Constitutive deletion of *translin* (*Tsn*) enhances locomotor response to amphetamine: role of developmental effects

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Abstract

The translin/trax microRNA-degrading enzyme mediates activity-induced changes in translation that underlie several long-lasting forms of cellular plasticity. As translin and trax are expressed in dopaminergic and striatal neurons, we investigated whether deletion of *Tsn* blocks amphetamine sensitization, a long-lasting, translation-dependent form of behavioral plasticity.

Although we had hypothesized that constitutive *Tsn* deletion would impair amphetamine sensitization, we found, instead, that it enhances the hyperlocomotion produced by the initial dose of amphetamine. Since these mice display elevated adiposity, which alters pharmacokinetics of many drugs, we measured brain levels of amphetamine in *Tsn* knockout mice and found that these are elevated. As conditional *Tsn* deletion in adulthood does not impact adiposity, we monitored the locomotor response to amphetamine following this manipulation. Acute and sensitized responses to amphetamine are not altered in these mice, indicating that the enhanced amphetamine response displayed by constitutive *Tsn* knockout mice is due to *Tsn* absence during development.

Introduction

Recent studies have focused attention on the role of the microRNA system in the pathophysiology of psychiatric diseases [1-4]. However, our understanding of how microRNA signaling pathways operate normally in the nervous system is quite limited, making it difficult to assess how altered microRNA signaling may contribute to these disorders. To help elucidate the physiological function of microRNA signaling in brain, we are investigating the role of the translin (TN)/trax (TX) microRNA-degrading enzyme, an RNase that targets a small population of microRNAs [5, 6]. We are focusing on this enzyme because expression of TN and TX is enriched in brain, where it is localized preferentially to neurons [7].

Characterization of *translin* (*Tsn*) knockout (KO) mice, which lack the TN/TX complex, has enhanced our understanding of the role of this microRNA-degrading enzyme *in vivo*. These studies have revealed several prominent phenotypes: blockade of several forms of long-lasting synaptic plasticity [8, 9], decreased anxiety [10], protection from vascular stiffening [11], and development of robust adiposity in the context of normal body weight [12]. Furthermore, defining the mechanism mediating these phenotypes suggests that TN/TX operates in the following manner: TN/TX activation by cellular stimulation triggers rapid degradation of a small population of microRNAs. Degradation of these microRNAs reverses silencing of their target mRNAs, allowing *de novo* protein translation to proceed. Thus, the TN/TX complex appears to be well positioned to mediate translation-dependent forms of cellular plasticity.

In pilot studies, we noted that TN and TX are expressed in midbrain dopaminergic (DA) neurons and striatal neurons, among other brain regions. These neuronal populations play key roles in mediating the action of psychostimulants [13-15], including sensitization, a long-term form of behavioral plasticity that is dependent on translation and thought to underlie the reinforcing properties of these drugs [16-18] Furthermore, recent studies have yielded compelling evidence demonstrating that the microRNA system plays a prominent role in mediating behavioral responses to these drugs [19-23]. Accordingly, we hypothesized that the TN/TX RNase mediates this form

of plasticity and proceeded to check the locomotor response to amphetamine in *Tsn* KO mice.

Methods and Materials

Animals

All mice were housed in ventilated racks and maintained on a 14-h/10-h light/dark cycle with access to standard chow (2018SX Teklad Global, Frederick, MD) and water *ad libitum*. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Johns Hopkins Animal Care and Use Committee.

The *Tsn* KO mice used in these studies are from the line generated in Dr. Kasai's laboratory [24] and provided by the JCRB Laboratory Animal Resource Bank of the National Institute of Biomedical Innovation (translin KO: Nbio055).

Mice with floxed alleles of *Tsn* or *Tsnax* were generated on a C57BL/6J background using CRISPR/Cas9 technology as described in Fu et al.[25]. The following Cre driver or reporter lines were obtained from JAX labs or MMRRC (Drd1-Cre (EY217); Ai9 #007909; DAT-Cre #006660; Adora2a-Cre #036158; Ubiquitin C-CreERT2 #007001). Genotyping was performed on tail snips by Transnetyx, Inc. (Cordova, TN).

Immunostaining

Three to four month-old male mice were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.) for transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate-buffered saline pH 7.4 (PBS). Their brains were dissected and stored in 4% paraformaldehyde for at least 24 h, and then washed with PBS and transferred to 30% sucrose in PBS. After the brains totally sank, 30 μm sections were cut with a freezing microtome.

For TN immunostaining, sections underwent heat-induced antigen retrieval by incubating them in 10mM NaCitrate for 30 min at a temperature of 70°C. After the sections cooled down to room temperature, they were washed with PBS three times,

blocked with 3% BSA and 0.1% Triton X-100 in PBS for 1h, and then incubated with primary antibodies against TN [26] and tyrosine hydroxylase (TH; MilliporeSigma, Burlington, MA) overnight at 4°C. After three washes, the sections were incubated with Alexa Fluor 488 goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA) and Alexa Fluor 594 goat anti-mouse (Jackson ImmunoResearch Laboratories) in 1.5% Normal Goat Serum (Vector Laboratories, Burlingame, CA) in PBS for 1h at room temperature. After three additional washes with PBS, the sections were mounted on slides, and coverslipped.

For TX immunostaining, sections were washed with PBS three times and blocked with 3% BSA and 0.1% Triton X-100 in PBS for 1h, followed by incubation with primary antibodies against trax [27] overnight at 4°C. After three washes, the sections were incubated with biotinylated goat anti-rabbit (Vector Laboratories) in PBS for 1h at room temperature. After three washes with PBS, the sections were incubated with AB solution (ABC kit, Vector Laboratories) for 90 min. Sections were washed with TBS three times, and incubated with tyramide (TSA kit, PerkinElmer) for 10 min. After one wash with TBS and three washes with PBS, sections were mounted on slides, and coverslipped.

All images were obtained with a Zeiss LSM 800 confocal microscope.

Western Blotting

Forebrain, nucleus accumbens and dorsal striatum tissue samples were homogenized in RIPA buffer (Cell Signaling Technology, Danvers, MA) containing a cocktail of protease and phosphatase inhibitors (MilliporeSigma). The concentration of total protein was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Equal amounts of total protein were separated electrophoretically on an SDS-PAGE gel, transferred to a PVDF membrane (Bio-Rad, Hercules, CA) and immunoblotted with antibodies: anti-DAT (MilliporeSigma), anti-D2R (MilliporeSigma), anti-TH (MilliporeSigma), and anti-tubulin (Cell Signaling Technology), anti-Translin and anti-Trax. Blots were developed with the ECL system (Thermo Fisher Scientific). Band intensities were quantified from digital images by densitometry using ImageJ.

Open-Field Locomotor Activity

Changes in locomotor activity in response to amphetamine were assessed in an open field arena. 3 to 4-month old male mice were first allowed to habituate to the arena (50 × 50 cm) for 30 minutes. Following habituation to the context, mice were given an i.p. injection of saline and then placed back in the center of the open field and allowed to explore the arena for another 30 minutes. At this point, mice were given an intraperitoneal (i.p.) injection of D-amphetamine hemisulfate (2.5mg/kg; MilliporeSigma), placed back in the arena and then monitored for an additional 40-50min.

Tissue Dopamine Levels

Mice were sacrificed, their brains removed and placed on ice. Dorsal striatum and nucleus accumbens were dissected and weighed, then flash frozen and stored at -80°C. Tissue samples were ultrasonicated in 0.1 M perchloric acid, and stored at -80°C until extraction. Upon thawing, the samples were homogenized in 0.1 M perchloric acid and centrifuged at 25,000 g for 12 minutes. Dopamine levels were measured by HPLC with electrochemical detection. The analytical column was a SunFire C18 5 lm (4.6 · 150.0 mm) from Waters (Milford, MA, USA). The mobile phase was 0.01 M sodium dihydrogen phosphate, 0.01 M citric acid, 1.2 mM sodium EDTA, 1.2 mM sodium 1-heptane sulfonic acid, 10% methanol, pH 3.5; the flow rate was 1.0 mL/min and the column temperature was 34°C. The installation consisted of a Waters 717 Plus automated injection system, a Waters 1525 Binary pump, and an ESA Coulochem III detector (Dionex, Sunnyvale, CA, USA). Waters Breeze system was used for analysis.

Body Composition

Body composition was determined by using a nuclear magnetic resonance scanner (EchoMRI-100, Houston, TX).

Plasma and Brain Amphetamine Levels

Amphetamine levels in plasma and brain samples were measured using high-performance liquid chromatography with tandem mass spectrometry (LC/MS-MS). Methanol, containing 0.5µM losartan as an internal standard, was used to extract amphetamine from plasma and brain. Standards were prepared by spiking amphetamine in naïve mouse tissue from 0.01 – 100 µmol/g in a half log dilution series. Plasma (20µL) and brain samples and corresponding standards were placed in low retention microfuge

tubes with 5 µL/mg of extraction solution for protein precipitation. Samples were vortex mixed, followed by centrifugation at 16,000 x g for 5 minutes at 4°C. The supernatants (80 µL) were transferred to a 96 well plate and 2 µL was injected for analysis. Samples were analyzed on an UltiMate 3000 UHPLC coupled to Q Exactive Focus orbitrap mass spectrometer (Thermo Fisher Scientific). Samples were separated on an Agilent Eclipse Plus C18 RRHD (1.8 µm) 2.1 × 100 mm column. The mobile phase consisted of water + 0.1% formic acid (A), and acetonitrile + 0.1% formic acid (B). Separation was achieved at a flow rate of 0.4 mL/min using a gradient run, from 97.5/2.5 (A/B) to 5/95 (A/B) over 1.5 minutes, maintaining at 5/95 (A/B) for 1 minute, and then re-equilibrating for 1 minute. Samples were introduced to the source through heated ion spray with the capillary temperature setting at 350°C and spray voltage of 3.5 kV. Nitrogen was used as the sheath and auxiliary gas with the settings of 30 and 5 respectively. Quantification was performed in product-reaction monitoring (PRM) mode with collision energy setting of 10. Transitions 136.1121 m/z to 119.0876 m/z and 91.0563 m/z (amphetamine) and 423.1695 to 377.1522 m/z and 207.0915 m/z (losartan) were monitored. Data were acquired and quantified with Xcalibur software.

Tamoxifen Treatment

Tamoxifen (10mg/ml in corn oil; MilliporeSigma) was administrated daily (100 mg/kg, i.p.) for 6 consecutive days to Tsn $^{fl/fl}$ and UBC-Cre $^{+/-}$/Tsn $^{fl/fl}$ mice. Three weeks later, these mice were used to monitor the locomotor response to amphetamine administration.

Statistical Analysis

Data are presented as mean ± SEM. Statistical significance was evaluated using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). Student's t-test was used to compare groups in single variable experiments. Repeated measures (RM) two-way ANOVA was used to analyze multiple variable experiments. Pairwise comparisons were made using Bonferroni's post-hoc test. Differences were considered significant at $p<0.05$.

Results

TN and TX are expressed in midbrain DA and striatal neurons

In prior immunostaining studies characterizing the expression of TN and TX in hippocampus and cortex [7], we noticed prominent neuronal staining in striatum and several brain stem areas. To check if TN and TX are expressed in midbrain DA neurons, we performed double immunostaining for TH and either TN or TX. These studies demonstrate clear co-expression of TN and TX in DA neurons (Figure 1A). TX staining in striatum is present in both D1R-positive and D2R-positive neurons, as we found a high degree of co-expression of TX in neurons stained for DARPP-32, which is expressed in both populations [19]. Furthermore, we conducted TX staining in tdTomato reporter mice (Ai9) that express this marker selectively in D1R-positive neurons, under the control of Drd1-Cre. We found that TX is expressed in both D1R-positive neurons, which are labeled with tdTomato, as well as in presumptive D2R-positive neurons, which are not (Figure 1B).

Tsn KO mice display increased locomotor response to amphetamine

To investigate the role of the microRNA system in regulating behavioral responses to amphetamine, we compared its ability to stimulate open field locomotor activity in *Tsn* KO mice and WT littermates (Figure 2A). Although we had hypothesized that *Tsn* deletion might impact responses elicited by repeated amphetamine administration, such as locomotor sensitization, which are mediated by changes in protein translation [17], we found, unexpectedly, that the locomotor response to the first injection of amphetamine is markedly enhanced in *Tsn* KO mice. In light of this observation, we checked if *Tsn* deletion impacts the midbrain DA pathway, which plays a major role in mediating the behavioral effects of amphetamine [14, 15].

Qualitatively, staining of midbrain and striatal sections from WT and *Tsn* KO mice for tyrosine hydroxylase (TH), showed a comparable pattern of expression of this enzyme in dopamine neuronal cell bodies and striatal projections between the two genotypes (Figure 2B). To obtain a quantitative assessment of the status of dopamine neurons, we measured tissue dopamine levels in nucleus accumbens (NAc) and dorsal striatum (STR) by HPLC (Figure 2C), as well as protein levels of TH (Figure 2D), dopamine transporter (DAT) and D2 dopamine receptor (D2R) by immunoblotting (Figure 2E), in both the NAc and STR. We did not detect any differences between samples obtained from *Tsn* KO mice and WT littermates in these assays.

Increased brain levels of amphetamine in Tsn KO mice: correlation with increased adiposity As *Tsn* KO mice display robust adiposity and adiposity impacts the pharmacokinetics of many drugs [28-30], we hypothesized that their enhanced locomotor response to amphetamine might be due to increased brain amphetamine levels. Since the amphetamine dose (2.5 mg/kg) used in monitoring locomotor response is in the mid-portion of the ascending part of the dose-response curve [31], increased levels of amphetamine in *Tsn* KO mice could account for their enhanced response in this assay. Consistent with this scenario, we noted a positive correlation between locomotor response to amphetamine and adiposity (%fat mass) that is pronounced in WT mice (Figure 3A), but also present when including both WT and *Tsn* KO mice. To test this directly, we collected both plasma and brain samples at 30 minutes following administration of amphetamine (2.5 mg/kg, i.p.). We found that brain levels of amphetamine are increased in *Tsn* KO mice, with a correlation between adiposity and amphetamine levels in either brain or plasma (Figure 3B and 3C). Thus, these findings: 1) indicate that the increased locomotor response to amphetamine in *Tsn* KO mice reflects increased brain levels of this drug, and 2) suggest that the alteration in amphetamine pharmacokinetics is related to the elevated adiposity, or other correlated metabolic abnormalities, displayed by *Tsn* KO mice.

Since *Tsn* KO mice exhibit a prominent increase in adiposity (~2-3 fold) without an increase in total body weight [12], both WT and *Tsn* KO mice received comparable amounts (2.5 mg/kg of total body weight) of amphetamine in experiments monitoring its effects on locomotor activity. On the other hand, *Tsn* KO mice received a higher dose with respect to their reduced lean body weight. As lipophilic compounds, such as amphetamine, are preferentially distributed to tissues with high blood flow, such as brain [32], it is conceivable that amphetamine would be preferentially delivered to brain at the expense of adipose tissue, which has a very low perfusion rate. These findings suggest that calculating the amount of amphetamine administered to each mouse based on their lean body mass, rather than total body weight, should eliminate the disparity in brain amphetamine levels between WT and *Tsn* KO mice. To test this inference, we administered a dose of 3.5 mg/kg amphetamine, ip, based on lean body weight, and collected plasma and brain samples 30 minutes later. As expected, this approach produced similar levels of amphetamine in plasma or brain of WT and *Tsn* KO mice

(Figure 3D).

Global, conditional deletion of Tsn in adulthood does not alter locomotor response to single or repeated administration of amphetamine

In recent studies, we have found that global, conditional deletion of *Tsn* in adulthood, using tamoxifen-induced activation of the UBC-CreERT2 allele, does not produce increased adiposity [25]. Therefore, we used this manipulation to examine the effect of *Tsn* deletion on the locomotor response to amphetamine in the absence of increased adiposity. To control for possible effects of the floxed *Tsn* allele and tamoxifen treatment, we tested *Tsn* $^{fl/fl}$ mice that had also received tamoxifen in parallel with *Tsn* $^{fl/fl}$ mice that are hemizygous for the UBC-CreERT2 allele. We did not find a significant difference between the locomotor responses of these groups of mice to a single dose of amphetamine (Figure 4A), indicating that the increased response to amphetamine displayed by constitutive *Tsn* KO mice is due to developmental effects of constitutive *Tsn* deletion, such as elevated adiposity. Of note, the baseline level of locomotor activity displayed by UBC-Cre X *Tsn* $^{fl/fl}$ mice is reduced compared to *Tsn* $^{fl/fl}$ mice (Figure 4C).

Recent studies have demonstrated that the TN/TX microRNA-degrading enzyme mediates long-term changes in synaptic plasticity that are dependent on *de novo* protein synthesis [33]. Therefore, we also wanted to check whether global, conditional deletion of *Tsn* during adulthood might impair sensitization of the locomotor response elicited by repeated administration of amphetamine, a form of drug-induced plasticity that is also dependent on protein synthesis [17]. To this end, we monitored the locomotor response of Tsn $^{fl/fl}$ and UBC-Cre $^{+/-}$/Tsn $^{fl/fl}$, that had been pre-treated with tamoxifen, to two more challenges with the same dose of AMPH (2.5 mg/kg, i.p.) administered at weekly intervals. We found that both groups of mice display comparable increases in locomotor response to amphetamine following these additional treatments (Figure 4). In addition, the difference in baseline locomotor activity persisted in these subsequent open field trials.

Selective, constitutive deletion of Tsn from DA neurons or Tsnax from D2R-positive striatal neurons does not alter amphetamine response or adiposity

As midbrain DA neurons are involved in regulating metabolic homeostasis [34-36] and mediate the locomotor response to amphetamine, we checked whether selective constitutive deletion of *Tsn* might mimic the increased adiposity and heightened response to amphetamine produced by global, constitutive deletion of *Tsn*. To assess this possibility, we tested mice that are homozygous for the floxed *Tsn* allele, *Tsn* $^{fl/fl}$, and hemizygous for the DAT-Cre allele [37]. Double immunostaining for TN and TH demonstrated complete loss of TN staining in midbrain DA neurons (Figure 5A). Furthermore, as found in *Tsn* KO mice [38], conditional deletion of *Tsn* from DA neurons also induces loss of TX protein.

First, we checked the effect of selective deletion of *Tsn* from DA neurons on body weight and composition and found that they are unchanged compared to Tsn $^{fl/fl}$ mice (Figure S1). We then proceeded to evaluate the impact of selective deletion of *Tsn* from DA neurons on the locomotor response to amphetamine. Since these mice are both hemizygous for the DAT-Cre allele and homozygous for the *Tsn* floxed allele, we also tested control mice that have each of these genotypic changes separately. Comparison of the effect of amphetamine on locomotor activity in these three groups indicated that conditional deletion of *Tsn* from DA neurons does not increase the locomotor response to amphetamine above that displayed by mice that are hemizygous for the DAT-Cre allele (Figure 5B). However, these results need to be interpreted cautiously, since, consistent with the recent report by Chohan et al. [39], control mice that are hemizygous for the DAT-Cre allele exhibit a reduced response to amphetamine compared to that of the other control group, mice that are homozygous for the *Tsn* floxed allele.

Previous studies have demonstrated that deletion of D2 receptors from D2R-positive striatal neurons blocks the locomotor response to methamphetamine [40, 41]. Furthermore, TX binds to the C-terminal tail of the A2a adenosine receptor, which is selectively expressed in D2R-positive striatal neurons [27]. Accordingly, we used A2a adenosine receptor-Cre mice (Adora2a-Cre KG139) to check whether conditional deletion of *Tsnax*, the gene encoding TX, from D2R-positive striatal neurons affects the locomotor response to amphetamine. We confirmed that these mice lack TX staining in

about half of striatal neurons, as expected (Figure 5C). Furthermore, we checked that conditional deletion of Tsnax from D2R neurons does not affect body weight or composition compared to Tsnax $^{fl/fl}$ mice (Figure S1). The locomotor response to amphetamine in these mice was identical to that observed in Tsnax $^{fl/fl}$ mice demonstrating that the increased response to amphetamine observed in constitutive Tsn KO mice is not mediated by loss of the TN/TX complex from D2R-positive striatal neurons (Figure 5D).

Discussion

Recent studies have provided compelling evidence that the TN/TX microRNA-degrading enzyme plays a key role in regulating several long-lasting forms of hippocampal synaptic plasticity [5, 8]. These findings support the view that activation of the TN/TX RNase promotes rapid degradation of selected microRNAs which, thereby, triggers *de novo* translation of transcripts that had been subjected to miRNA-mediated silencing. Since our immunohistochemical studies revealed that TN and TX are expressed in DA and striatal neurons, we hypothesized that the TN/TX RNase might regulate activity-induced changes in translation that underlie plasticity in these circuits. Amphetamine sensitization is a well-established long-term plasticity paradigm that is dependent on *de novo* translation [17] and has been implicated in mediating the powerful reinforcing properties of this drug [16, 18]. Therefore, we set out to assess whether *Tsn* deletion impairs this classic, drug-induced plasticity paradigm. We found that the initial dose of amphetamine, itself, elicits a stronger locomotor response in *Tsn* KO mice than WT littermates. Conceivably, this heightened response might reflect occlusion, i.e. that constitutive *Tsn* deletion might elicit a sensitized response to amphetamine even without prior exposure to amphetamine. However, prior to considering this explanation, it was important to rule out the possibility that the heightened response to amphetamine might reflect altered pharmacokinetics of amphetamine in *Tsn* KO mice. Investigating this potential mechanism seemed particularly warranted since these mice display robust adiposity, a feature that has been associated with altered pharmacokinetics of many drugs [28-30]. This line of research demonstrated that *Tsn* KO mice display higher brain levels of amphetamine providing a clear explanation for their heightened locomotor response to amphetamine.

It is likely that the elevated brain levels of amphetamine observed in *Tsn* KO mice are due to their robust adiposity in the context of normal body weight. As a result, they received a higher dose per lean body mass, which could yield higher brain levels. Consistent with this view, we confirmed that calculating the dose of amphetamine based on lean body mass corrects the disparity in brain amphetamine levels. However, we cannot rule out the alternative possibility that the higher brain levels displayed by *Tsn* KO mice are due to other changes caused by constitutive deletion of this gene. From this point of view, calculating the dose based on lean body mass, which preferentially reduces the total amount of amphetamine administered to Tsn KO mice, would produce a commensurate reduction in brain amphetamine levels. Although we favor the view that elevated brain amphetamine levels in *Tsn* KO mice reflect altered distribution of this drug due to their robust adiposity, it is conceivable that other effects of constitutive Tsn deletion mediate this effect. However, we can conclude with confidence that this effect is due to developmental effects of *Tsn* deletion, since global deletion of *Tsn* in adulthood does not alter the locomotor response to amphetamine.

As global deletion of *Tsn* in adulthood does not elicit the robust increase in adiposity found in constitutive *Tsn* KO mice, this manipulation enabled us to confirm that *Tsn* deletion, in the absence of adiposity, does not affect the locomotor response to a single dose of amphetamine. Furthermore, we found that amphetamine sensitization is intact in these mice. Given these negative findings, it will be important in future studies to assess whether the TN/TX RNase may mediate other forms of plasticity in DA or striatal neurons. Of note, a detailed study of multiple long-term forms of hippocampal synaptic plasticity has revealed that *Tsn* deletion blocks some of them, such as synaptic tagging and “spaced” LTP, but not others, such as mGluR-induced LTD and “massed” LTP [33]. DA and nucleus accumbens mount a plasticity response to chronic stress that regulates susceptibility to depression-like states [41, 42]. As single nucleotide polymorphisms in the *Tsnax* gene are associated with depression [42, 43], future studies aimed at investigating whether the TN/TX RNase mediates this form of plasticity are warranted.

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Author Contributions

XF contributed to the design of experiments, the acquisition, analysis and interpretation of the data and writing of the manuscript. APS helped design experiments, collect and analyze data, and edit the manuscript. TMM, BL, JA, DF, MN, JLC and RR contributed to data acquisition. AS revised the manuscript. GT contributed to the design of experiments. JMB was responsible for overseeing all parts of the study and directly contributed to the interpretation of the results and writing of the manuscript. All co-authors provided final approval of the manuscript to be published.

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Figure Legends

Figure 1: Localization of TN and TX to DA neurons and striatal neurons

(A) Double immunostaining of midbrain sections for TH (red) and TN or TX (green) shows TN or TX expression located in the VTA or substantia nigra. (B) Immunostaining in striatal medium spiny neurons shows prominent nuclear TX staining (green) in DARPP-32 positive neurons (red) (top row). TX staining of sections from mice expressing tdTomato in D1R+ neurons demonstrates that TX is expressed in both D1R-positive neurons as well as in D1R-negative neurons (bottom row). Scale bar, 30 μm.

Figure 2: *Tsn* KO mice display increased locomotor response to amphetamine (AMPH) but normal levels of tissue DA, TH, DAT and D2R

(A) Amphetamine-induced (2.5 mg/kg, i.p.) locomotor activity is increased in KO mice. Locomotor activity was monitored every 5 minutes and arrowheads indicate time of injections. n = 8/group. (B) Immunostaining shows comparable TH staining of DA neurons (left panel) and striatal terminals (right panel) in WT and translin KO mice. Scale bar, 500 μm. (C) Tissue DA levels in the nucleus accumbens (NAc) and striatum (STR) are similar in WT and translin KO mice. n = 5-6/group. (D and E) Immunoblotting shows normal TH, DAT, D2R expression levels in NAc and STR in translin KO mice. Image J was used for quantification. n = 6/group. Data are expressed as mean ± SEM. Statistical significance was assessed by Student's t-test, or two-way ANOVA with repeated measures followed by Bonferroni's post-hoc analysis. **$p<0.01$, and ***$p<0.001$.

Figure 3: Correlation of fat mass with AMPH-induced locomotor activity and AMPH levels in plasma and brain

(A) The total locomotor activity induced by AMPH (2.5 mg/kg i.p.) was correlated with % fat mass ($r = 0.5571$, $P = 0.025$). n= 8/group. (B) Plasma AMPH levels (in nmol/mL)

(left), and correlation with % fat mass (right; r =0.744, P =0.0055). 2.5mg/kg AMPH was injected i.p. based on body weight. n = 6/group. (C) Brain AMPH levels (in nmol/g) (left), and correlation with % fat mass (right; r =0.919, p<0.0001). 2.5mg/kg AMPH was injected i.p. based on body weight. n = 6/group. (D) Plasma and brain AMPH levels when 3.5 mg/kg AMPH was injected i.p. based on lean mass. Data are expressed as Mean ± SEM. Statistical significance was assessed by Student's t-test. **p<0.01.

Figure 4: Global, conditional deletion of *Tsn* in adulthood does not affect locomotor response to AMPH

(A) UBC-Cre X Tsn $^{fl/fl}$ and Tsn $^{fl/fl}$ mice display comparable locomotor responses to three challenges with AMPH (2.5 mg/kg i.p.), given at weekly intervals. UBC-Cre X Tsn$^{fl/fl}$ finished tamoxifen treatment three weeks prior to amphetamine testing to induce global deletion of Tsn. Tsn $^{fl/fl}$ mice that also underwent tamoxifen treatment were used as a control group. (B) The locomotor sensitization to AMPH (2.5 mg/kg i.p.) is unaffected in UBC-CreERT2 xTsn$^{fl/fl}$ mice. (C) Baseline locomotor activity is reduced in UBC-Cre X Tsn$^{fl/fl}$ mice compared to Tsn$^{fl/fl}$ mice. (D) Repeated amphetamine administration increased total locomotor activity to a similar extent in both UBC-CreERT2 xTsn$^{fl/fl}$ mice Tsn$^{fl/fl}$ mice. n=8/group. Data are expressed as mean ± SEM. Statistical significance was assessed by two-way ANOVA with repeated measure followed by Bonferroni's post-hoc analysis. No significant effect of genotype was detected, *p<0.05. **p<0.01. ***p<0.001.

Figure 5: Conditional deletion of *Tsn* from DA neurons or Tsnax from D2R-positive neurons

(A) Double immunostaining of midbrain sections for TH (red) and TN or TX (green) shows that TN or TX expression was totally absent in DAT-Cre xTsn$^{fl/fl}$ mice. Scale bar, 50 µm. (B) AMPH-induced (2.5 mg/kg i.p.) locomotor activity in DAT-Cre xTsn$^{fl/fl}$ mice was not significantly different from the response of mice that are hemizygous for the DAT-Cre allele. n =8/group. (C) Immunostaining of striatal sections for TX (green) shows TX expression is absent from about half of cells in A2a-Cre xTsnax$^{fl/fl}$ mice. Red arrows point to striatal neurons that lack TX staining. Scale bar, 20 µm. (D) AMPH-induced (2.5 mg/kg i.p.) locomotor activity is unaffected in A2a-Cre xTsnax$^{fl/fl}$ mice compared to Tsnax$^{fl/fl}$ mice. n =8/group. Data are expressed as mean ± SEM. Different letters indicate

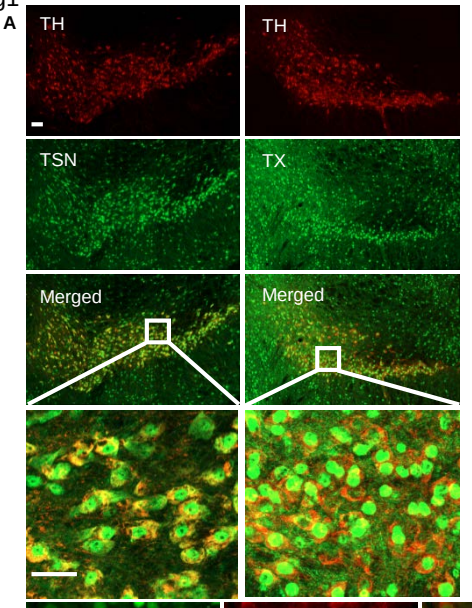
statistically significant differences ($p < 0.05$) by two-way ANOVA with repeated measures followed by Bonferroni's post-hoc test.

Supplementary Figures

Figure S1: Conditional deletion of *Tsn* or *Tsnax* from DA or D2R-positive neurons does not affect adiposity

(A) Body weight and fat mass were normal in DAT-Cre x$Tsn^{fl/fl}$ mice. n=7/group.
(B)A2a-Cre x $Tsnax^{fl/fl}$ mice showed the same body weight and fat mass as $Tsnax^{fl/fl}$ mice. n=8/group. Data are expressed as Mean ± SEM. Student's t-test was used for statistical analysis.

Fig1



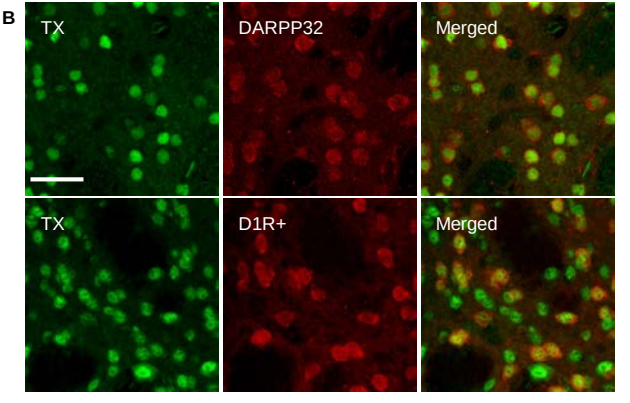
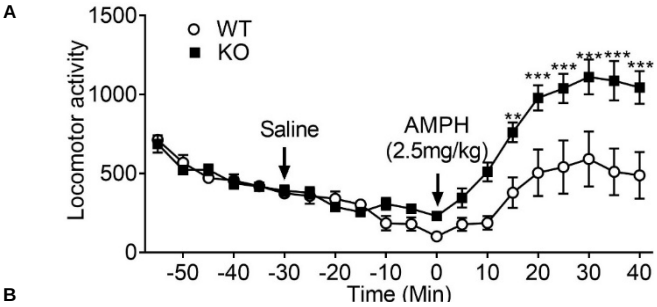
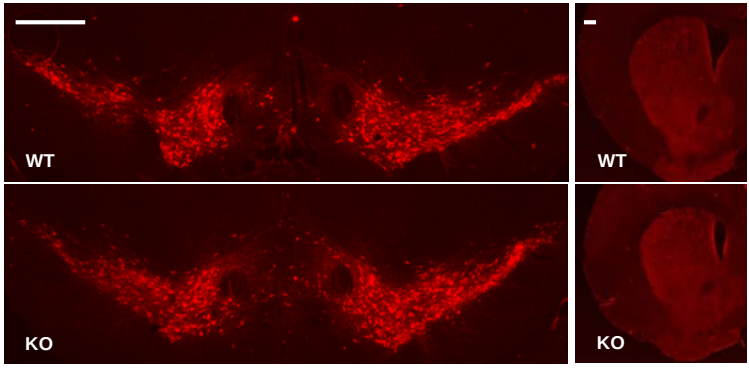
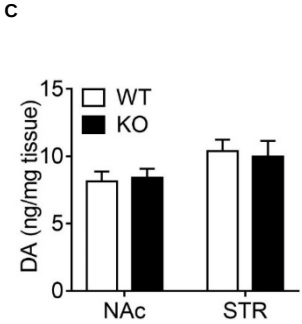
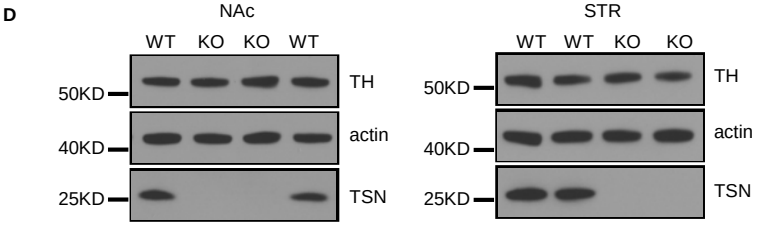


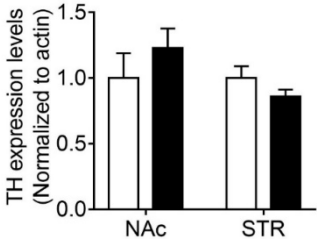
Fig2

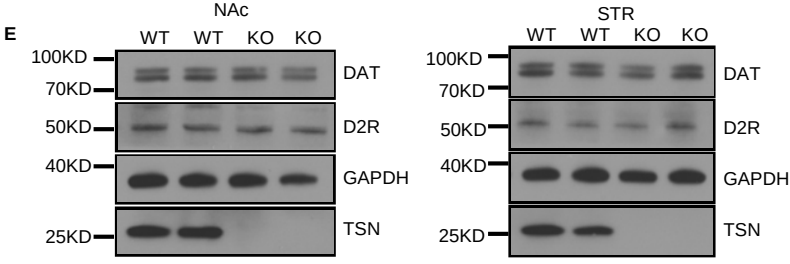

A
WT
KO
Locomotor activity
1500
1000
500
0
Saline
AMPH
(2.5mg/kg)
-50 -40 -30 -20 -10 0 10 20 30 40
Time (Min)

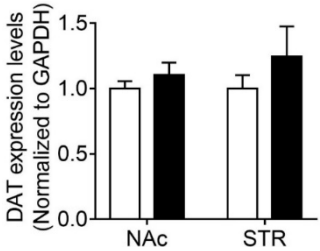

B
WT
KO
WT
KO


C
WT
KO
DA (ng/mg tissue)
15
10
5
0
NAc
STR


D
NAc
WT KO KO WT
50KD
40KD
25KD
TH
actin
TSN
STR
WT WT KO KO
50KD
40KD
25KD
TH
actin
TSN


TH expression levels
(Normalized to actin)
1.5
1.0
0.5
0.0
NAc
STR


E
NAc
WT WT KO KO
100KD
70KD
50KD
40KD
25KD
DAT
D2R
GAPDH
TSN
STR
WT WT KO KO
100KD
70KD
50KD
40KD
25KD
DAT
D2R
GAPDH
TSN


DAT expression levels
(Normalized to GAPDH)
1.5
1.0
0.5
0.0
NAc
STR

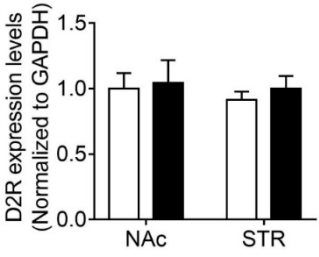

D2R expression levels
(Normalized to GAPDH)
1.5
1.0
0.5
0.0
NAc
STR

Fig3

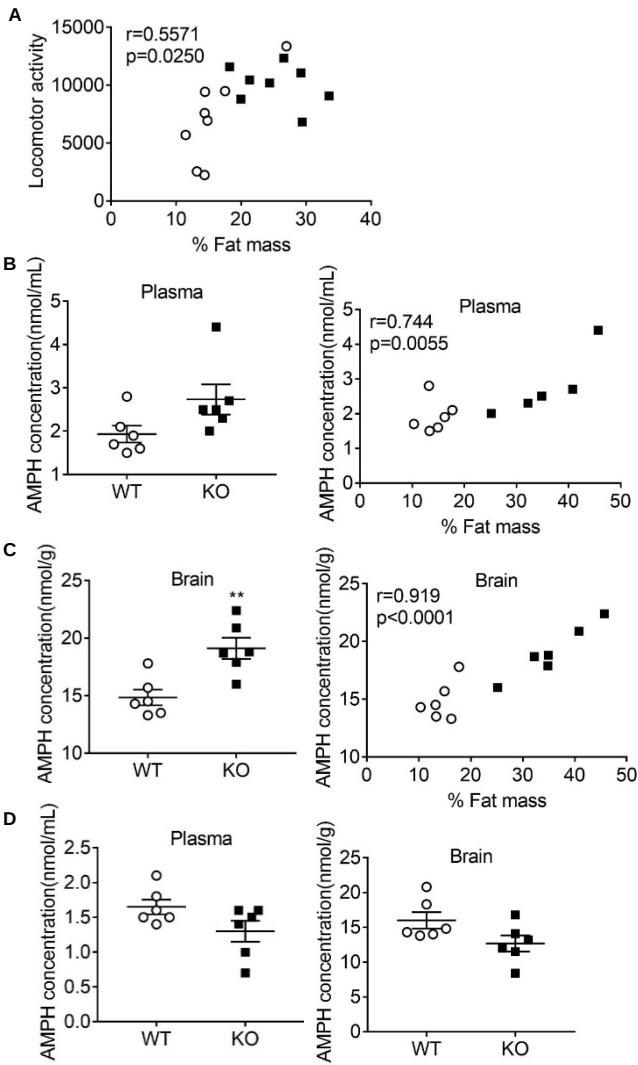
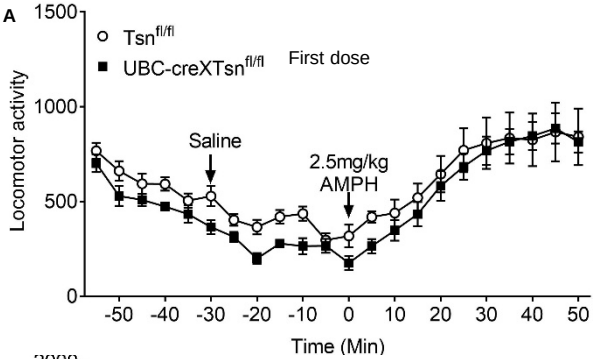
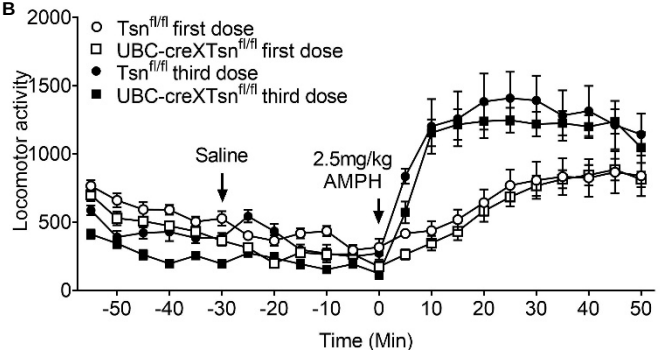
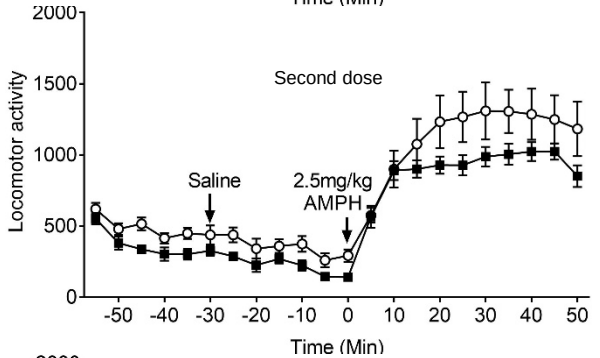
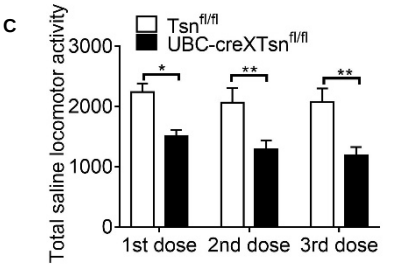

A
r=0.5571
p=0.0250
Locomotor activity
% Fat mass
B
Plasma
AMPH concentration(nmol/mL)
WT
KO
Plasma
r=0.744
p=0.0055
AMPH concentration(nmol/mL)
% Fat mass
C
Brain
**
AMPH concentration(nmol/g)
WT
KO
Brain
r=0.919
p<0.0001
AMPH concentration(nmol/g)
% Fat mass
D
Plasma
AMPH concentration(nmol/mL)
WT
KO
Brain
AMPH concentration(nmol/g)
WT
KO

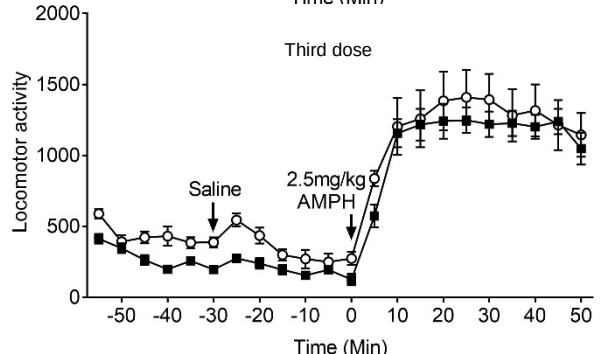
Fig4


A
Tsn$^{fl/fl}$
UBC-creXTsn$^{fl/fl}$
First dose
Saline
2.5mg/kg AMPH
Locomotor activity
Time (Min)


B
Tsn$^{fl/fl}$ first dose
UBC-creXTsn$^{fl/fl}$ first dose
Tsn$^{fl/fl}$ third dose
UBC-creXTsn$^{fl/fl}$ third dose
Saline
2.5mg/kg AMPH
Locomotor activity
Time (Min)


Second dose
Saline
2.5mg/kg AMPH
Locomotor activity
Time (Min)


C
Tsn$^{fl/fl}$
UBC-creXTsn$^{fl/fl}$
Total saline locomotor activity
1st dose
2nd dose
3rd dose


Third dose
Saline
2.5mg/kg AMPH
Locomotor activity
Time (Min)

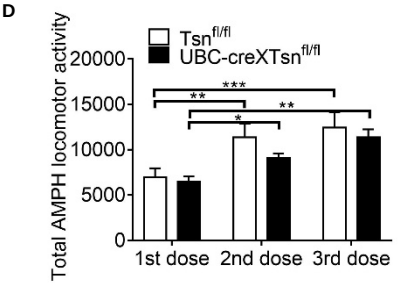
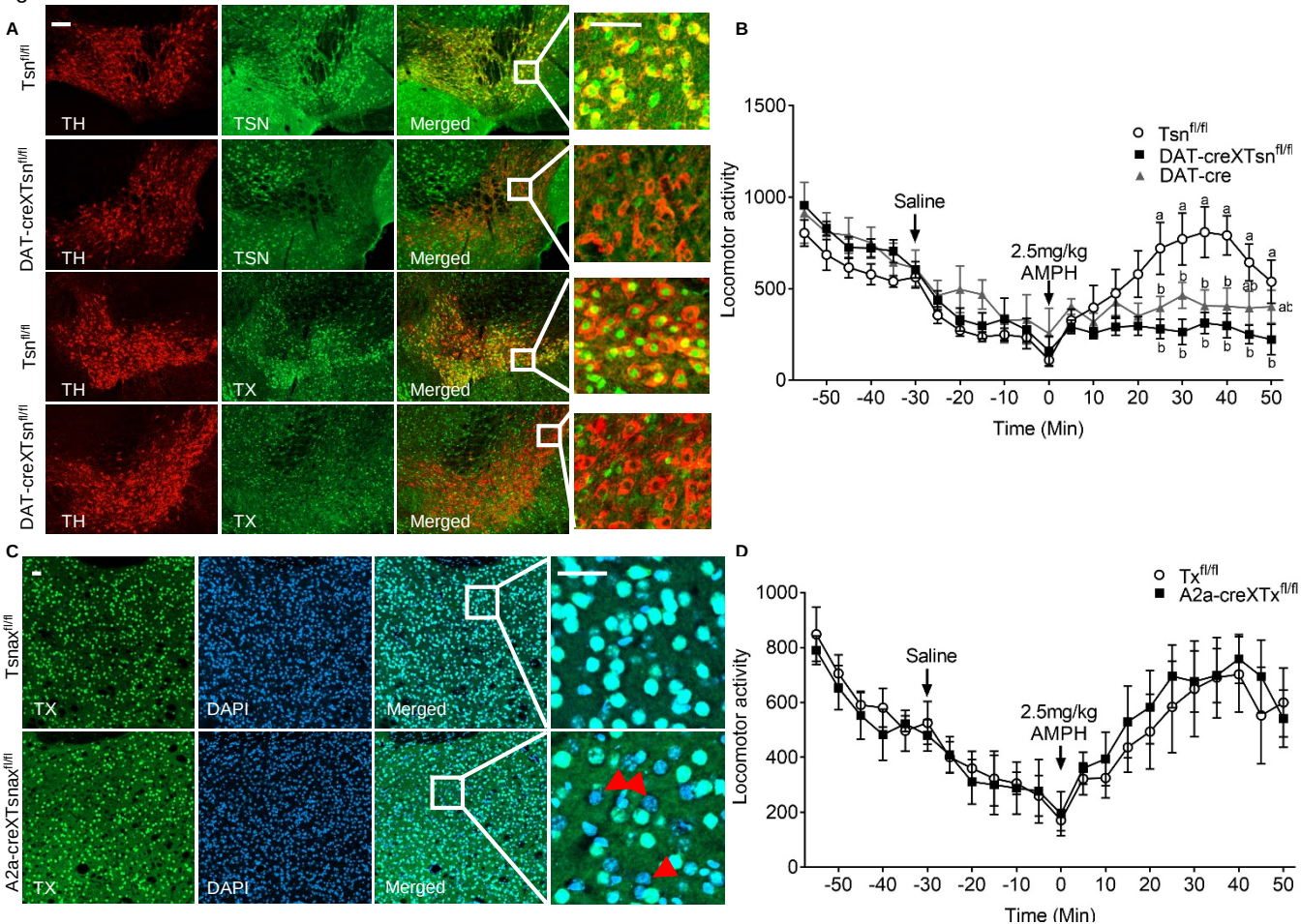
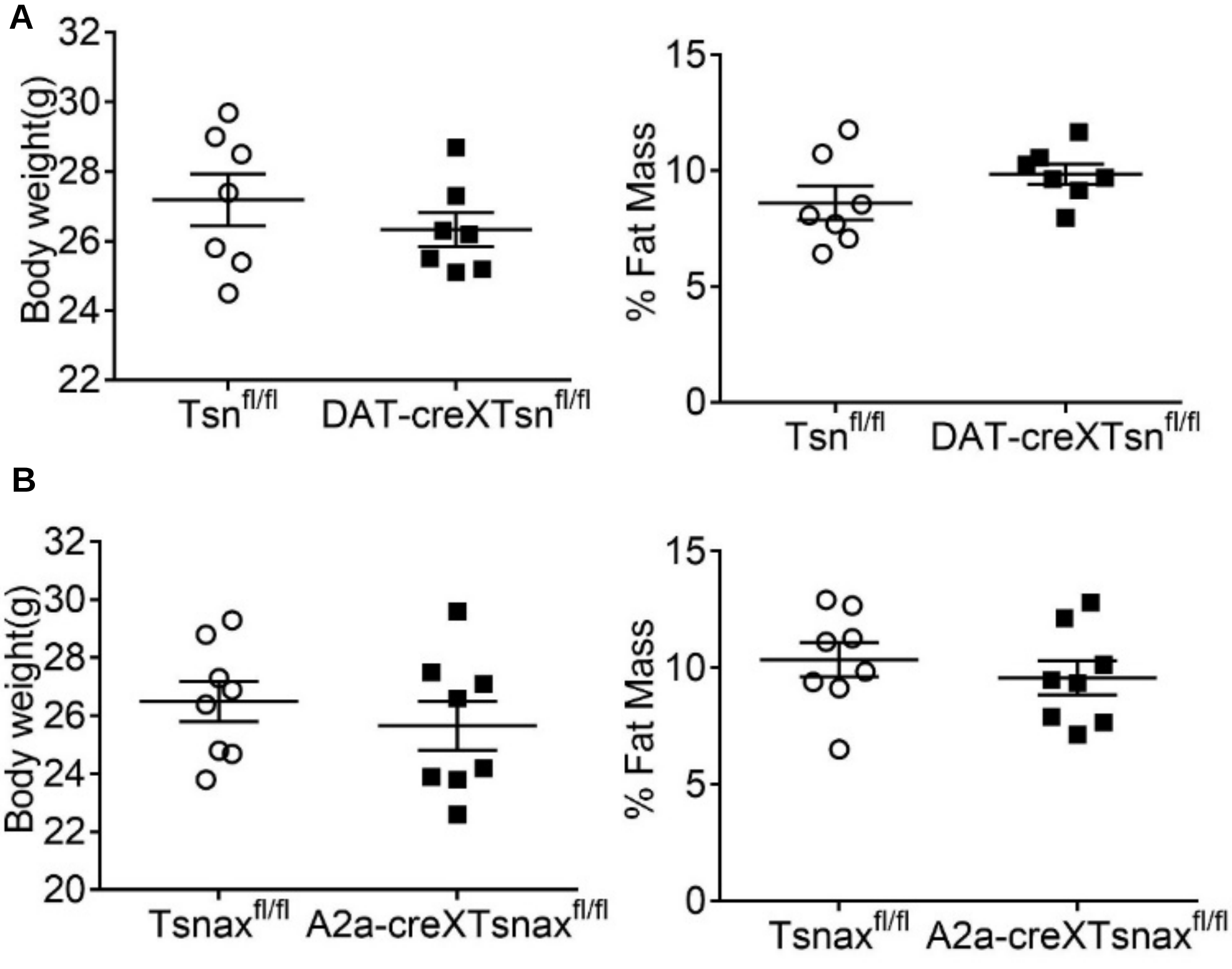

D
Tsn$^{fl/fl}$
UBC-creXTsn$^{fl/fl}$
Total AMPH locomotor activity
1st dose
2nd dose
3rd dose

Fig5


A
Tsn fl/fl
DAT-creXTsn fl/fl
Tsn fl/fl
DAT-creXTsn fl/fl
TH
TSN
Merged
TH
TSN
Merged
TH
TX
Merged
TH
TX
Merged
B
Tsn fl/fl
DAT-creXTsn fl/fl
DAT-cre
Saline
2.5mg/kg
AMPH
Locomotor activity
Time (Min)
C
Tsnax fl/fl
A2a-creXTsnax fl/fl
TX
DAPI
Merged
TX
DAPI
Merged
D
Tx fl/fl
A2a-creXTx fl/fl
Saline
2.5mg/kg
AMPH
Locomotor activity
Time (Min)

FigS1


A
Body weight(g)
32
30
28
26
24
22
Tsn fl/fl
DAT-creXTsn fl/fl
% Fat Mass
15
10
5
0
Tsn fl/fl
DAT-creXTsn fl/fl
B
Body weight(g)
32
30
28
26
24
22
20
Tsnax fl/fl
A2a-creXTsnax fl/fl
% Fat Mass
15
10
5
0
Tsnax fl/fl
A2a-creXTsnax fl/fl