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Characterization of dopamine transporter transgenic mice (DAT-tg)

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1. INTRODUCTION:

The neurotransmitter dopamine is synthesized by mesencephalic neurons of the substantia nigra and ventral tegmental area, and by hypothalamic neurons of the arcuate and periventricular nuclei (Cooper *et al.*1996). Dopaminergic neurons located in these areas project their axons to the striatum (nigrostriatal pathway), neocortex (mesocortical pathway), limbic system (mesolimbic pathway) and hypophysis (tuberoinfundibular pathway), thereby controlling a wide array of physiological functions including regulation of locomotor activity, cognitive processes, neuroendocrine secretion and the control of motivated behaviors such as emotion, affect and reward mechanisms (Carlsson 2001; Greengard 2001; Bozzi and Borrelli 2006). The effects of dopamine are mediated through its interaction with G-protein-coupled membrane receptors. The dopamine receptor family contains five members that, according to structural and pharmacological similarities, are divided into two subfamilies: the D_1-like family, comprising D_1 and D_5 receptors; and the D_2-like family, which includes D_2, D_3 and D_4 receptors (for review see Jackson *et al*. 1994; and Missale *et al.*1998). The understanding of the global dopaminergic signaling remains still incomplete due mainly to the complexity generated

by the presence of multiple dopaminergic pathways activated by the various dopamine receptors. Nevertheless, dysfunctions in the DA system are believed and have been shown to contribute to the development of several neurological, neuroendocrine and psychiatric conditions such as Parkinson's disease, depression, schizophrenia, attention deficit hyper-activity disorder (ADHD), Tourette syndrome and drug addiction (Table 1) (Carlsson 1987; Roth and Elsworth 1995; Koob and Le Moal 1997; Greengard 2001; Adinoff 2004). With the aim to elucidate the mechanisms of all these pathological conditions, the dopaminergic system has been the focus of much research over the past 50 years. Besides the numerous studies oriented in understanding the molecular structure, the different functions and the pharmacology of all the dopamine receptors subtypes, lots of research in the last 15 years has been focused on the role exerted by the plasma membrane DA transporter (DAT). This transporter in fact determines the intensity and duration of DA signaling at synapses driving the re-uptake of the transmitter back into nerve terminals. Re-uptake through the DAT is the primary mechanism for the regulation of synaptic DA concentrations and thus the most effective way of determining DA actions at post-synaptic and pre-synaptic receptors (Gainetdinov and Caron 2003).

1.1 Structure and functional role of the dopamine transporter

The gene encoding the DAT was first cloned in 1991 by several groups (Kilty *et al.* 1991; Giros *et al.* 1991; Shimada *et al.* 1991; Usdin *et al.* 1991). This transporter belongs to the Na^+/Cl^- dependent family of neurotransmitter transporters, which also includes transporters for the related biogenic amines norepinephrine and serotonin (NET and SERT respectively), as well as for the inhibitory neurotrasmitters GABA and glycine (Masson *et al.* 1999; Torres *et al.* 2003). The topological arrangement of these transporters consist of 12 transmembrane domains (TMs), a large glycosylated loop between TMs 3 and 4 and intracellular amino and carboxy terminal domains. In the case of DAT, three N-glycosylation sites are found on the second extra-cellular loop -between transmembrane domain 3 and 4- which have been shown to be important for the proper surface expression of the DAT (Torres *et al.* 2003a). Once at the plasma membrane, DAT co-transports two Na^+, one Cl^- and one DA molecule from the outside to the inside of the neuron utilizing the ionic gradient established by the Na^+/K^+ ATPase as driving force.

Furthermore**,** DAT can be considered a specific marker for dopaminergic neurons because it is expressed exclusively in neurons that synthesize DA as neurotransmitter (Cilax et *al.* 1995; Hoffman *et al.* 1998). Psychostimulants, such as cocaine and amphetamine, exert their action either by respectively inhibiting or reversing the DA transport of DAT. Indeed, cocaine and other classical re-uptake blockers act as competitive inhibitors of DAT, while amphetamine and its related molecules are direct substrates of DAT. Once inside the terminal, amphetamine-like molecules enter the DA containing vesicles resulting in the redistribution of DA from its vesicular localization to the cytoplasm. Ultimately, the increased cytoplasmic concentration of DA leads to the reversal of the transporter and a massive efflux of DA from the intracellular to the extracellular space (Seiden *et al.* 1993; Sulzer *et al.* 1995, Jones *et al.* 1998). DAT is also the molecular target for therapeutic agents used in the treatment of mental disorders such as ADHD and depression (Barker and Blakey 1995).

1.2 Dopamine transporter deficient mice

It is not surprising that mice lacking the DAT have attracted continued interest, and a considerable amount of data on transporter function and pharmacology have been gained using this model. As reviewed by Gainetdinov and Caron in 2003, the DAT knockout (DAT-KO) mice, generated through genetic deletion of DAT by homologus recombination, display distinct behavioural phenotypes. They are hyperactive, dwarf, display cognitive and sensorimotor gating deficits and sleep dysregulation. Abnormalities in skeletal structure and altereted regulation of gastrointestinal tract motility have also been described in DAT-KO mice. Moreover, these animals demonstrated the crucial role of DAT in determining the duration of action of extra-cellular DA. Indeed, using cyclic voltametry approach, it was shown that there is a 300 fold increase in the extra-cellular lifetime of DA in DAT-KO animals compared to their wild type littermates (Jones *et al.* 1998a). In addition to the changes observed in the extra-cellular dynamics of DA, profound alterations both in the pre and post-synaptic neurons of DAT-KO animals have also been documented. In fact, it has been reported that in the basal ganglia of DAT-KO animals, the levels of both D1 and D2 post-synaptic receptors are reduced by ~50% (Giros *et al.* 1996). Moreover, the levels of post-synaptic density-95 (PSD-95), a key

molecule involved in synaptic plasticity, were also found decresed in the striatum and nucleus accumbens of DAT-KO animals. With regards to the pre-synaptic neurons, it has been documented that tissue levels of DA, reflecting the intraneuronal concentration of the neurotransmitter, are reduced by 95% in DAT-KO animals. In addition, the levels of tyrosine hydroxylase (TH), the rate limiting enzyme in DA synthesis, are also reduced by ~90% in DAT-KO animals making these animals extremely sensitive to ʹϒ-methyl-*p*-tyrosine (ʹϒMT), an irreversible inhibitor of TH (Jones *et al.* 1998, Sotnikova *et al.* 2005). In fact, treatment of DAT-KO animals with ʹϒMT results in a complete depletion of striatal DA content resulting in behavioral phenotypes reminiscent of an acute mouse model of parkinson's disease (PD) (Sotnikova *et al.* 2005).

2. RESULTS

2.1 DAT Transgenic mice over-express DAT

To further investigate the function of DAT in the physiology of the dopamine system and its role in the above mentioned pathological conditions, a transgenic model of DAT mice (DAT-tg) was developed with the aim to over-express DAT in C57Bl/6J mice. Since the promoter region of the DAT is not well characterized (Giros *et al.* 1991)), a BAC transgenic approach was used for the over-expression of DAT. It has been shown that BAC transgenesis reliably produces proper spatio-temporal expression of the transgene, essentially acting as a gene duplication event. The BAC chosen for the generation of the transgene in this study contains the murine DAT locus (40kb) and 80Kb of up and downstream genomic sequence surrounding the DAT locus (Figure1A). A BAC transgenic founder line was generated by pro-nuclear injection and characterized for the extent of DAT over-expression achieved. First, the number of transgene copies integrated in the genome of the mice was calculated by southern blot analysis using a probe specific for DAT. As shown in figure 1B, the increased intensity of the genomic fragment from DAT-tg animal samples indicates that there is indeed genomic integration of the transgene. Moreover, the intensity of the band on the southern blot is directly proportional to the number of copies of DAT within the genome. Densitometric analysis shows that there is a 3 fold increase in the levels of genomic DAT in the transgenic line

compared to the wild-type, indicating that there are 4 copies of transgene integrated in the genome, bringing the total number of DAT copies to 6 in the DAT-tg animals. The observation of simple mendelian inheritance of the trasngene indicates that the 4 transgene copies have integrated in tandem at a single locus. In accordance with this increased genomic copies of DAT, there is a 3 fold increase in the levels of DAT protein in the transgenic animals compared to wild-type controls as measured by western blot on striatal preparations (Figure 1C). Next, the expression pattern of DAT within the transgenic line was examined and found to be restricted to dopaminergic neurons, as for the endogenous protein, confirming that the BAC transgenic approach leads to the proper spatial expression of the transgene in essence acting as gene duplication (Gong *et al.* 2003). As shown in figure 2, in both WT and DAT-tg animals, DAT antibody labels dopaminergic neurons that originate in substantia nigra and ventral tegmental area with major projections to the striatum.

2.1 DAT Transgenic mice show an increased level of both D1 and D2 post synaptic receptors

Different studies have shown how the two major DA receptors (D1 and D2) are down-regulated in DAT-KO mice. In particular, a decrease of both D1 and D2 mRNA levels, about 55% and 45% respectively, was observed in the basal ganglia of DAT-KO mice (Giros *et al.* 1996). These results were independently confirmed when a down-regulation in mRNA levels of both D1 (34% in caudate putamen; 45% in nucleus accumbens) and D2 (36% in caudate putamen; 33% in nucleus accumbens) was detected in the same animal model (Fauchey *et al.* 2000). Therefore, it would be expected that in DAT-tg mice expressing increased levels of DAT, D1 and D2 should be both up-regulated compared to wild-type littermates. To address this point, we performed different saturation binding experiments utilizing either D1 or D2 antagonists on striatal membrane extacts of DAT-tg mice compared with both wild-type and DAT-KO. To perform D1 saturation binding, we utilized as radioligand the specific D1 antagonist ^{3}H-SCH23390 and as cold Flupenthixol an antipsychotic which blocks D1, D2 and serotonin receptors. The data obtained from these experiments show that the DAT-KO mice have a decrease of 45% in the levels of D1 compared to the wild-type, while the DAT-tg show an increase of 32% (Fig. 1). D2

saturation binding experiments were conducted utilizing as radioligands two different specific D2 antagonist, ^{3}H-Spiperone and ^{3}H- Raclopride, and the antipsychotic haloperidol as cold which preferentially binds to D2 receptors. For the radioligand ^{3}H-Spiperone, we found a 51% decrease of D2 levels in the DAT-KO mice and an increase of 68% in the DAT-tg mice compared to the wild type animals. These results were confirmed using the radioligand ^{3}H-Raclopride; in DAT-KO mice we found the levels of D2 to be decreased by 39%, while in DAT-tg mice were increased by 72%.

3. REFERENCES

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Table 1

Pathways	**Brain areas involved**	**Dopamine alterations**	**Associated disease**
Nigrostriatal	Neurons from substantia nigra (SN) innervate the striatum.	↓ Dopamine ↑ Dopamine	Parkinson's disease Huntington's disease ADHD Schizophrenia Tourette's syndrome
Mesocortical	Links the ventral tegumental area (VTA) to medial prefrontal, cingulate and entorhinal cortices.	↑ Dopamine	ADHD Schizophrenia Tourette's syndrome
Mesolimbic	Ventral tegumental area (VTA) cells project to the nucleus accumbens and other limbic areas.	↓ Dopamine ↑ Dopamine	Drug addiction Obesity Depression Epilepsy
Tuberoinfundibular	Projections from arcuate and periventricular nuclei of the hypothalamus to the pituitary gland.	↑ Dopamine	Pituitary tumors

Table 1: Description of dopamine pathways and implications of their alteration in neurological, neuroendocrine and psychiatric disease.

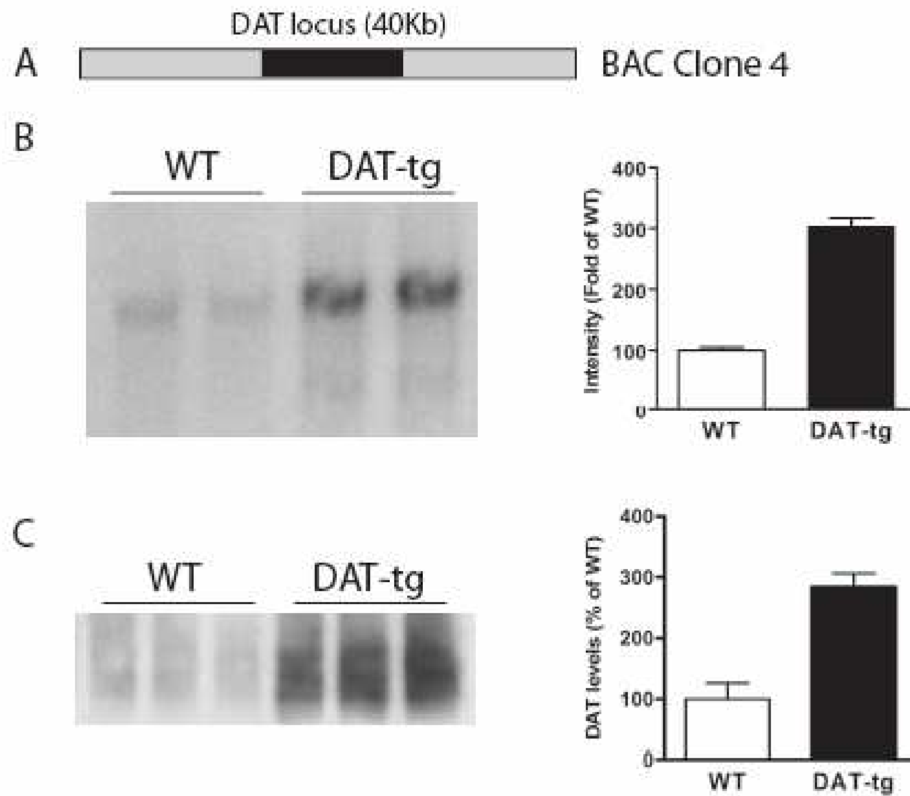


Figure 1: Generation of DAT-tg animals. A) Schematic representation of the BAC clone used for the generation of the DAT-tg animals. The DAT locus (40Kb) is flanked by 80kb of upstream and downstream genomic sequence. B) Representative DAT southern blot analysis from WT and DAT-tg animals. Data are means ± SEM (4 per group). C) Representative DAT western blot anaylsis of striatal tissue from WT and DAT-tg animals. Data are means ± SEM (5 per group).

Figure 2

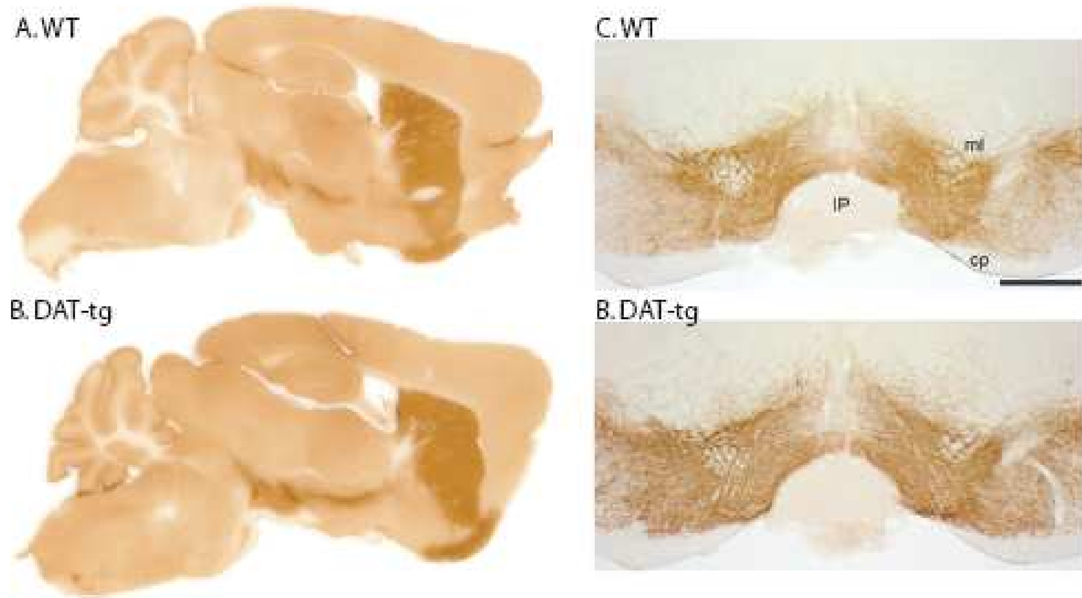
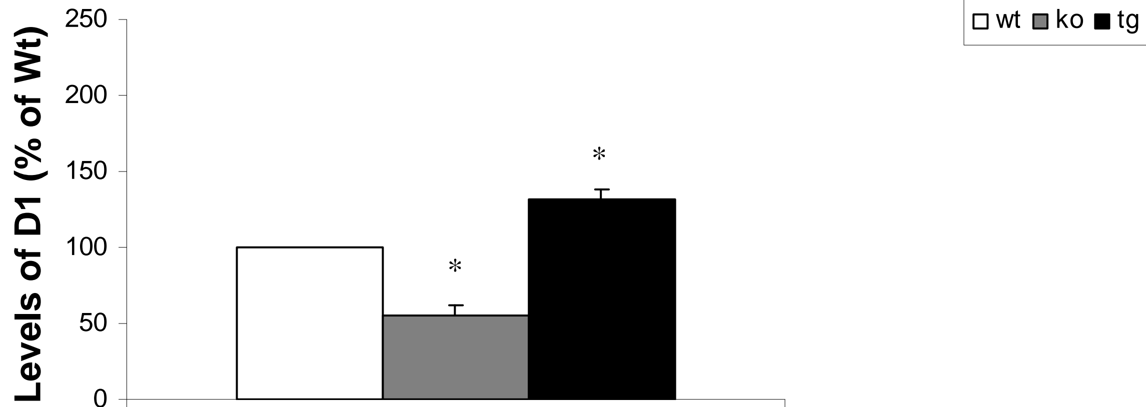


Figure 2: Immunohistochemistry anaylsis. Sagital sections of WT and DAT-tg showing the expression pattern of DAT by immunoperoxidase labeling (A and B). Coronal sections through the ventral midbrain of WT and DAT-tg mice showing immunoperoxidase labeling for DAT. Abbreviations: cp, cerebral peduncle; ml, medial lemniscus. Scale bar represents 250 um.

Figure 3



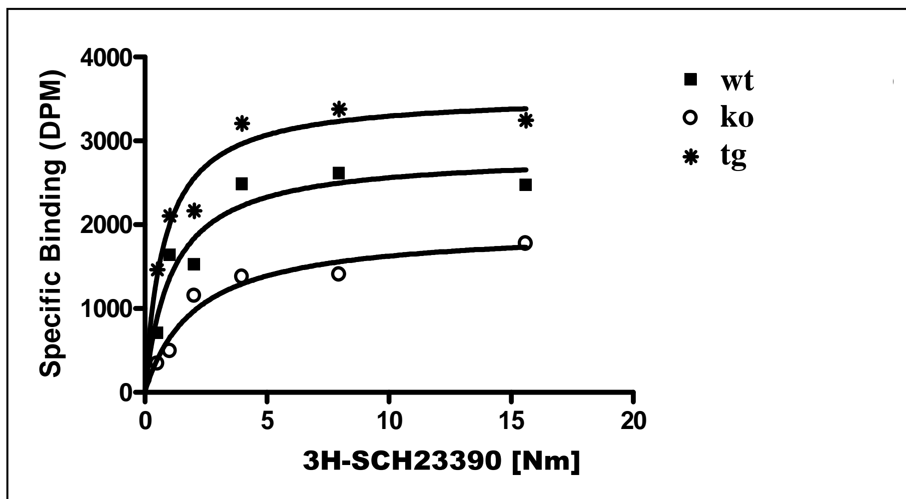
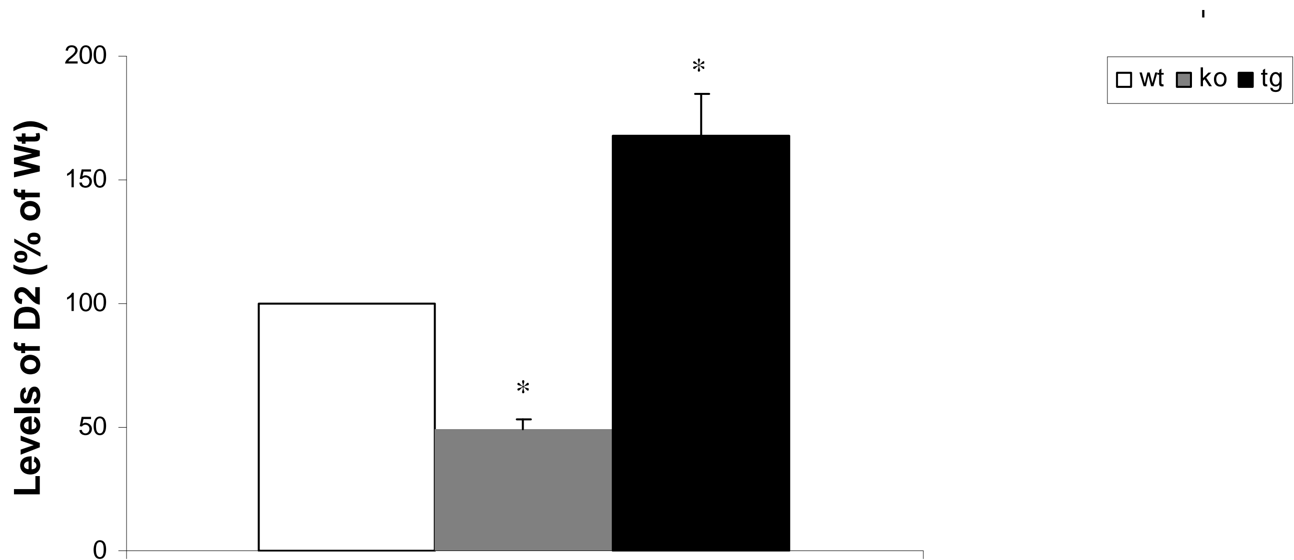


Figure 3: (A) Quantification of D1 receptors following 3H-SCH23390 saturation binding experiments. Five mice per group were analyzed and experiments performed in triplicate. The data were analyzed by Graph Pad program and results expressed as percentages of D1 level relative to wild type (Wt). Confidence intervals were calculated and tested for significance. *, P < 0.01. (B) Representative curve of a 3H-SCH23390 saturation binding experiment conducted on striatum of wild type (Wt), DAT-KO and DAT-tg mice. Specific binding is expressed in disintegrations per minute (DPM).

Figure 4



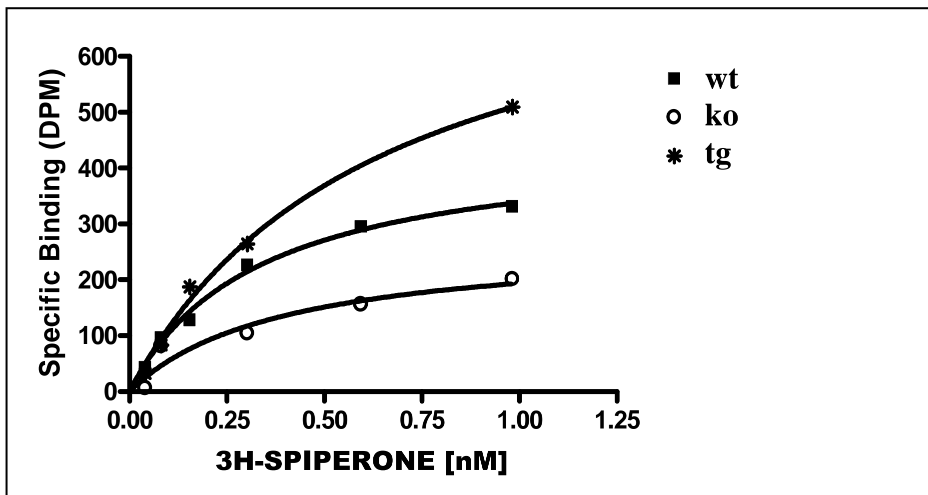
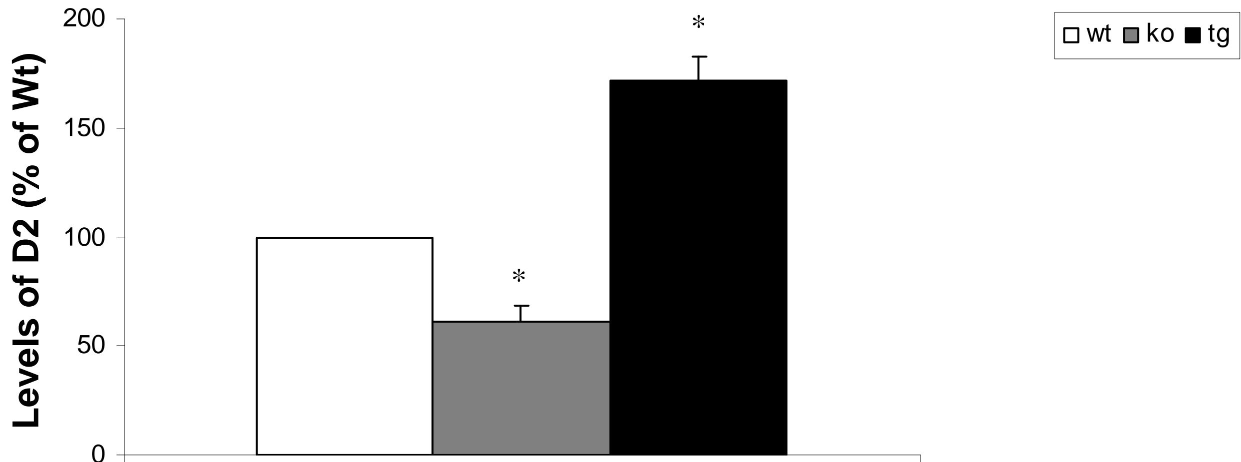


Figure 4: (A) Quantification of D2 receptors following 3H-Spiperone saturation binding experiments. Five mice per group were analyzed and experiments performed in triplicate. The data were analyzed by Graph Pad program and results expressed as percentages of D2 level relative to wild type (Wt). Confidence intervals were calculated and tested for significance. *, $P < 0.01$. (B) Representative curve of a 3H-Spiperone saturation binding experiment conducted on striatum of wild type (Wt), DAT-KO and DAT-tg mice. Specific binding is expressed in disintegrations per minute (DPM).

Figure 5



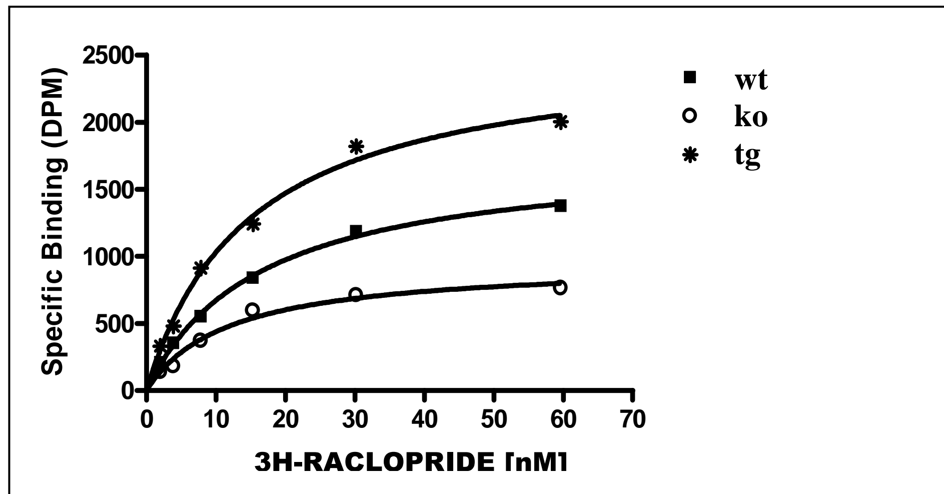


Figure 5: (A) Quantification of D2 receptors following 3H-Raclopride saturation binding experiments. Five mice per group were analyzed and experiments performed in triplicate. The data were analyzed by Graph Pad program and results expressed as percentages of D2 level relative to wild type (Wt). Confidence intervals were calculated and tested for significance. *, P < 0.01. (B) Representative curve of a 3H-Raclopride saturation binding experiment conducted on striatum of wild type (Wt), DAT-KO and DAT-tg mice. Specific binding is expressed in disintegrations per minute (DPM)