (1 mg/kg, Tocris #1910) was dissolved in sterile saline solution and then administered IP. This hormone was administered 20 minutes before starting experimental

procedure (0.1ml/10 gr of mice).

Behavioral Analysis

Locomotor activity was assessed using an open field apparatus (Omnitech Electronics) and quantified as infrared beam-breaks. Mice were allowed to habituate to the behavioral room for 30 minutes before testing. They were then placed individually into the locomotor monitoring cage (18.5" x 14.5" x 8"). Activity was recorded for 1 hr and quantified as infrared beam breaks using Fusion software (Omnitech Electronics).

Elevated plus maze, *rotarod*, and *pre-pulse inhibition of startle* were measured as described previously by our group (3, 5, 6).

Social interaction. A modified version of three-chamber box was used to quantify social interaction, as previously described (7). The apparatus consisted in an opaque white Plexiglas box (60 x 38 x 20 cm), open to the top, with overhead video monitoring using ANY-Maze software (Stoelting). All procedures were performed under moderate diffuse illumination (40 lux). Male and female mice were tested in separate sessions. Two circular zones (15 cm diameter) were defined within the open field, side by side, separated from each other and from the walls by 10 cm. A wire cylinder, closed on the top, was placed in the center of each of these zones. Two removable solid white dividers were placed into the box between these two zones, creating a rectangular central area separated from both, in which mice were placed at the beginning of the session. The whole apparatus was carefully cleaned between sessions.

Social interaction quantifies the preference of a 'test' mouse for an unfamiliar conspecific 'stranger' mouse relative to an inanimate novel object. Stranger mice were unused siblings from the same cohort and were matched to test mice for sex and age but had no contact with test mice before behavioral testing. Target mice were placed within one of the inverted wire cylinders in the open field arena for 20 min/day for 2 days before social interaction testing, to habituate them to the environment.

Test mice were first habituated for 60 min to the testing room in their home cage. They were then placed into the arena between the dividers. The dividers were removed, allowing the test mouse to explore the arena for 10 min. Exploration in the zones around the two wire

cylinders was quantified; mice showing any marked bias (>30 sec dwell time) for one target zone over the other were removed and re-tested on a subsequent day. None of the animals used in this manuscript required a third test due to bias. The test mouse was then removed and the dividers replaced.

Next, a target mouse was placed in one of the two wire cylinders (selected pseudo-randomly), and an inanimate object (Duplo® blocks, black, of similar size to a mouse) was placed in the other. The test mouse was again placed between the dividers, which were then removed. The test mouse was allowed to explore the apparatus for 10 min. Entries into and time within the social target zone and the corresponding nonsocial target zone were quantified.

Stereotypic behavior. Stereotypic time (grooming and sniffing) was scored from video using an automated system (Cleversys HomeCage Scan); we have previously shown that this quantification correlates well with blinded manual scoring (3). Stereotypical beam-breaks were defined as repetitive breaks of a single infrared beam within one second (8-10). Beam-break data were collected and analyzed using Fusion software (Omnitech Electronics).

Operant conditioning. Operant testing was conducted in standard aluminum chambers (Med Associates). One side of the chamber was equipped with a photocell pellet-delivery magazine and three photocell-equipped noseport apertures that could be illuminated internally positioned on the opposite side of the chambers. Operant boxes were housed inside sound-attenuating cubicles. Background white noise was broadcast throughout all sessions.

Two weeks prior to beginning operant testing, mice underwent a mild dietary restriction and were maintained at ~85% of their free feeding weight for the duration of the behavioral experiment.

Following two days of exposure to rewards (14 mg sucrose pellets; BioServ, Flemington, NJ), mice were trained to retrieve sugar pellets from the food magazine in a single, 30 min session. A single pellet was delivered when a mouse made a response into the magazine aperture or 30 s has lapsed without an entry into the magazine; sessions terminated once 30 rewards had been earned or 30 min had lapsed, whichever occurred first.

The next day, mice were returned to the operant chamber. The magazine was illuminated at the beginning of each trial. A single nosepoke into the illuminated magazine

triggered the illumination of the two outermost of the three noseport apertures on the opposite wall. A subsequent nosepoke response into either of the illuminated noseports resulted in delivery of single reward and a 1s illumination of the magazine receptacle. Noseport responses into the non-illuminated noseport resulted in a 60 s timeout in which all cues were extinguished. Following a 5 s intertrial interval, the magazine was illuminated and mice could initiate another trial. Sessions terminated when mice completed 60 trials or 60 min had elapsed, whichever occurred first. Mice completed one daily sessions until accuracy was greater than 80% and the number of completed trials was greater than 48 across two consecutive days.

Next, mice were trained in a cue discrimination task. In these sessions, successfully initiated trials resulted in the illumination of a single noseport aperture (randomly determined left or right). Responses into the illuminated noseport aperture resulted in delivery of a single reward, while responses into the non-illuminated noseport apertures did not. Sessions terminated when mice completed 60 trials or 60 min had lapsed, whichever occurred first. Mice completed once daily sessions until accuracy in a single session was greater than 80%.

The day after reaching the performance criterion in the cue discrimination task, the ability of mice to shift strategies was assessed. In these sessions, trial initiation resulted in illumination of a single noseport aperture (left or right), but only responses to the left noseport aperture were reinforced, regardless of whether that noseport aperture was illuminated or not. The shift from cue discrimination to spatial discrimination used here is similar to the extra-dimensional shifts that are implemented in set-shifting tasks (45). Sessions terminated when mice completed 60 trials or 60 min lapsed, whichever occurred first.

The primary dependent measure in the noseport training sessions and the two task conditions (cue discrimination and spatial discrimination) was the total number of trials that were required to reach the performance criterion.

The number of trials required to reach criterion in the three behavioral assessments (noseport training, cue discrimination, and spatial discrimination) were analyzed using general estimating equations with a Poisson loglinear distribution in SPSS (version 22; IBM) with experimental group as the between subjects factor. Significant effects were subsequently analyzed using Bonferroni corrected pairwise comparisons.

Immunohistochemistry

Following behavioral testing, mice were heavily sedated using ketamine/xylazine (100/10 mg/kg) and transcardially perfused with saline followed by paraformaldehyde (PFA; 4% in PBS). Brains were post-fixed in PFA at 4°C for 24 h and then transferred to 30% sucrose at 4°C for 48 h. Brains were sliced coronally on a cryostat (Leica) at 30 µm. Slices were stored at 4°C in a solution of 30% (vol/vol) ethylene glycol and 30% (vol/vol) glycerol.

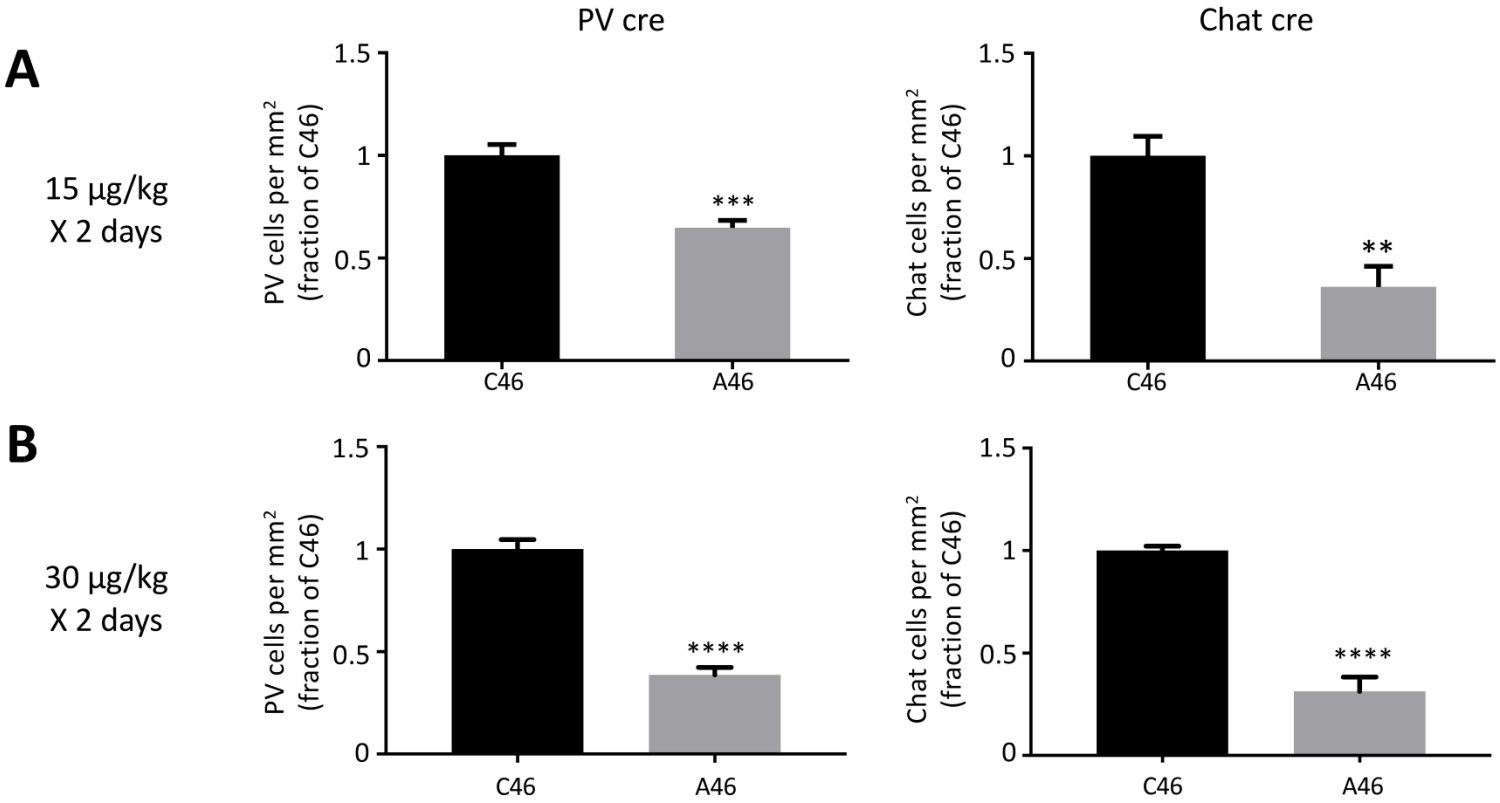
For staining, slices were washed 3x 5 min at room temperature in TBS, incubated for 20 min in Bloxall solution (Vector Laboratories, USA), washed 3x 10 min in TBS, and then blocked for 30 min at room temperature in 3% blocking buffer (3% normal goat serum or normal rabbit serum, corresponding to the secondary antibody used) in TBS with 0.3% Triton X-100. Samples were then incubated overnight in blocking buffer with one of the following primary antibodies: goat anti-choline acetyltransferase (1:100, Millipore); rabbit anti-PV (1:1000, Abcam); rabbit anti- P-S235/236-rpS6 (1:400, Cell Signaling Technology); rabbit anti-P-S9- GSK3β (1:100, Cell Signaling Technology); rabbit anti-P-S10 histone H3 (1:200, Millipore); or rabbit anti-P-T581 MSK1 (1:250, Cell Signaling Technology). The next day, slices were washed 2x in TBS with 0.1% Triton X-100 and once with TBS at room temperature, and then incubated with biotinylated goat anti-rabbit, goat anti-mouse, or rabbit anti-goat secondary antibody (1:300; all secondaries from Vector Laboratories) for 1 hr at room temperature. Slices were then washed 3x with TBS containing 0.1% Triton X-100 and then incubated for 1 hr at room temperature with avidin-coupled horseradish peroxidase complex in TBS (ABC Elite Kit, Vector Laboratories), and then developed using diaminobenzidine (DAB; Vector Laboratories), according to the manufacturer's instructions. Finally, slices were washed 4 x with TBS, counterstained with nuclear fast red (Vector Laboratories), and mounted on slides.

Immunopositive cells were counted from a single field in the dorsal striatum on each side in representative slices as was previously described (11, 12). Mounted sections were visualized using a Leica DM1000 microscope. Images in the dorsal striatum were collected at 20×. Several images were collected for each mouse, but only one field was collected for each section, to prevent overlap. Cells were manually counted with the Photoshop counter tool in

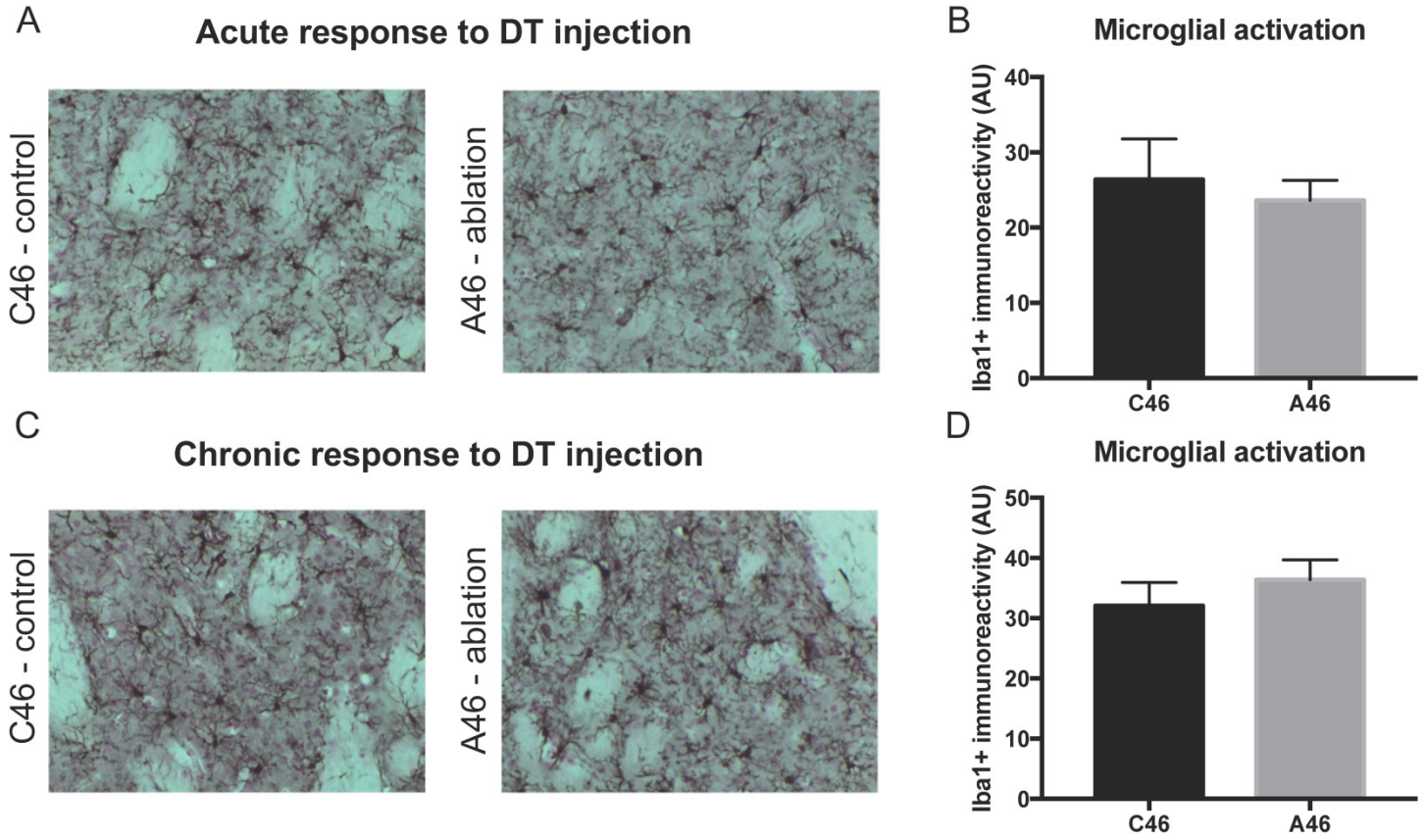
each image by an investigator blind to experimental condition, and averaged across all sections from that mouse. Cell counts were normalized per unit area and then averaged across each animal before statistical analysis; N in these experiments thus reflects number of animals, not number of slices.

Data Analysis

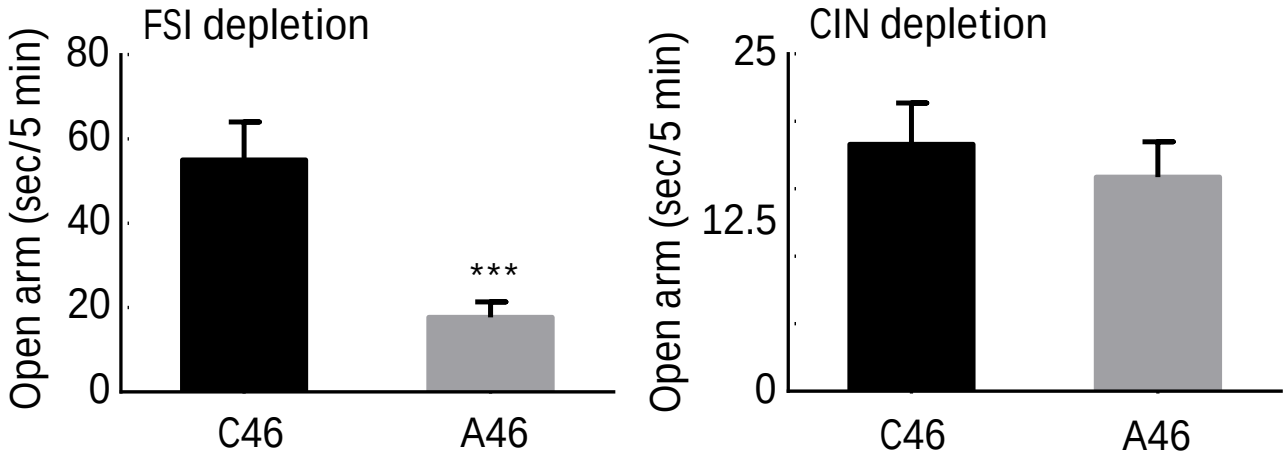
All quantification was performed blind to experimental condition. Statistical analysis was performed using Prism 6.0 (GraphPad) or, for 3-way analyses, Minitab 17 (Minitab, Inc.) or SPSS 22.0 (IBM). Group values are presented as mean ± SEM. Pairwise comparisons were performed using Student's t-test. More complex comparisons were performed using ANOVA with Sidak's post-hoc. Differences between conditions were considered statistically significant at $p < 0.05$.



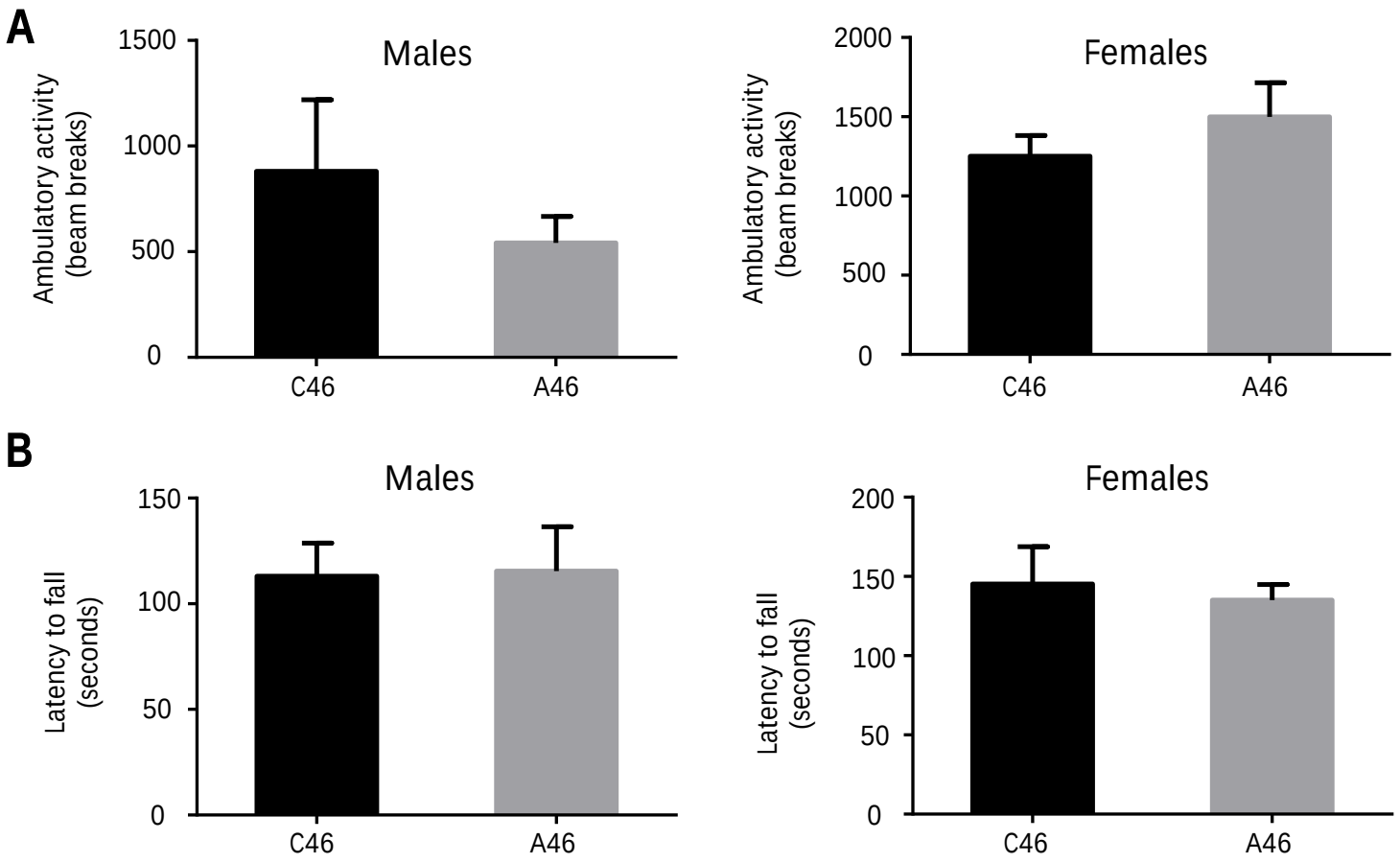
Supplemental Figure S1. Single striatal interneuron type depletion in two cohorts of PV-cre and Chat-cre mice.
Cre-dependent DTR virus (A46) and control virus (C46) were infused bilaterally into single-transgenic male PV-cre and ChAT-cre single-transgenic male mice to control for effects of single interneuron-type depletion. **A.** In a first cohort, moderate depletion was achieved by a single injection of 15 µg/kg DT/dy x 2 days: PV-cre: $t[8]=5.5$; $p=0.0006$, C46 n=5, A46 n=5; ChAT-cre: $t[8]=4.6$; $p=0.0018$, C46 n=5, A46 n=5. This replicates previous work (10, 11). **B.** In a second cohort we administered 30 µg/kg DT/dy x 2 days and achieved more substantial interneuron depletion, especially of FSIs: PV-cre: $t[10] = 10.4$, $p < 0.0001$, $n = 6, 6$; ChAT-cre: $t[11] = 8.7$, $p < 0.0001$, $n = 6, 7$.



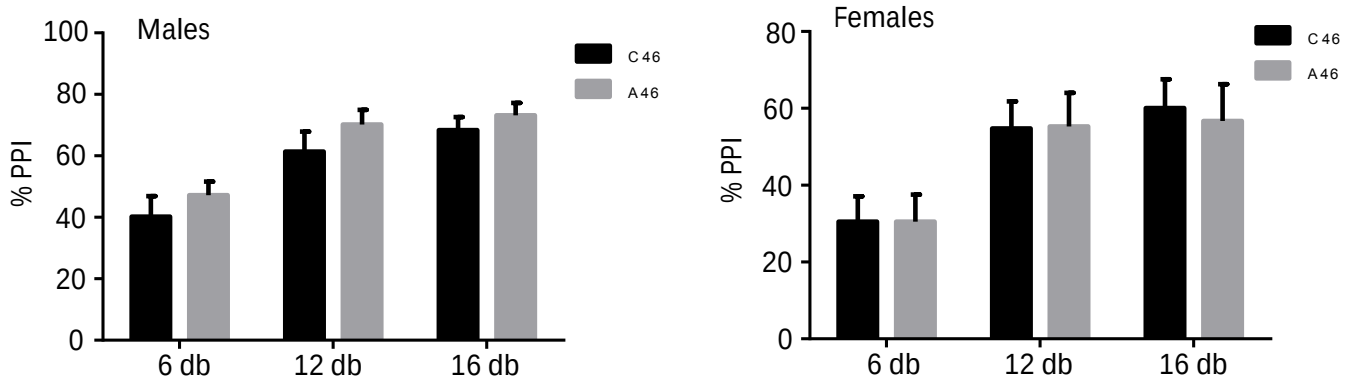
Supplemental Figure S2. Inflammatory changes after viral infusion and DT administration do not differ in A46 and C46 conditions. One day (acute) or 2-3 weeks (chronic) following interneuron depletion (double-transgenic male mice; 15 µg/kg DT x 2 days), microglial activation was similar in A46 and C46 groups, suggesting that there is no difference in inflammatory changes after interneuronal depletion. Microglia were visualized using immunohistochemistry; total surface area occupied by Iba1 immunoreactivity was quantified using ImageJ as in our recent work (13). Acute: $t[3] = 0.52$, $p = 0.64$, $n = 2, 3$; chronic: $t[6] = 0.86$, $p = 0.42$, $n = 4,4$.



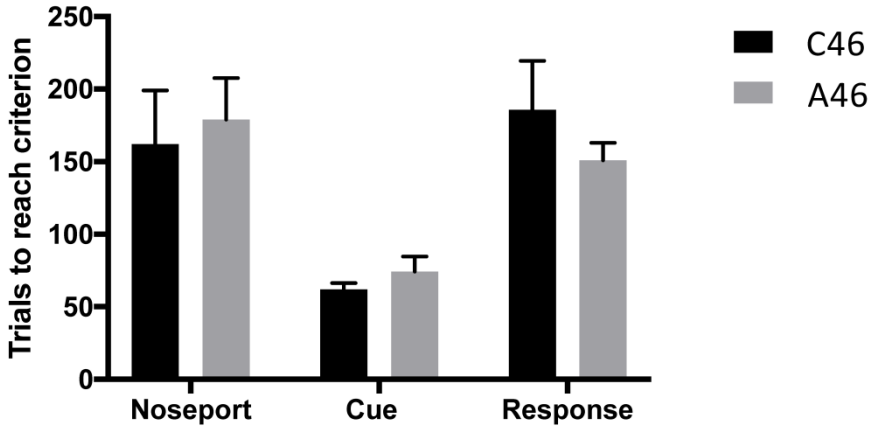
Supplemental Figure S3. Anxiety after FSI depletion but not after CIN depletion.
PV-cre mice with specific depletion of FSIs (15 µg/kg DT x 2 days; see Figure 1A) exhibited elevated anxiety in the elevated plus maze, shown here as decreased time spent in the open arm. $t[8] = 3.85$, $p = 0.005$, $n = 5, 5$. This replicates previous work (2). In contrast, ChAT-cre mice with specific depletion of CINs (15 µg/kg DT x 2 days; see Figure 1) exhibited no change in anxiety, also consistent with previous work (3). $t[8] = 0.60$, $p = 0.56$, $n = 5,5$. (Note that ChAT- cre single-transgenic mice are on a slightly different genetic background, which is likely to explain the difference in baseline.)



Supplementary Figure S4. Exploratory locomotion and motor coordination are not affected by conjoint FSI and CIN depletion.
A. Depletion of FSIs and CINs (15 µg/kg DT x 2 dys; see Supplemental Figure S1A) did not significantly affect ambulatory activity in an unfamiliar open field environment in males (t[19]=0.98, p=0.34, N = 10 C46, 11 A46) or females (t[16]=0.99, p=0.34, N = 9,9). **B.** Conjoint interneuron depletion did not affect latency to fall in a single session on the accelerating rotorod, a measure of motor coordination. Males: t[19] = 0.88, p = 0.93; females: t[16] = 0.40, p = 0.69.

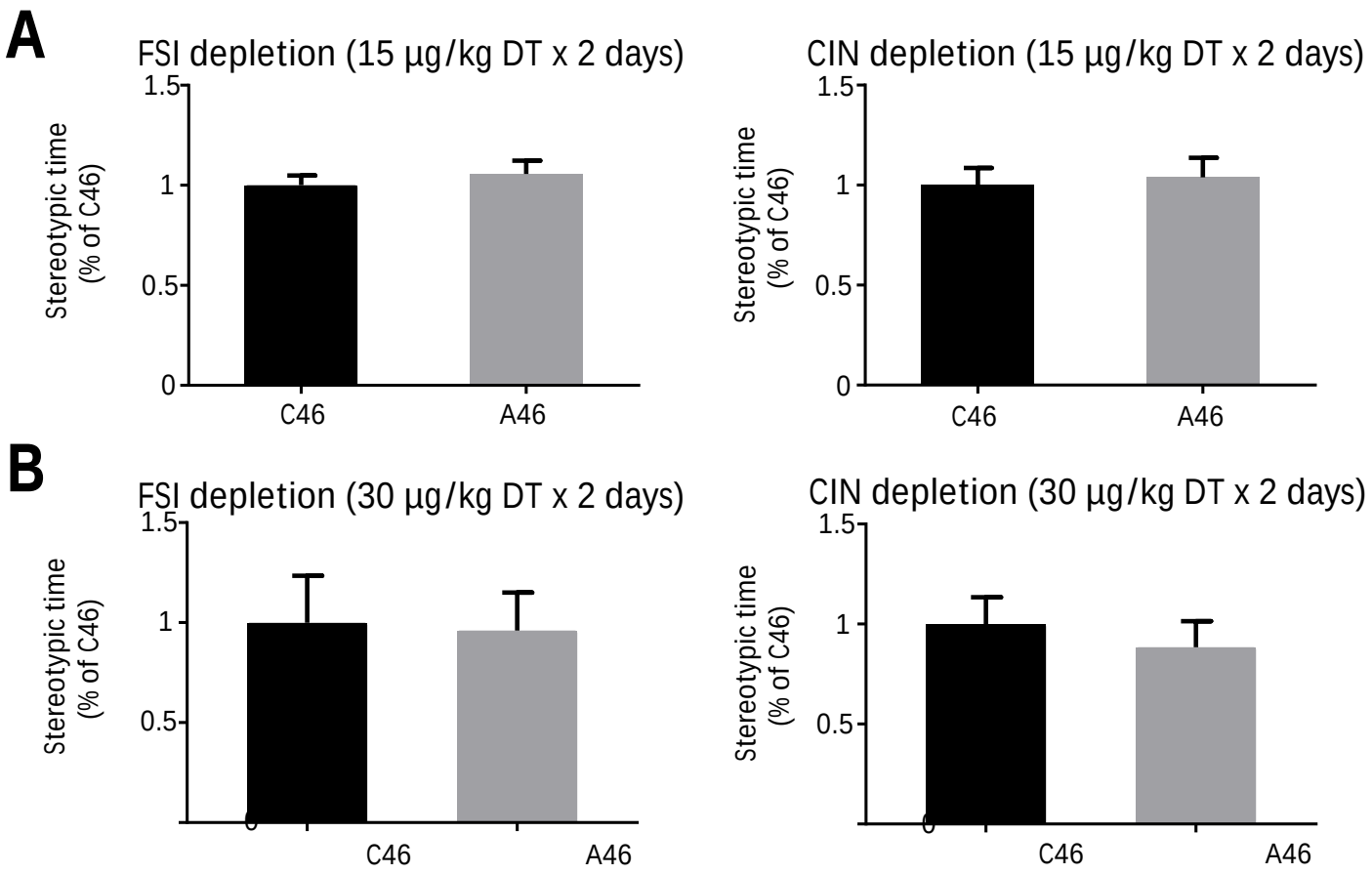


Supplementary Figure S5. Prepulse inhibition (PPI) after conjoint interneuron depletion.
PPI of the acoustic startle response, a measure of sensorimotor gating, was measured across three prepulse intensities as described previously (5, 6). There were no abnormalities in PPI in either males (left) or females (right) after interneuron depletion (15 µg/kg DT x 2 dys; see Supplemental Figure S1A). Males: 2-way repeated measures ANOVA, main effect of prepulse intensity, $F[2,38] = 59.1$, $p < 0.0001$; main effect of virus, $F[1,19] = 1.09$, $p = 0.31$; interaction: $F[2,38] = 0.2999$, $p = 0.7427$. Females: 2-way repeated measures ANOVA, main effect of prepulse intensity, $F[2,32] = 32.2$, $p<0.0001$; main effect of virus, $F[1,16] =0.009$, $p=0.95$; interaction:, $F[2,32] = 0.16$, $p=0.86$.



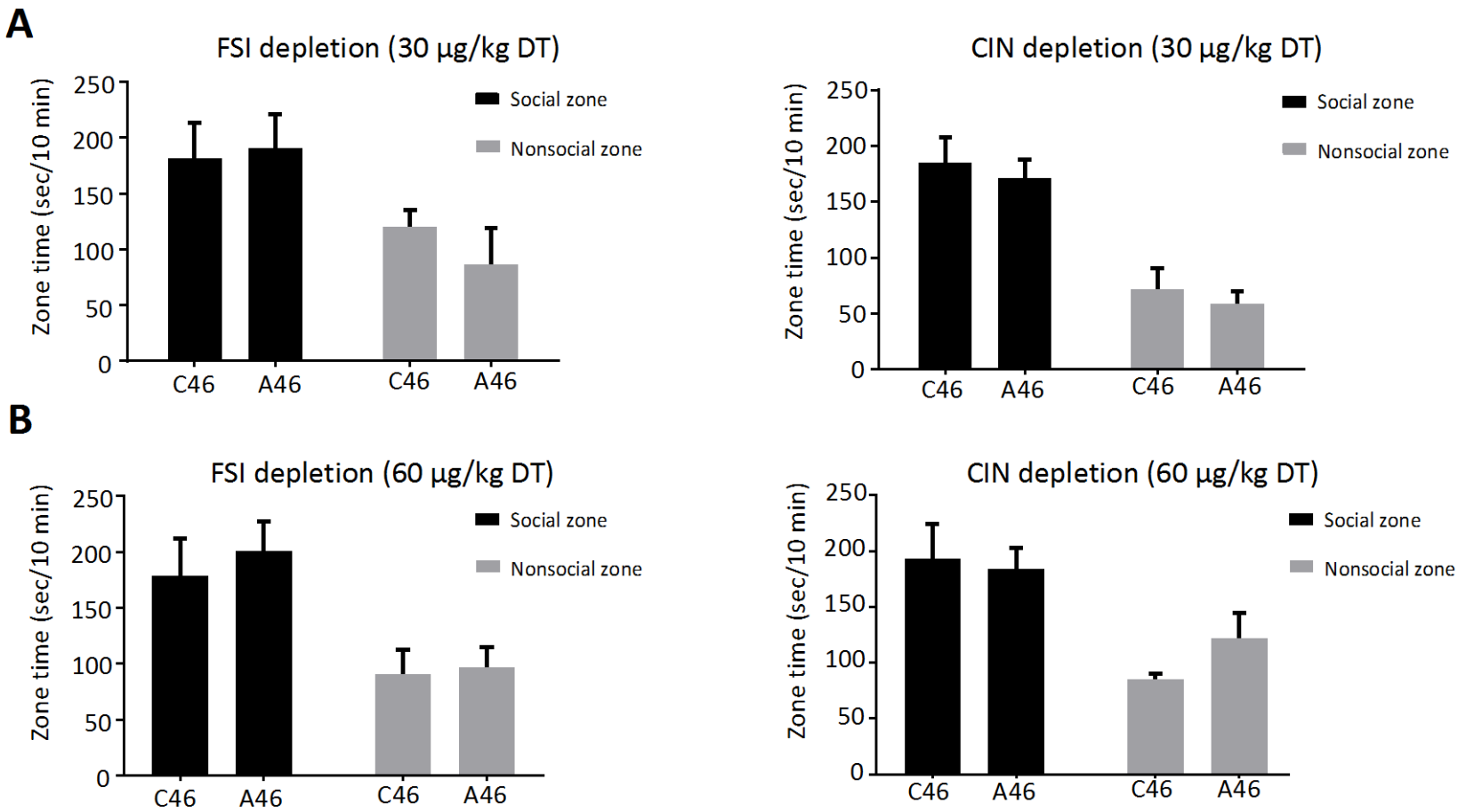
Supplemental Figure S6. Normal behavior in an instrumental learning, discrimination, and reversal task after conjoint FSI and CIN depletion.

Male bitransgenic mice with interneuron depletion (15 µg/kg DT x 2 dys; see Supplemental Figure S1A) underwent a three- phase instrumental task, as described further in the Supplemental Methods. In the first, 'noseport' phase they learned to nose-poke to receive a food reward. In the second, 'cue' phase they learned to track a visual cue by making a nose-poke into only the illuminated noseport in order to receive a food reward. In the third, 'response' phase they had to switch from using a cue strategy to using a spatial strategy, as only responses on the left noseport, regardless of whether or not it was illuminated, resulted in reward. The dependent measure in all phases was trials to reach a predetermined performance criterion. There was no difference between A46 and C46 mice in any phase: main effect of experimental group, χ^2=0.07; p=0.79; group x phase interaction, χ^2=3.62; p=0.16. There was a significant effect of experimental phase (χ^2=53.29; p<0.001), indicating that mice (in both groups) found a shift in strategy (i.e. the response phase) more difficult than learning the cue discrimination.

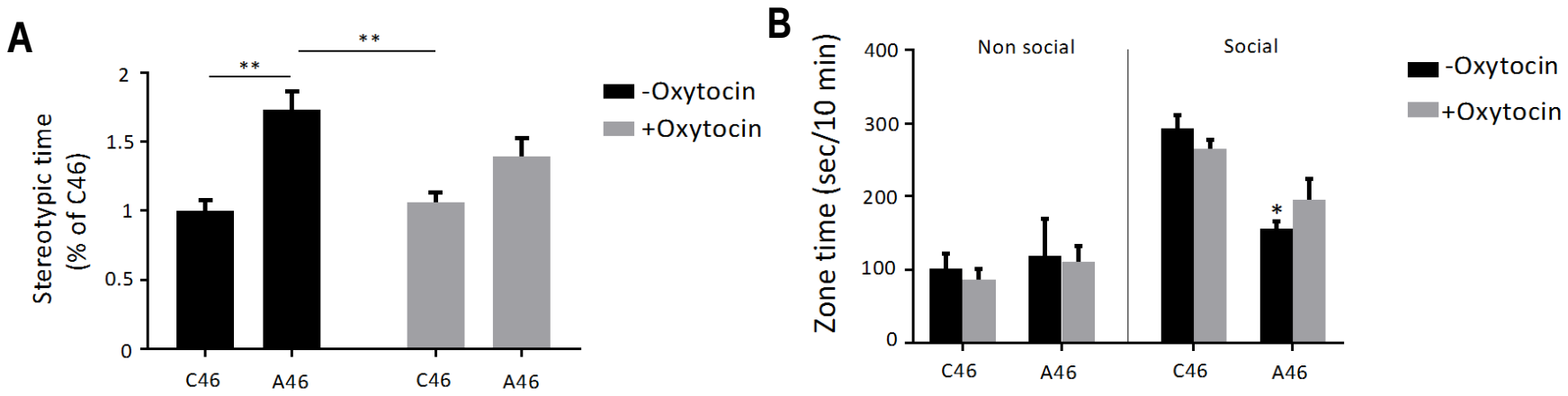


Supplemental Figure S7. Lack of spontaneous stereotypy after single depletion of FSIs or CINs.

A. Mice with depletion of only FSIs (see Supplementary Figure S1A) did not show elevated baseline stereotypic movements, confirming previous results (2) (t[8] = 0.31, p=0.77, C46 N=5, A46 N=5). Similarly, mice with depletion of only CINs (see Supplementary Figure S1A) did not exhibit elevated baseline stereotypic movements (t[8]=0.69, p=0.51, C46 N=5, A46 N=5) (3). **B.** Higher depletion of FSIs (t[11]=0.13, p=0.90, C46 N=6, A46 N=7) or CINs (t[11]=0.62, p=0.55, C46 N=6, A46 N=7) (see Supplementary Figure S1B) did not induce increased stereotypy.

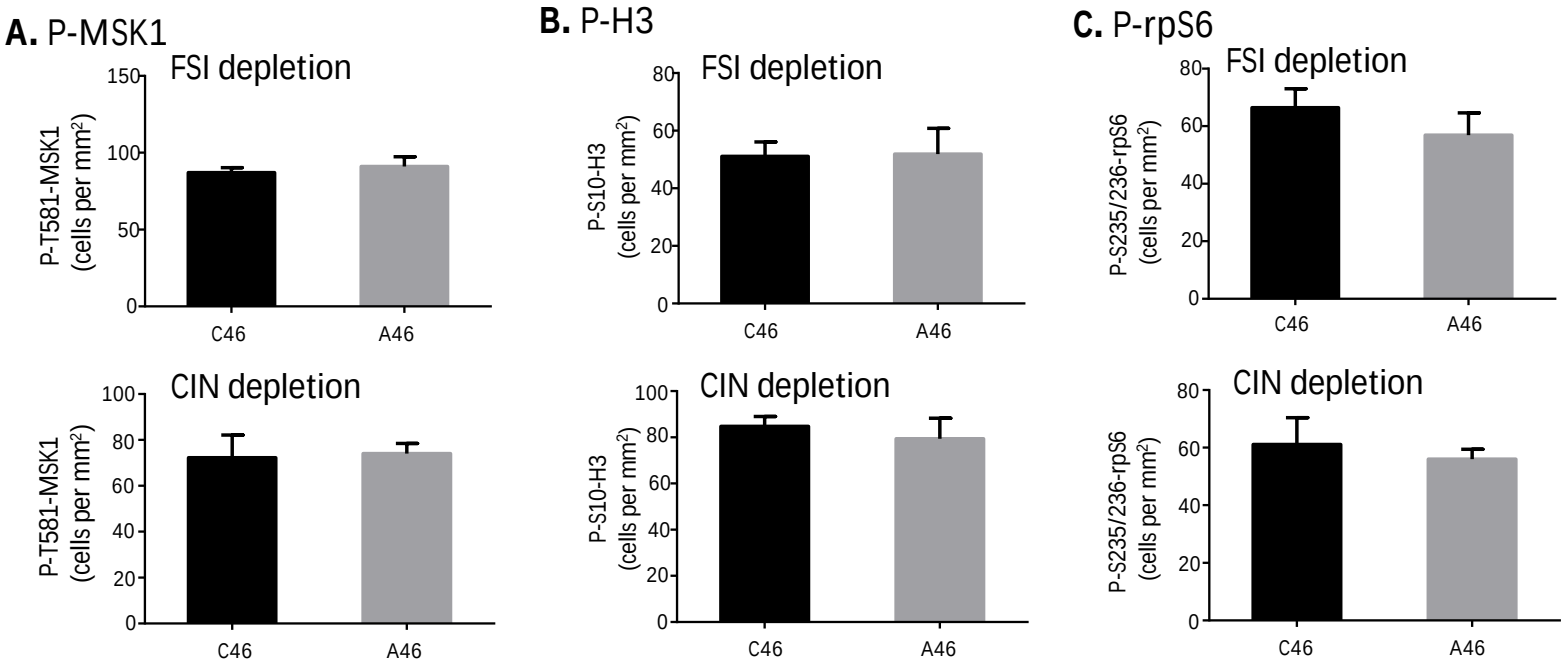


Supplemental Figure S8. Targeted depletion of FSIs or CINs alone does not produce social deficits.
A. FSI (main effect of virus F[1,16]=0.196, p=0.6639; main effect of zone F[1,16]=8.358, p=0.0098; interaction virus x zone F[1,16]=0.5738, p=0.4598; C46 N=5, A46 N=5) and CIN (main effect of virus F[1,16]=0.5624, p=0.4642; main effect of zone F[1,16]=40.13, p<0.0001; interaction virus x zone F[1,16]=0.0009, p=0.9762; C46 N=5, A46 N=5) depletion (see Suppl. Figure S1A) did not alter time spent in the social zone or in the non-social zone. **B.** Higher depletion ratios of CIN or FSI (see Suppl. Figure S1B) failed to induce social deficits (FSIs, main effect of virus F[1,22]=0.3176, p=0.5787; main effect of zone F[1,22]=14.24, p=0.010; interaction virus x zone F[1,22]=0.094, p=0.7616; C46 N=6, A46 N=7) (CINs, main effect of virus F[1,22]=0.4011, p=0.530; main effect of zone F[1,22]=15.41, p<0.001; interaction virus x zone F[1,22]=1.138, p=0.2977; C46 N=6, A46 N=7).



Supplemental Figure S9. Oxytocin administration modestly mitigates social and stereotypic behaviors in CIN and FSI depleted males.

A. Oxytocin administration showed mild effects on stereotypic behavior after striatal (2 way ANOVA, main effect of virus $F[1,17]= 22.48$, $p=0.0002$; main effect of treatment $F[1,17]= 1.511$, $p=0.2357$; interaction $F[1,17]=3.155$, $p=0.09$). N=6 C46 saline, n=6 A46 saline, n=4 C46 oxytocin, n=6 A46 oxytocin. Sidak's post hoc comparison corrected for multiple comparisons. **p<0.01. **B.** Social interaction in CIN and FSI conjoint depleted mice was minimally improved after oxytocin injection (3 way ANOVA, main effect of virus $F[1,55]= 4.6$, $p=0.037$; main effect of zone $F[1,55]=461$, $p<0.001$; main effect of treatment $F[1,55]=0.001$, $p=0.983$; interaction virus x zone $F[1,55]= 13.59$, $p=0.001$; interaction virus x treatment $F[1,55]= 1.55$, $p=0.220$; interaction zone x treatment $F[1,55]= 0.08$, $p=0.772$; interaction virus x zone x treatment $F[1,55]=0.45$, $p=0.506$). N=7 C46 saline, n=7 C46 oxytocin, n=6 A46 saline, n=8 A46 oxytocin. Sidak's post-hoc comparison: *p=0.014 C46 Social zone - Oxytocin vs A46 non-social one - Oxytocin.



Supplemental Figure S10. Single depletion of FSIs or CINs does not produce basal alterations in markers of MSN activation.

A. (Top) Depletion of FSIs (see Supplemental Figure S1) did not produce measurable changes in P-T581-MSK1 positive cells ($t[8]=0.58$, $p=0.58$; n = 5,5). (Bottom) Similarly, there was no alteration in P-T581-MSK1-T581 after specific CIN depletion ($t[8]=0.16$, $p=0.88$; n=5,5). **B.** Neither FSI depletion (Top; $t[8]=0.078$, $p=0.94$; n = 5,5) nor CIN depletion (Bottom; $t[8]=0.55$, $p=0.60$) produced alterations in P-S10-H3 positive cells. **C.** Neither FSI depletion (Top; $t[8] = 0.93$, $p=0.38$; n = 5,5) nor CIN depletion (Bottom; $t=0.51$, $p=0.62$) produced alterations in P-S235/236-rpS6 positive cells. All data are from males. The increases in these activity-dependent phosphorylation events seen after conjoint FSI/CIN depletion are therefore the result of synergistic effects of pathology in both interneuron populations.

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