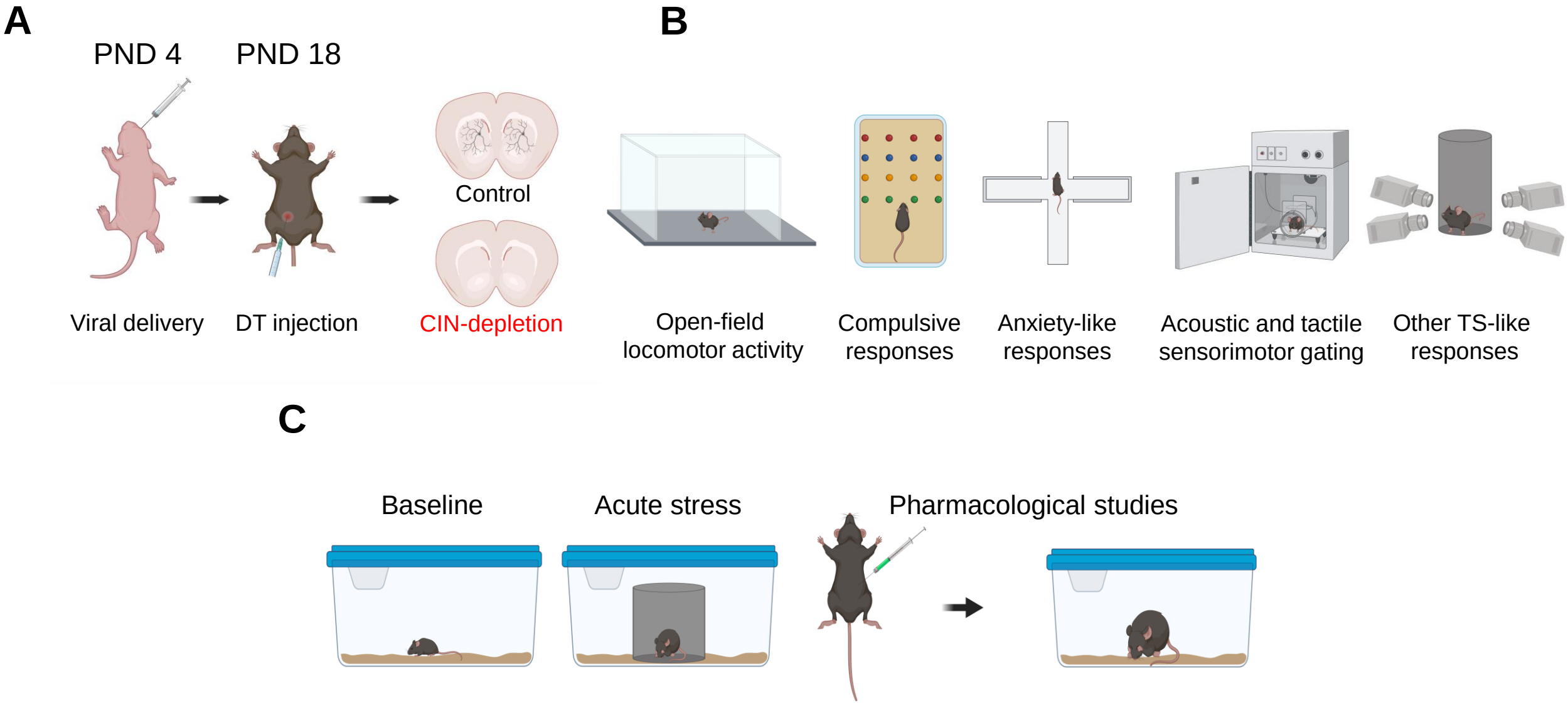
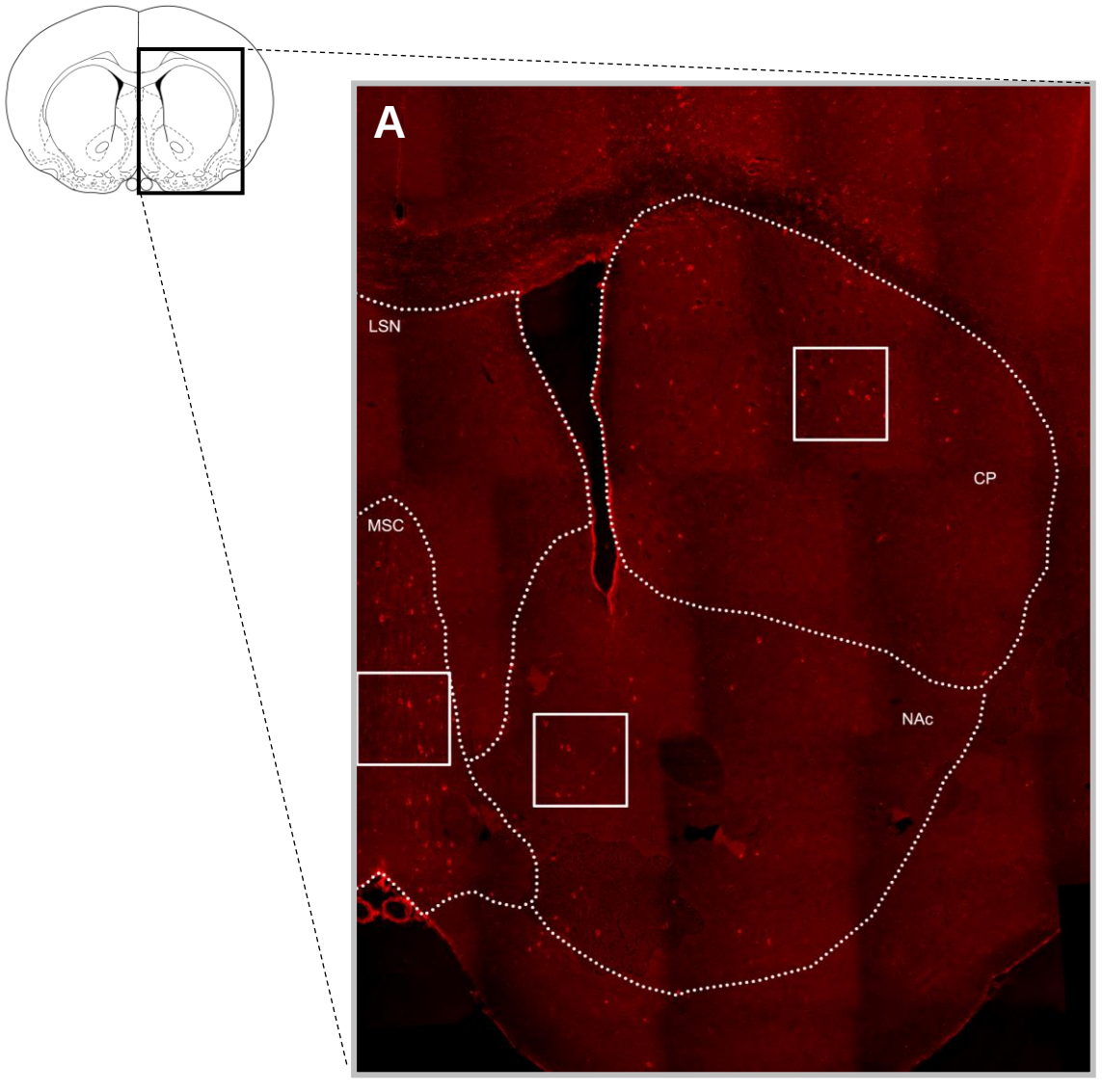
Supplemental Figure 1



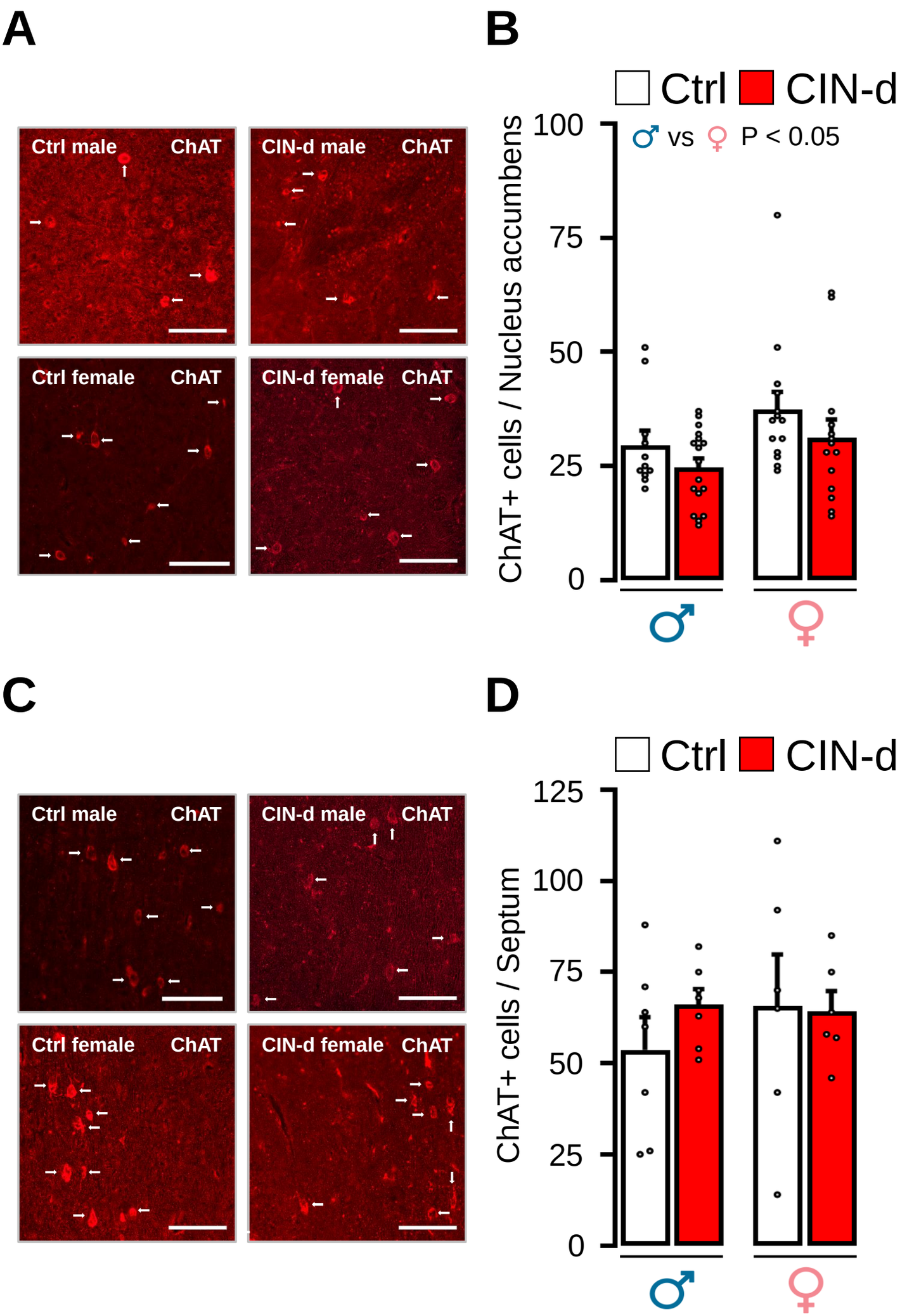
**Supplemental Figure 1. Schematic representation of the procedures used in the present study.** (A) Male and female pups at postnatal day (PND) 4 underwent bilateral stereotaxic infusion of either a viral construct harboring the simian diphtheria toxin (DT) receptor (A06) or its control (C06); mice were left undisturbed in their home cage until PND 18 when they received a single intraperitoneal (IP) injection of DT. (B) Juvenile mice (35-45 day-old) underwent a battery of behavioral tasks aimed at capturing responses relevant to Tourette syndrome (TS) and comorbid disorders. Testing was performed both in baseline conditions and in response to acute stress. (C) Stereotypy- and tic-like behaviors were scored both in freely moving conditions and in response to spatial confinement (SC) stress; the efficacy of anti-stereotypy and anti-tic treatments was evaluated in SC mice. Pharmacological studies included the analysis of different treatments that effectively reduce tic severity in TS-affected individuals and the impact of various neurosteroid-based therapies.

Supplemental Figure 2



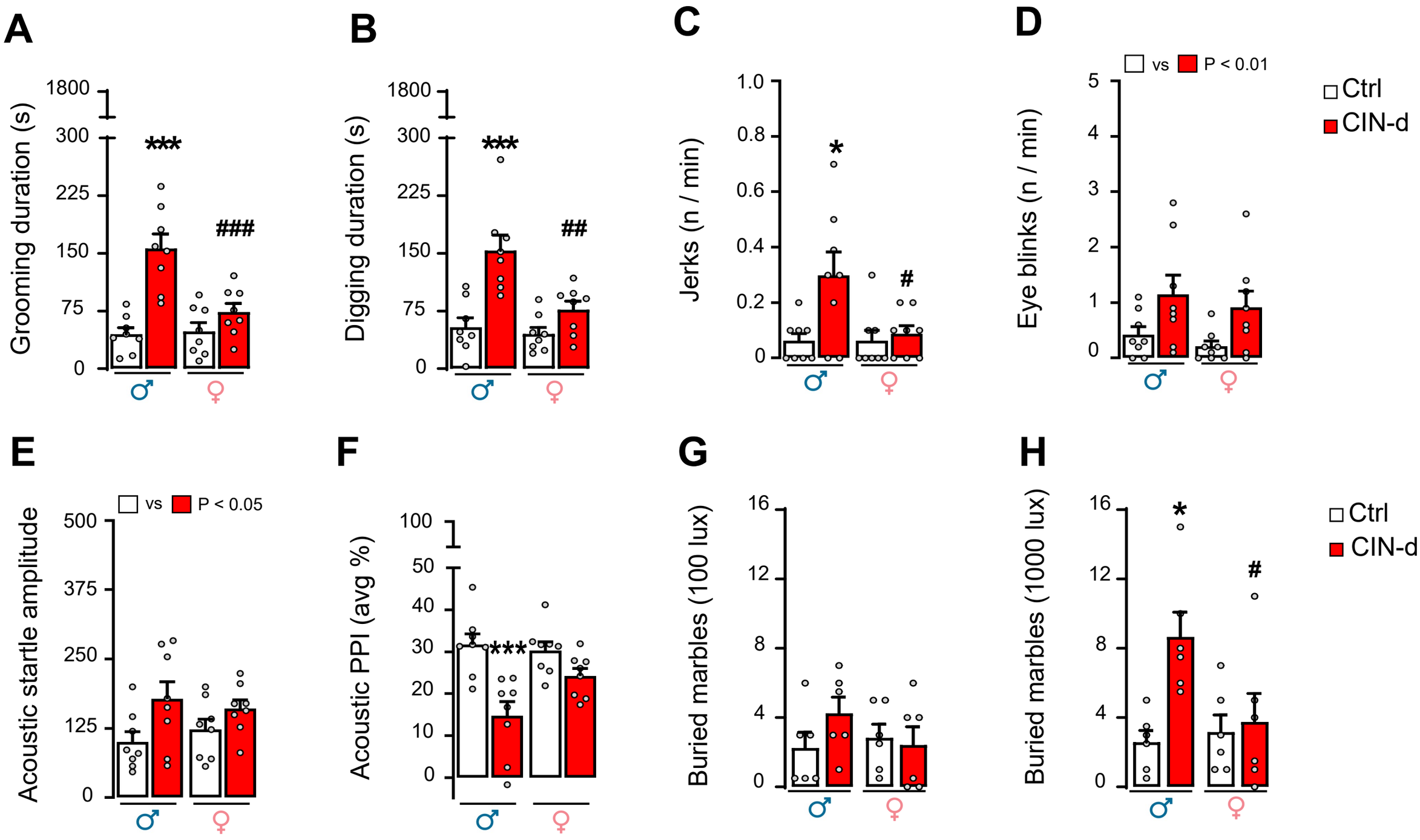
**Supplemental Figure 2.** (A) Representative image showing the regions where choline acetyltransferase- (ChAT) positive cells were counted, marked in a full reconstruction including the dorsolateral striatum (Caudate Putamen; CP), the nucleus accumbens (NAc) and the septum, including the medial septal complex (MSC) and the lateral septal nuclei (LSN). The corresponding coronal section was adapted from The Allen Mouse Brain Atlas (coronal level: bregma 0.70 mm). (B-J) Representative immunostaining of DAPI, ChAT and control merge in CP (B-D), NAc (E-G) and septum (H-J) of C06-infected control animals. Scale bars equal 50 µm.

Supplemental Figure 3



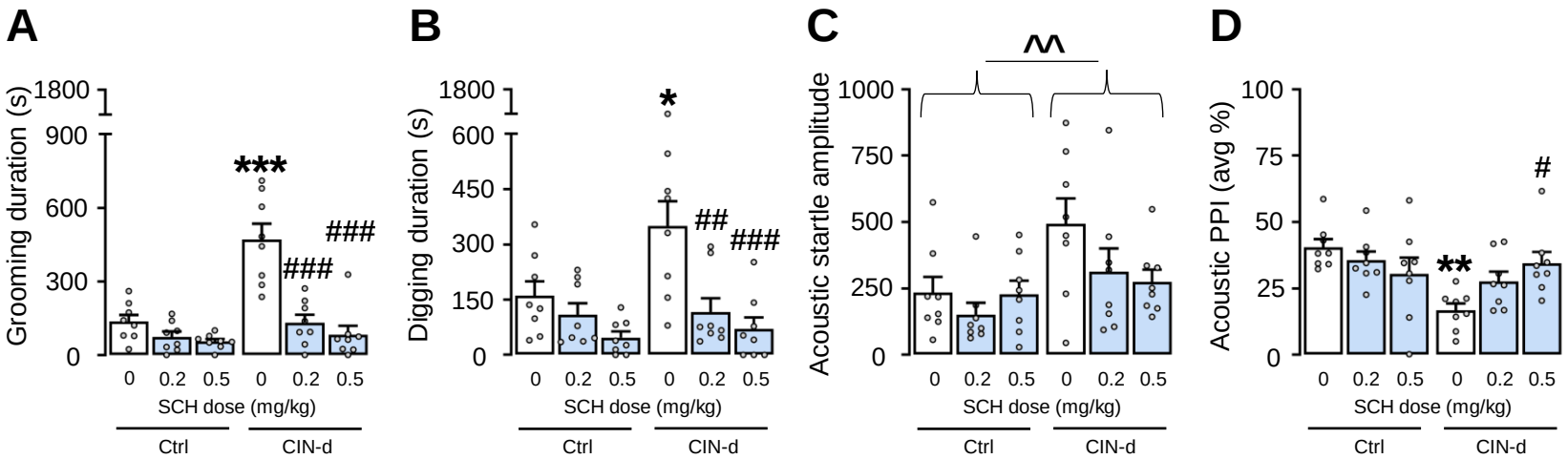
**Supplemental Figure 3. Effects of early-life cholinergic interneuron depletion (CIN-d) in the nucleus accumbens (NAc) and septum of male and female mice.** (A) Density of ChAT-positive interneurons in the NAc of A06-targeted mice, relative to C06-infected controls (Ctrl), after systemic administration of the simian diphtheria toxin (DT). (B) Immunostaining for ChAT-positive cells in the NAc revealed a marginal, yet not significant loss of CINs in A06+DT mice across both sexes. The number of CINs was significantly higher in the NAc of females, irrespective of the experimental group. (C) Density of ChAT-positive interneurons in the septum of CIN-d and Ctrl mice. (D) The number of CINs was not modified in the septum. Data were analyzed by two-way ANOVAs. All data are shown as means ± SEM. For more details, see text and Supplemental Table 1F.

Supplemental Figure 4



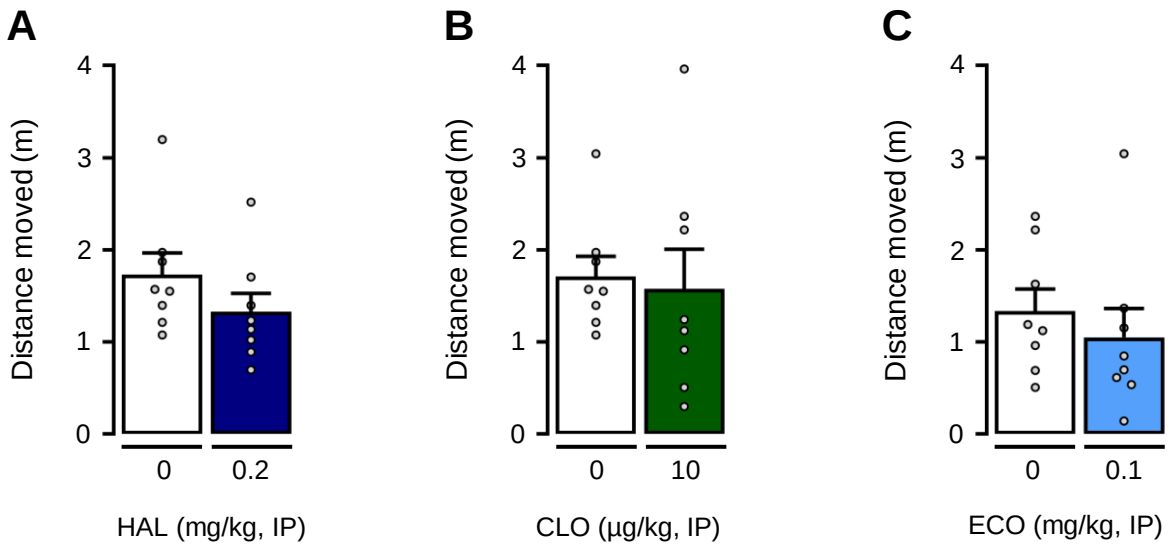
**Supplemental Figure 4. Effects of acute stress on Tourette syndrome- (TS) relevant responses of CIN-depleted (CIN-d) and control (Ctrl) male and female mice, during the light phase.** In mice exposed to spatial confinement (SC) during the light phase (40-min duration), (A) self-grooming and (B) digging responses were significantly higher in male, but not female, CIN-d mice. In a separate experiment, subjecting mice to a 10-min SC session led to (C) a greater frequency of head jerks in CIN-d males, as compared with Ctrl males and CIN-d females. Eye blink frequency was increased in CIN-d mice of both sexes (D). SC (E) increased acoustic startle reflex in CIN-d male and female mice and (F) disrupted acoustic prepulse inhibition (PPI) in CIN-d males, as compared with Ctrl males. Under normal ambient light, CIN-d and Ctrl mice exhibited (G) similar marble-burying behavior. In a distinct group of mice, bright ambient light elicited (H) a significant enhancement in marble-burying behaviors in male, but not female, CIN-d mice. All data were analyzed by two-way ANOVAs. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ for comparisons between CIN-d male vs Ctrl male; $^{\#}$, $P<0.05$; $^{\#\#}$, $P<0.01$ for comparisons between CIN-d females vs CIN-d males. All data are shown as means ± SEM. n=6-8/group. For more details, see text and Supplemental Table 1G.

# Supplemental Figure 5



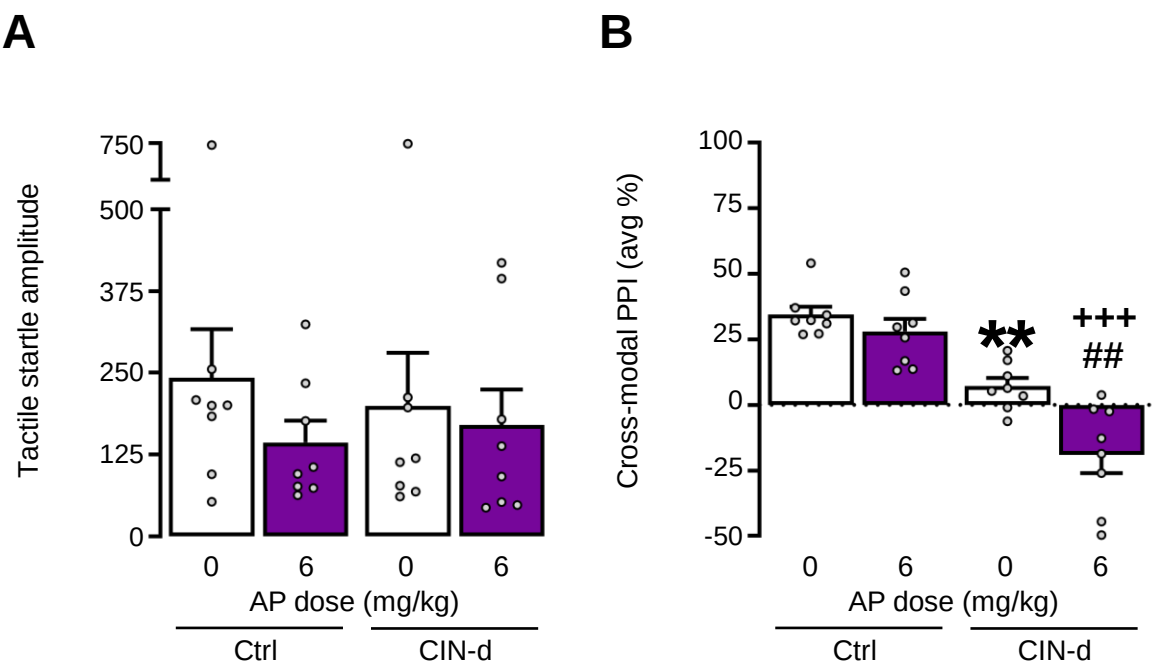
**Supplemental Figure 5. Effects of the prototypical $D_1$ receptor antagonist SCH23390 (SCH; 0.2 and 0.5 mg/kg, IP) on repetitive (perseverative) behaviors, as well as prepulse inhibition (PPI) deficits in CIN-depleted (CIN-d) and control (Ctrl) male mice exposed to spatial confinement (SC).** SCH reduced (A) grooming and (B) digging exacerbated by stress. While CIN-d mice showed increased acoustic startle amplitude (C), this effect was not affected by SCH treatment. Conversely, this drug reversed the PPI deficits induced by SC in CIN-d mice (D). All data were analyzed by two-way ANOVAs. ^^, $P<0.01$; main effect for CIN-d. *, $P<0.01$; **, $P<0.01$; ***, $P<0.001$ for post-hoc comparisons between treatment vehicle (represented by white bars)-treated CIN-d and Ctrl mice. $^{\#}$, $P<0.05$; $^{\#\#}$, $P<0.01$; $^{\#\#\#}$, $P<0.001$ for comparisons between CIN-d mice injected with SCH or vehicle. All data refer to SC-exposed CIN-d and Ctrl male mice and are shown as means ± SEM. Treatment doses are indicated under colored bars. n=8/group. For more details, see text and Supplemental Table 1H.

# Supplemental Figure 6



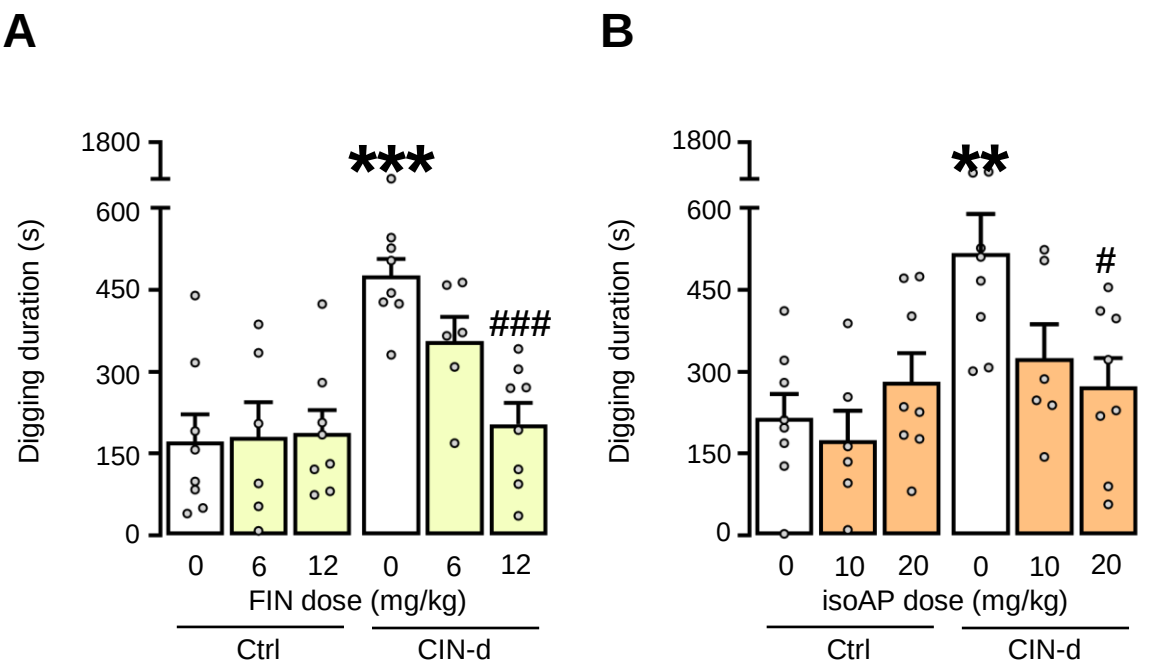
**Supplemental Figure 6. Locomotor effects of (A) haloperidol (HAL), (B) clonidine (CLO), and (C) ecopipam (ECO) in control male mice at doses that reversed the effects of spatial confinement in CIN-depleted male mice.** Data were analyzed by one-way ANOVA and are shown as means ± SEM. Treatment doses are indicated under colored bars. n=8/group. For more details, see text and Supplemental Table 1I.

# Supplemental Figure 7



**Supplemental Figure 7. Allopregnanolone (AP; 6 mg/kg, IP) exacerbated cross-modal prepulse inhibition (PPI) deficits in CIN-depleted (CIN-d), but not control (Ctrl) male mice.** (A) The analysis of tactile startle reflex showed no significant difference between groups. Conversely, this steroid significantly worsened the spontaneous PPI deficits in CIN-d males (B), compared with vehicle-treated and AP-injected Ctrl counterparts. Data were analyzed by two-way ANOVAs. **, $P<0.01$ for post-hoc comparisons between vehicle (represented by white bars)-treated CIN-d and Ctrl mice. ##, $P<0.01$ for post-hoc comparisons between CIN-d mice injected with either AP or its vehicle; +++, $P<0.001$ for post-hoc comparisons between Ctrl and CIN-d mice treated with AP. AP doses are reported under colored bars. All data are shown as means ± SEM. n=8/group. For more details, see text and Supplemental Table 1J.

# Supplemental Figure 8



**Supplemental Figure 8. Opposing allopregnanolone (AP) synthesis and signaling normalized the elevation in digging behavior induced by spatial confinement (SC) stress in CIN-depleted (CIN-d) mice.** SC-induced elevation in total digging duration were dose-dependently countered by (A) the inhibitor of AP synthesis finasteride (FIN; 6 and 12 mg/kg, IP) and (B) the AP antagonist isoAP (10 and 20 mg/kg, IP). All data were analyzed by two-way ANOVAs. **, $P<0.01$; ***, $P<0.001$ for post-hoc comparisons between CIN-d and control (Ctrl) mice treated with vehicle (represented by white bars). $^{\#}$, $P<0.05$; $^{\#\#\#}$, $P<0.001$ for comparisons between CIN-d mice injected with either the treatment or its vehicle. All data refer to SC-exposed CIN-d and Ctrl male mice and are shown as means ± SEM. Treatment doses are indicated under colored bars. n=6-8/ group. For more details, see text and Supplemental Table 1K.