

Social withdrawal and gambling-like profile after lentiviral manipulation of DAT expression in the rat accumbens

Walter Adriani^{1*}, Frederic Boyer^{2*}, Damiana Leo³, Rossella Canese¹, Franca Podo¹,
Carla Perrone-Capano^{3,4}, Jean-Luc Dreyer² and Giovanni Laviola¹

¹ Sections of Behavioral Neuroscience and of Molecular & Cellular Imaging, Department Cell Biology & Neurosciences, Istituto Superiore di Sanità, Roma, Italy

² Department of Medicine, University of Fribourg, Switzerland

³ Institute of Genetics and Biophysics 'A. Buzzati Traverso', CNR, Naples, Italy

⁴ Department of Biological Sciences, University of Naples 'Federico II', Naples, Italy

Abstract

Dysfunction of brain dopamine transporter (DAT) has been associated with sensation seeking and impulse-control disorders. We recently generated a new animal model by stereotaxical inoculation of lentiviral vectors, which allowed localized intra-accumbal delivery of modulators for DAT gene: GFP (green fluorescent protein) control, silencers (Sil), a regulatable enhancer (DAT+), or both (DAT+Sil). Wistar male rats were followed both for socio-emotional profiles and for propensity to seek risky, uncertain rewards. Elevated anxiety and affiliation towards an unfamiliar partner emerged in Sil rats. Interestingly, in DAT+Sil rats (and Sil rats to a lesser extent) levels of playful social interaction were markedly reduced compared to controls. These DAT+Sil rats displayed a marked 'gambling-like' profile (i.e. preference for a large/uncertain over a small/sure reward), which disappeared upon doxycycline-induced switch-off onto DAT enhancer, but consistently reappeared with doxycycline removal. MRI-guided ¹H-MRS (at 4.7 T) examinations *in vivo* (under anaesthesia) revealed changes in the bioenergetic metabolites (phosphocreatine and total creatine) for DAT+Sil rats, indicating a functional up-regulation of dorsal striatum (Str) and conversely a down-regulation of ventral striatum (i.e. nucleus accumbens, NAc). A combined profile of (1) enhanced proneness to gambling and (2) strong social withdrawal is thus associated with altered DAT-induced balance within forebrain dopamine systems. In fact, risk of developing a gambling-prone, social-avoidant psychopathology might be associated with (1) dominant semi-automatic strategies and/or habits, developed within Str circuits, and (2) reduced NAc function, with poorer feedback adjustment on decisions by aversive experiences.

Key words: Dopamine transporter, impulse control, *in-vivo* gene modulation, light/dark emersion, probabilistic delivery task, social behaviour.

Introduction

Pathological gambling (PG), rapidly emerging as a mental health concern among Western civilizations, is essentially an impulse-control disorder, frequently comorbid with attention deficit hyperactivity disorder (ADHD; Sood *et al.* 2003). Comorbidity to other impulse-control disorders is common, especially to

compulsive buying and compulsive sexual behaviour (Black & Moyer, 1998). More than half of PG people have obsessive-compulsive, avoidant/antisocial and/or schizotypal/paranoid personality, as well as substance abuse/dependence and bipolar disorders (Hollander *et al.* 2005a; Lowengrub *et al.* 2006).

The aetiology of PG is multi-factorial (Joukhador *et al.* 2003). There is a relationship with risk-taking and sensation-seeking traits, as revealed by psychometric tests (Powell *et al.* 1999). Psycho-genetic studies (Comings *et al.* 2001) have revealed that, among several genes involved, one of the most significant for PG is the dopamine transporter (DAT). Abnormal DAT expression has also been detected in ADHD

Address for correspondence: Dr W. Adriani, Behavioural Neuroscience Section, Department of Cell Biology & Neurosciences, Istituto Superiore di Sanità, viale Regina Elena 299, I-00161 Roma, Italy.

Tel.: +39 06 4990 3171 Fax: +39 06 495 7821

Email: Walter.Adriani@iss.it

* These authors contributed equally to this work.

(Dougherty *et al.* 1999; Jucaite *et al.* 2005; Spencer *et al.* 2005), which is often comorbid with PG (Sood *et al.* 2003). Similar polymorphisms were also observed in association with pathological violence in adolescents and aggressive socio-pathology (Chen *et al.* 2005; Ladouceur *et al.* 1997; Parke & Griffiths, 2004). Hence, it appears that DAT is worthy of being explored as a putative key player in PG aetiology and therapy (Ibáñez *et al.* 2003; Tamiz *et al.* 2000).

Among forebrain areas and specifically within the motivational system, ventral striatum (i.e. nucleus accumbens, NAc) is a strong candidate for PG pathogenesis, being involved in 'wanting' to reach a salient prize and 'setting' the maximal affordable effort (Berridge, 2007; Berridge & Robinson, 1998; Ikemoto & Panksepp, 1999; Salamone & Correa, 2002; Salamone *et al.* 2003). Notably, a reduction of ventro-striatal activation has been observed in PG patients, compared to controls (Reuter *et al.* 2005), and disease severity was linked to the extent of hypo-activation. We addressed whether manipulation of accumbal functioning, through altered DAT expression, would result in a behavioural phenotype reproducing major PG and/or ADHD symptoms. To this end, we have recently developed a novel animal model (Adriani *et al.* 2009), i.e. brain inoculation of self-inactivating regulatable lentiviruses. These vectors allow the permanent incorporation of enhancers or silencers for DAT, enabling local switch-on or switch-off onto gene expression (Bahi *et al.* 2004a, b, 2005a, b). Behavioural changes were observed in the live animal, consisting of differential reactions in a task for gambling behaviour. Indeed, after DAT silencing or enhancement, respectively, rats showed either a 'conservative' or a more 'risk-prone' strategy (see Adriani *et al.* 2009). Moreover, data demonstrate that motivation to explore a novel environment was impaired in these rats.

In our study, this animal model was further investigated. First, we added a combined group where enhancer and silencers for DAT were acting simultaneously. Indeed, we hoped the final level of DAT function, achieved by balancing these two opposite modulators, would be responsible for the fine tuning of the DAT-related phenotype. Second, other endpoints were added, e.g. social and anxiety-related performance. Third, a deeper validation of the 'risk-prone' phenotype was performed, by explicitly probing its disappearance and reinstatement following a switch-off and switch-on again onto the regulatable DAT enhancer. Overall, present data confirm our model for the ability to reproduce both social avoidance and compulsive symptoms of ADHD, PG and impulse-control disorders.

Methods

All experiments were performed in accordance with the European Communities Council Directive (86/609/EEC) and Italian Law, and were formally approved by Institutional Animal Survey Board, on behalf of the Italian Ministry of Health. All efforts were made to reduce the number of animals used and to minimize their suffering and distress. Wistar male rats (Harlan, Italy) weighting 250 g at arrival were housed in pairs, under a normal 12-h light/dark cycle (lights off 21:00 hours). Two weeks after arrival, animals were inoculated bilaterally into the nucleus accumbens (NAc) with lentiviral vectors (Adriani *et al.* 2009; see Supplementary Material, available online), and allowed 2 wk of recovery. Four groups of ten rats were inoculated: 2 μ l lenti-GFP (green fluorescent protein, control), 2 μ l lenti-DAT (DAT+), 2 μ l lenti-DAT-siRNAs (Sil), and 2 μ l lenti-DAT plus 2 μ l lenti-DAT-siRNAs (DAT+Sil).

In-vivo experiments

Expt 1: Social-interaction test

In order to evaluate social motivation (Terranova *et al.* 1993), young adult rats underwent two social encounters (first, familiar reunion; second, unfamiliar partner), for 24 min (see Supplementary Material). Rats were individually housed 24 h before the first encounter (Terranova *et al.* 1993) and again housed individually for further 24 h before the second encounter. Members of the pair were always from the same inoculation group. The following social behaviours (Cirulli *et al.* 1996; Laviola *et al.* 2004) were scored. *Affiliative*: (1) Social rest; (2) Allogrooming. *Investigative*: (1) Ano-genital sniff; (2) Body sniff; (3) Mutual circle. *Soliciting*: (1) Crawl under; (2) Crawl over, (3) Pouncing, i.e. attempting to nose or rub the nape of the partner's neck. *Play*: (1) Wrestling, i.e. rough-and-tumble; (2) Follow, i.e. chasing the partner; (3) On top, i.e. standing over the partner lying with its back on the floor.

Expt 2: Light/dark test for anxiety

Classical 'light/dark' procedures (Metzenauer *et al.* 1992; Bourin and Hascoët, 2003; see Supplementary Material) were performed.

Expt 3: Operant Probabilistic-delivery task

Animals were tested by a probabilistic-delivery protocol, involving a choice between a small, certain reinforcer and a larger, uncertain one (Adriani &

Table 1. Slope of the gambling curves, as measured in adult male rats with a previous focal inoculation of a lentiviral vector. This tool allowed DAT gene (enhancer and silencers) transfer into the nucleus accumbens

	First replication	Second replication (+ Doxy)	Third replication
'Risk-prone' subpopulation			
GFP control	-28.4 ± 8.2	-31.9 ± 1.8	-33.5 ± 6.7
DAT +	$-13.7 \pm 8.4^\dagger$	$-30.6 \pm 8.5^*$	$-33.9 \pm 6.4^*$
Sil	-28.1 ± 4.6	-38.8 ± 7.4	$-57.0 \pm 8.0^{*\dagger}$
DAT + Sil	-20.7 ± 6.1	$-48.1 \pm 11.9^{*\dagger}$	-34.8 ± 6.4
Conservative subpopulation			
GFP control	-64.7 ± 4.2	-64.9 ± 4.7	-64.8 ± 4.5
DAT +	-52.0 ± 7.8	-64.0 ± 4.8	-58.0 ± 6.3
Sil	-66.2 ± 11.6	-77.1 ± 11.2	-71.7 ± 11.4
DAT + Sil	-57.7 ± 8.5	$-86.4 \pm 4.2^{*\dagger}$	-72.1 ± 6.3

DAT +, Dopamine transporter enhancer; GFP, green fluorescent protein; Sil, silencer. Animals belonging to the more conservative subpopulation (i.e. quickly shifting to small/sure, $n=5$) are also shown, but discussion is focused on the specific 'risk-prone' subpopulation (i.e. large/luck-linked bound animals, $n=5$).

* $p < 0.05$ compared to first replication; † $p < 0.05$ compared to GFP control rats.

Laviola, 2006). Hence, animals had to make a choice between a small/sure (SS) or a large/luck-linked (LLL) reward (session length 25 min; timeout 15 s; see Supplementary Material for more details). This probabilistic-delivery schedule was replicated three times: a switch-off regulation of DAT enhancer was performed during the test phase of second replication, when doxycycline (0.02% in tap water) bottles were provided to rats in their home cage.

We demonstrated previously (Adriani *et al.* 2009) the emergence of two distinct subpopulations (see also Adriani *et al.* 2003). Using a criterion such as LLL preference more *vs.* less than 33% at the end of the task two subgroups were defined (i.e. five animals per subgroup). The present paper aimed to deal with the phenotype of risk proneness, and hence data for the 'conservative' subgroup are not relevant. The typical curves which can be obtained from either subpopulation are discussed in our previous work (Adriani *et al.* 2009). Here, we decided to briefly show these data by means of slope values (Table 1). The difference between 'risk-prone' and 'conservative' rats is apparent.

Expt 4: Magnetic resonance imaging-guided ^1H spectroscopy

Eight animals per group (i.e. except those that were later used for immunohistochemistry) underwent the MRI-guided ^1H -MRS analyses (Adriani *et al.* 2007). Single-voxel localized ^1H -MR spectra (PRESS,

TR/TE=4000/23 ms, n.s.=256) were collected (see Supplementary Material) from different brain areas: prefrontal cortex (PFC) (31.5 μl), dorsal striatum (Str) (36.4 μl) and NAc (21 μl). MR spectra were analysed by LCModel (Provencher, 2003) and data were processed according to our previous work (see Adriani *et al.* 2007).

Ex-vivo experiments

At the end of behavioural analyses, a drinking regimen of doxycycline (or tap water) was semi-randomly assigned to rats (see Supplementary Material). For some rats, whole brains were removed at sacrifice, and quickly frozen in isopentane stored for structural analyses. For all other rats, NAc tissues were dissected out bilaterally on wet ice, and hemispheres were either used for fresh preparation of synaptosomes (to assess [^3H]DA uptake), or assigned to RT-PCR/Western blotting (to assess DAT mRNA and protein).

Expt 1: [^3H]DA high-affinity uptake by striatal synaptosomes

Striatal complexes (including both dorsal and ventral portion), coming from water-exposed animals, were immediately processed for synaptosomal [7,8- ^3H]DA uptake (for details, see Supplementary Material). DA uptake was calculated as the amount of radioactivity accumulated at 37 °C minus blank values.

Expts 2 and 3: DAT mRNA transcripts and DAT proteins – quantification

NAC samples, frozen at sacrifice, were processed 2 months later (see Supplementary Material). The relative abundance of DAT transcripts was calculated as the ratio between this target gene and cyclophilin F levels (see Adriani *et al.* 2009; Bahi *et al.* 2004*a,b*). Western blots were incubated for DAT and for β -actin detection (see Adriani *et al.* 2009; Bahi *et al.* 2004*a,b*).

Expt 4: Immunohistochemistry, fluorescence microscopy

Immunohistochemistry assays were run on rat brains quickly frozen in isopentane. The coronal sections were incubated with primary antibody (against DAT) and then with secondary antibody (Texas Red-conjugated). To observe the staining, a fluorescence microscope (Axioplan 2, Germany) was used (for more details, see the Supplementary Material).

Design and data analysis

Data were analysed by split-plot ANOVA using StatView software (version 5.0.1, SAS Institute Inc., USA). Data in all figures and text are presented as means \pm standard errors of the mean (S.E.M.). The general design of all experiments was a four-level (between subjects) ‘group’ factor (GFP, DAT, Sil, DAT+Sil) \times repeated measures on the same individual rat (within-subject factors). Repeated-measure factors varied depending on the experimental protocol. For social encounters, a two-level ‘sociality’ factor (familiar *vs.* unfamiliar) and a three-level ‘interval’ factor (8-min bins) were added. For light/dark test, ‘time’ was the repeated-measure factor (two 5-min bins). For the Gambling task, a multi-level ‘probability’ factor (20% to 6%) and a three-level ‘interval’ factor (500-s bins) were added. Multiple *post-hoc* tests were performed with Tukey’s HSD test, and threshold for significance was set at $p < 0.05$ for all comparisons. Separate analyses with single one-way factorial ANOVAs were also used when required and allowed (see Supplementary Material for more details).

Results

In-vivo experiments

Overall, the levels of general locomotion and arousal were identical across groups, as suggested by several indexes in various tests. For instance, no significant group effect emerged for: ‘explore’ and ‘rearing’ during social encounters; for ‘activity’ rate (i.e. number of photocell interruptions per time unit) during

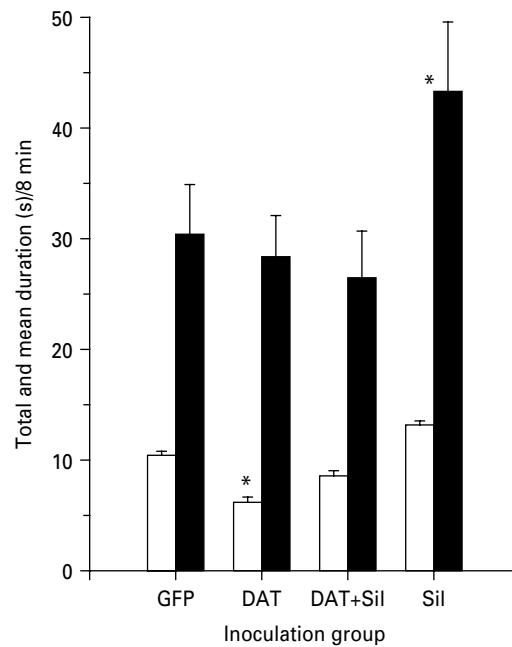


Fig. 1. Allogrooming (unfamiliar partner). Mean (□) and total (■) duration (s) of the allogrooming episodes observed during a social encounter between two unfamiliar rats that were isolated 24 h before the session. The 24-min session was divided into three 8-min intervals. * $p < 0.05$ compared to green fluorescent protein (GFP) controls ($n = 10$).

light/dark test; for ‘ineffective’ nose-poking (i.e. number of hole visits expressed during the timeout interval, when they had no scheduled consequence). This rules out a potential ‘bias’ due to altered activity, as already reported in our previous work (Adriani *et al.* 2009). No evidence of stereotypy or repetitive patterns of behaviour were observed.

Expt 1: Social-interaction test

No significant effect emerged in the ANOVAs for: Social rest; Crawl under and over; Follow; all non-social parameters (Explore, Rearing, Self-grooming). A complete description of the significant effect for social behaviours, together with complete ANOVA findings and *post-hoc* analyses, is reported in the Supplementary Material (see also Figs 1 and 2).

Social-behaviour summary. Following the concomitant expression of DAT enhancer and silencers in the NAC, rats expressed much lower levels of social investigation (see Fig. 2*a*, white bars), solicitation to play (see Fig. 2*b*, white bars), and actively elicited much less play behaviour (see Fig. 2*a,b*, dark bars for both panels). DAT + Sil (and Sil rats in some cases)

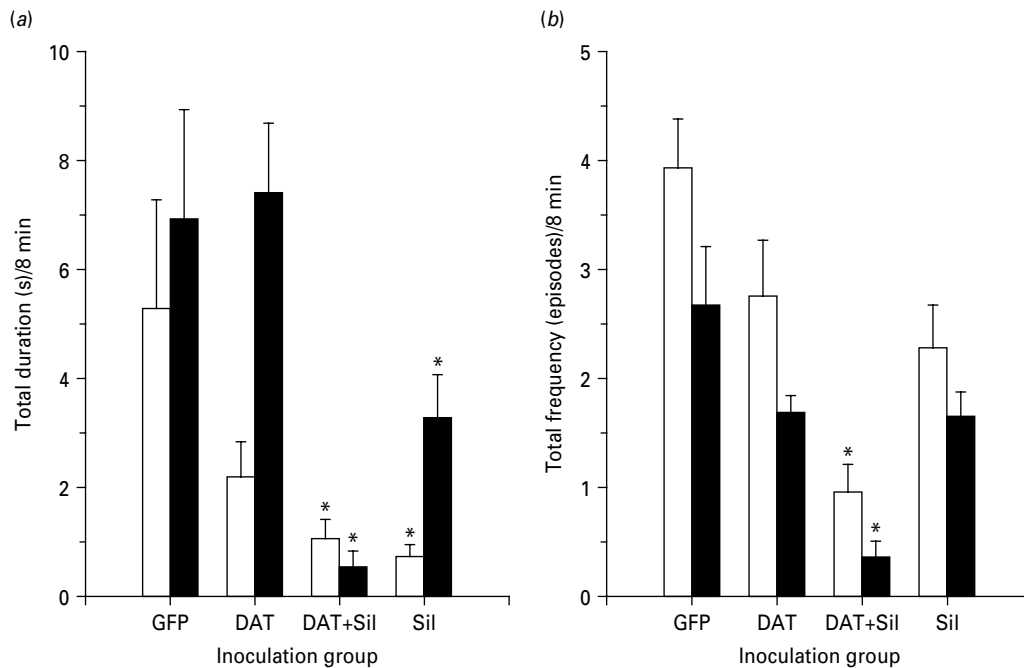


Fig. 2. (a, b) Interaction (familiar reunion). (a) Duration (s) and (b) frequency of several behaviours observed between two familiar (cage-mate) rats that were isolated 48 h before the social encounter. The histograms denote investigation/soliciting (□) and social play (■). The whole 24-min session was divided into three 8-min intervals. * $p < 0.05$ compared to green fluorescent protein (GFP) controls ($n = 10$).

expressed a striking reduction in social playful interaction, despite this being usually observed during familiar encounters. Suppression of accumbal DAT alone slightly affected play behaviour. Simultaneous enhancement of DAT even worsened such social performance, possibly due to a non-regulatable, disruptive final level of DAT expression.

In contrast, when encounters were between unknown individuals, rats expressed affiliation towards the stranger (see Fig. 1). However, DAT+ rats expressed a similar duration of total contact with a lower mean duration of each episode, thus implying much more episodes ($4.6 \pm 0.5/8$ min) compared to controls ($2.9 \pm 0.4/8$ min). Conversely, Sil rats expressed higher total duration, but the mean duration of each episode was similar, so that the number of contacts was slightly increased ($3.3 \pm 0.4/8$ min). Hence, DAT+ rats appeared somewhat restless (i.e. more but much shorter episodes) while Sil rats spent longer on social-contact time (i.e. more frequent, but not longer episodes). Since allogrooming denotes an affiliative, non-aggressive approach, we propose that levels of DAT expression appear to be inversely related to motivation for an amicable interaction during challenging social situations. It is worthy of note that, despite potential social stress, no 'agonistic/

defensive' postures nor indexes of 'threat/fight' were ever observed in any of the encounters.

Expt 2: Light/dark test for anxiety

The ANOVA for crossing between compartments (Fig. 3a) yielded a trend for a main effect of group [$F(3,35) = 2.19$, $0.05 < p < 0.10$], and for a group \times time interaction [$F(3,35) = 2.08$, $0.05 < p < 0.10$], with the Sil group expressing lower crossing between compartments during the second 5-min interval ($p < 0.05$, compared to all other groups). Such a profile was confirmed with *post-hoc* comparisons, performed for time spent in the bright chamber (Fig. 3b). Indeed, Sil rats spent significantly less time in the bright compartment during the second 5-min interval ($p < 0.05$, compared to GFP controls), thus showing more anxiety than all other groups. Clearly, these findings demonstrate a marked anxiety within Sil rats, which retreated to the dark chamber and showed no further exploration of the bright compartment.

The time-dependent change of behavioural parameters during the course of the session was only found for this specific test. In the other cases, i.e. for the social encounters and the Probabilistic-delivery task, the various behavioural parameters were quite

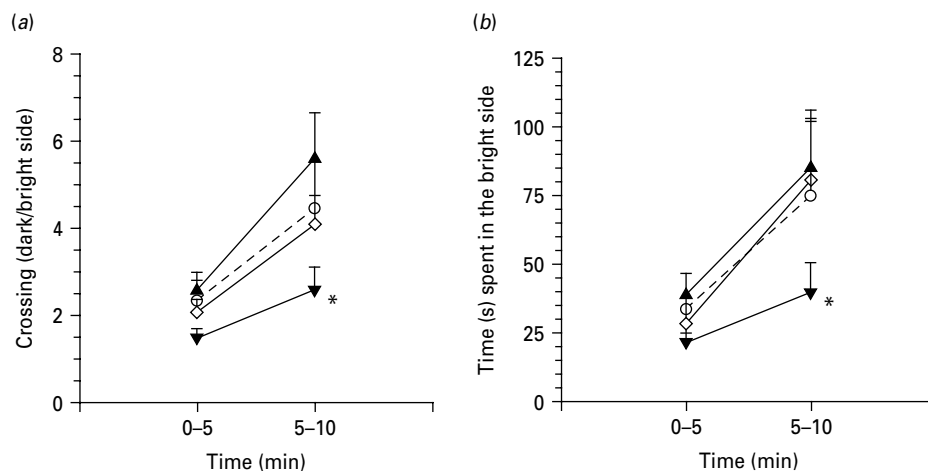


Fig. 3. Anxiety-related behaviour in the light/dark test. (a) Crossing between compartments and (b) time (s) spent in the bright chamber during a light/dark test. Rats were placed into the dark chamber and allowed to freely explore both compartments for 10 min. The first and second 5-min halves are shown. Groups: DAT+ (▲); Sil (▼); DAT+Sil (◇); green fluorescent protein (GFP) control (○). * $p < 0.05$ compared to GFP controls ($n = 10$).

constant throughout each daily session, regardless of session length. Indeed, sessions were always divided into three 8-min or 500-s intervals, but this repeated-measure factor failed to reach significance in any of the ANOVAs performed.

Expt 3: Operant Probabilistic-delivery task

The possibility of replicating the Probabilistic-delivery task with and without doxycycline allowed us to probe the influence of genetic manipulation on behaviour. Since differences between the two subpopulations were striking, these were analysed separately. As for the more conservative rats, no effect or interactions emerged among group, replication and probability (see Table 1 and Supplementary Material).

The most interesting data were, however, evidenced from the 'risk-prone' subgroup. The general ANOVA yielded significance for group effect [$F(3, 49) = 3.55$, $p < 0.05$], and for replication \times probability interaction [$F(10, 245) = 2.49$, $p < 0.01$], suggesting that group differences were modulated across the three replications by doxycycline exposure and removal. When observing the four group curves averaged across replications (Fig. 4, left of panel), it is evident that DAT and DAT+Sil groups had higher overall values of LLL preference than control and Sil rats. Multiple comparisons revealed that the DAT+Sil group showed higher LLL preference across nearly all ' p ' values, suggesting increased engagement in a gambling-like strategy. A similar gambling-like profile was found for DAT+ rats but to a lesser extent. Indeed, LLL preference was significantly enhanced ($p < 0.05$, compared

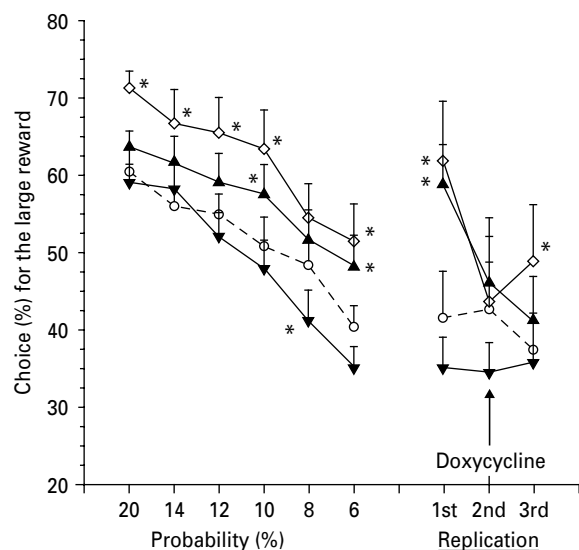


Fig. 4. Gambling in the Probabilistic-delivery task. Choice (%) of the large but uncertain reward. Rats of the four groups [DAT+ (▲); Sil (▼); DAT+Sil (◇); GFP control (○)] had choice between one food pellet for certain (SS) or five uncertain food pellets (LLL). The task was replicated three times and the average of the three replications is shown (left side of panel). The last point at $p = 6\%$ is also presented for the three replications (right side of panel). During the second replication, exposure to doxycycline was to switch-off the exogenous DAT over-expression. * $p < 0.05$ compared to green fluorescent protein (GFP) controls ($n = 5$).

to controls) for $p = 10$ and $p = 6\%$. This last point is of particular importance, since only rats with a considerable proneness to take risks would continue LLL

Table 2. Metabolic parameters, measured in the dorsal striatum and the nucleus accumbens (NAc) of adult male rats with previous focal inoculation of a lentiviral vector. This tool allowed DAT gene (enhancer and silencers) transfer into the NAc ($n=6$)

	Phosphocreatine/ total (%)	Phosphocreatine	Total creatine
Dorsal striatum			
GFP control	65.4 ± 3.4	6.29 ± 0.54	9.86 ± 0.41
DAT +	63.0 ± 2.7	6.60 ± 0.30*	10.46 ± 0.10*
Sil	64.8 ± 2.6	6.24 ± 0.52	9.57 ± 0.46
DAT + Sil	65.5 ± 2.1	6.97 ± 0.35*	10.62 ± 0.29*
Nucleus accumbens			
GFP control	64.3 ± 1.5	6.49 ± 0.17	10.10 ± 0.26
DAT +	67.5 ± 2.3	6.72 ± 0.27	9.96 ± 0.22
Sil	59.7 ± 3.4*	5.63 ± 0.44*	9.56 ± 0.26
DAT + Sil	59.1 ± 3.6*	5.84 ± 0.32*	9.80 ± 0.26

DAT +, Dopamine transporter enhancer; GFP, green fluorescent protein; Sil, silencer. Levels of metabolites are given in arbitrary units, referred to the unsuppressed water signal.

* $p < 0.05$ compared to GFP control rats.

nose-poking in spite of successful delivery of the larger prize becoming a really rare event.

Such risk proneness is clearly depicted in Fig. 4 (right of panel), where we report the values of LLL preference at $p=6\%$ for each of the three replications. Indeed, in the first replication, a similar gambling-like profile was found for DAT and DAT+Sil rats, that showed a significantly enhanced LLL preference ($p < 0.05$, compared to controls). As expected for the second replication, when DAT over-expression was turned off by doxycycline exposure, the percent LLL choice for both DAT+ and DAT+Sil groups actually fell to control values. This piece of data is a clear demonstration that manipulation of accumbal DAT can influence reactions to a Probabilistic-delivery task. However, when DAT over-expression was again turned on by doxycycline removal during the third replication, the DAT+ group did not display the gambling-like profile and was similar to controls. In contrast, the DAT+Sil rats showed a significant elevation of LLL preference ($p < 0.05$ over controls), although they never reached the same levels of first replication. Such findings suggest a strong role of actual DAT gene expression levels in producing elevated LLL preferences. This kind of profile is confirmed by analyses for slope values (see Table 1). (These Results are fully described in the Supplementary Material.)

It is of interest that effects of DAT switch-on (induced by doxycycline removal in the third replication) were only evident in DAT+Sil rats, and not in DAT+

animals. To explain such profile, we considered the fundamental difference between these two groups. Under a doxycycline-induced switch-off onto the DAT enhancer (second replication), the DAT+Sil rats experienced a total silencing of any sources of DAT protein, at least for accumbal areas, thus becoming similar to Sil animals. Conversely, for the DAT+ group where silencers are absent, the constitutional DAT gene continued to work when the regulatable enhancer was switched off, with these animals becoming similar to controls. This consideration underlines the striking contrast between the two groups, in that effects produced by doxycycline may be more marked for DAT+Sil rats (becoming similar to Sil), compared to the DAT+ group (becoming similar to controls).

Expt 4: Magnetic resonance imaging-guided spectroscopy

Changes among groups were detected mainly in the Str and NAc (see Table 2), and were detected in either total creatine or in phosphocreatine alone. (These Results are fully described in the Supplementary Material.) No differences were evident for any other metabolites or within the PFC.

Ex-vivo experiments

Expt 1: [^3H]DA high-affinity uptake by striatal synaptosomes

We tested DAT protein function by the [^3H]DA high-affinity uptake in synaptosomes, freshly obtained

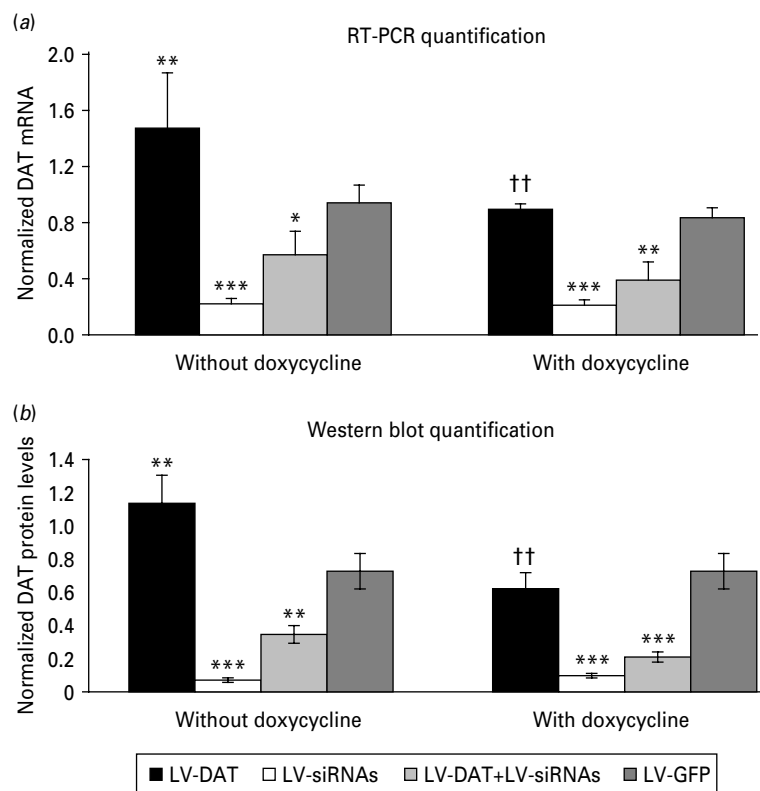


Fig. 5. (a) DAT mRNA and (b) DAT protein levels, extracted from nucleus accumbens of rats inoculated with lentiviruses. Following behavioural analysis, and before sacrifice, animals were exposed to a drinking regimen of either 0 or 0.02 % doxycycline in tap water (switch-on or -off, respectively). cDNA transcripts were quantified using real-time PCR, then normalized against cyclophilin F, with β -actin mRNA being also analysed as an endogenous control. From the Western blots, DAT signal was estimated using a multi-analyst software and normalized against the β -actin signal. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to the green fluorescent protein (GFP) control rats; †† $p < 0.01$ when comparing doxycycline presence vs. absence ($n = 4$).

from rats' striatal complexes (both the dorsal and the ventral portion). ANOVAs performed on data from synaptosomal [^3H]DA uptake revealed an effect of group [$F(3, 12) = 179$, $p < 0.0001$]. The [^3H]DA uptake of the DAT + group (73.2 ± 2.6 k) was similar to that of the GFP control (68.0 ± 3.1 k). Interestingly, a marked decrease of [^3H]DA uptake was observed in Sil (10.2 ± 0.99 k, 15% of the GFP control) and DAT + Sil (21.7 ± 2.3 k, 32% of the GFP control) groups ($n = 4$ per group). As expected, the [^3H]DA uptake was almost completely abolished (95% inhibition) in all groups by a selective uptake inhibitor, GBR-12909.

Expts 2 and 3: expression of DAT mRNA transcript and protein in the NAc

As expected from our previous study (Adriani *et al.* 2009), RT-PCR and Western-blot data were almost similar. [DAT gene and protein expression (see Fig. 5a,b) are fully described in the Supplementary Material.]

Expt 4: Immunohistochemistry, fluorescence microscopy

DAT fluorescence images were in agreement with RT-PCR and Western blot data (Supplementary Fig. S1A) and confirmed the accumbal localization of gene transfer, with no diffusion of viral infection (Supplementary Fig. S1B). This set of results can be found in the Supplementary Material.

Discussion

Abnormal brain expression of DAT has several implications for psychopathology as reduced DAT levels in forebrain areas has been proposed for ADHD aetiology (Dougherty *et al.* 1999; Jucaite *et al.* 2005). However, in light of the putative role of DAT gene in PG and aggressive socio-pathology (Parke & Griffiths, 2004), we probed DAT's role in social performance and in reaction to an operant Probabilistic-delivery task. Thus, for *in-vivo* silencing or over-expression of accumbal DAT function, we inoculated lentiviral

vectors which were locally and permanently integrated into the NAc (Boyer & Dreyer, 2008). We previously reported that motivation to explore a novel environment was impaired in these rats, and that an impulsive behavioural profile was produced (Adriani *et al.* 2009). Indeed, NAc cells became infected by our viral vectors, and the artificial DAT expression level did act on tuning of accumbal DA synaptic levels. This modulatory action over DAT was stable over months and limited within the NAc (Boyer & Dreyer, 2008).

We co-inoculated both vectors for simultaneous silencing and over-expression. When both opposite modulators were active, DAT gene silencing appeared to prevail over its enhancement. Indeed, in DAT+Sil rats, expression of DAT (both mRNA and protein) was found at intermediate levels between Sil and control GFP rats. Accordingly, DAT+Sil rats displayed almost double [³H]DA uptake values compared to Sil, and a 3-fold reduction compared to GFP controls. Thus, there was some residual DAT function in DAT+Sil rats (and also in Sil rats). Of note, however, this residual expression was intriguingly preserved under doxycycline-induced enhancer switch-off, when DAT+Sil animals could be expected to resemble Sil animals at least for molecular parameters. Indeed, despite the transient enhancer switch-off, which spared the silencers' action on endogenous DAT gene, DAT+Sil rats displayed almost double values for DAT mRNA and protein, when compared to Sil rats.

These molecular data may suggest that Sil and DAT+Sil rats could behave similarly, since 'hyper-dopaminergic' phenotypes might be hypothesized for both groups. Yet, considerable differences emerged between them, and the most striking behavioural anomalies were evidenced in DAT+Sil rats. Here, the regulatable enhancer transgene as well as the constitutional DAT gene are jointly silenced but there is still some residual DAT function coming from mRNA which overcomes silencing and is hence expressed into protein. Such residual DAT function, however, cannot be in any way considered as being similar to 'wild' DAT function, as is typical of control GFP subjects, since residual DAT in DAT+Sil rats is of mixed origin, deriving not only from endogenous but also from exogenous gene transcripts. The latter in particular may be insensitive to any feedback regulation, operated by epigenetic control factors, which may conversely operate on the former. This may explain to some extent the origin of detrimental behavioural regulation and inability to cope with external challenges, like those occurring during social life

and decisions, described for the present DAT+Sil group.

Repertoire of social alterations

DAT+Sil rats (and also Sil animals, but to a lesser extent) expressed strikingly reduced social investigation and solicitation behaviour upon reunion with their cage mate. Suppression of the accumbal DAT function slightly affected play behaviour, and the concomitant presence of artificial DAT enhancers led to poorer performance in this situation. As a possible explanation, the final DAT tone, reached under concomitant enhancer and silencers, may possibly not be regulated by feedback through epigenetic control factors. Hence, the 'artificial' DAT tone experienced by DAT+Sil rats may generate a disruptive social condition. It should be noted that the rats were not socially 'inattentive', nor 'inept', since they were able to behave correctly when facing a novel partner rat. Rather, the picture is reminiscent of social withdrawal observed with DA agonists (Beatty *et al.* 1982) in models of paranoid schizophrenia (Sams-Dodd 1995, 1998).

When rats were challenged with an unfamiliar partner, over-expression *vs.* silencing of the DAT gene produced diverging effects on affiliation, a friendly kind of approach expressed in the form of allogrooming episodes. Compared to controls, DAT over-expression led to more frequent (but shorter) episodes of interaction, somewhat reminiscent of a motoric hyperarousal, although none of the other classical indexes of locomotor activity was affected. Conversely, DAT silencing led to highest levels of affiliation, with more (frequent but not longer) reciprocal contact. Apparently, the extent of friendly approaches to an unfamiliar subject are directly proportional to basal accumbal DA levels, in that lower (or higher) reuptake of accumbal DA in Sil (or DAT+) rats favours a more (or less) affiliative social-coping strategy. Furthermore, Sil rats quickly retreated into the dark chamber, indicating a marked anxiety-related behaviour. An anxious tone may indeed be produced by excessive accumbal DA (Cancela *et al.* 2001).

It is noteworthy that both affiliation and anxiety gave similar results, being increased in Sil rats. It could be proposed that excessive anxiety-like traits may explain the need for an affiliative and more friendly approach displayed by Sil rats. Consistently, levels of both parameters fell to control baseline levels in DAT+Sil rats, and were indistinguishable between DAT+Sil rats and GFP controls. However, these two parameters are specific to the emotional domain, and

point to other non-DAergic structures, e.g. the amygdala. In contrast, the apparent dissociation between anxiety and affiliation on one side, *vs.* social investigation/solicitation on the other, underlines the suggestion that these two latter social parameters may be more directly linked to the accumbal DA tone, and hence likely to be subserved by the meso-limbic, motivational brain system. The loss of social motivation in DAT+ rats comes along with other 'depressive-like' and 'ADHD-like' symptoms: indeed, our previous data (see Adriani *et al.* 2009) demonstrate impaired motivation to explore a novel environment and impulsive choice in the Delay-intolerance task. Thus, these rats may be proposed as a model for social problems, e.g. conduct disorder, often observed among ADHD patients (Blum *et al.* 2000, 2008).

Repertoire of gambling-like traits

Rats underwent a Probabilistic-delivery operant task, specifically tailored to induce a kind of 'temptation' to gamble on the possibility of a rare but bigger prize over the certainty of a lower one (Adriani & Laviola, 2006). Control and Sil rats shifted to the latter option, as expected (Adriani *et al.* 2009). In the first replication, attraction for the rare but bigger prize was evidenced in both DAT and DAT+Sil groups. When the regulatable DAT enhancer was turned off (i.e. under doxycycline exposure during the second replication), a regular choice profile was entirely restored for both DAT and DAT+Sil groups, as would indeed be expected with complete switch-off over 'extra' DAT expression. Interestingly, when the regulatable DAT enhancer was turned on again (i.e. when doxycycline was removed during the third replication), only DAT+Sil rats came back to a clear LLL-preferring profile, similarly to that already shown during the first replication. Namely, a DAT switch-on in the DAT+Sil animals re-introduced a marked temptation to gamble for the large reward despite its rarity.

The risk-proneness induced by DAT switch-on, which was clearly evident in DAT+Sil rats, was not found in DAT+ animals, possibly due to a learning effect during the second and the third replication. Of note, a fundamental difference between these two groups relies on the internal states experienced when the DAT enhancer is turned off (under doxycycline exposure). In fact, DAT+ rats are transiently drawn to a control GFP-like state, whereas DAT+Sil rats transiently experience a full DAT silencing, as in the Sil group. Therefore, the contrast between internal states resulting when turning 'off' and 'on' again exogenous DAT expression (by doxycycline exposure

and removal), is somewhat amplified for DAT+Sil rats. The DAT+ rats undergo the second replication under a control GFP-like internal asset, and may thus learn the task contingencies associated with (poor) likelihood of delivery. Hence, a control-like strategy is developed and then expressed in the third replication despite enhanced DAT. Conversely, DAT+Sil rats do transiently become Sil-like and may express avoidance to the uncertain reward (Cardinal, 2006). These rats, however, are again attracted by the rare but bigger reward when their DAT tone is brought back to the previous 'artificial', disruptive condition. In fact, the DAT+Sil group was never allowed to perform the operant task under a normal, GFP-like state, and this may have altered their incentive-learning abilities. Specifically, although doxycycline-exposed DAT+Sil rats are able to display large-reward devaluation and extinction of LLL preference, these same rats might be unable to recall this previously learned information, and use it instead of actual salience assessment, as was the case for DAT+ animals when doxycycline was removed.

In-vivo screening for metabolic modulation

Brain MRS revealed a decrease in NAc phosphocreatine (both absolute and relative levels) in both groups inoculated with DAT silencers. Thus, a decreased energy metabolism followed accumbal DAT silencing. For the Str (that was not the primary site of inoculation), MRS revealed an increase in total creatine and phosphocreatine for DAT+ and DAT+Sil rats. Thus, a DAT enhancer in the NAc led to increased energy metabolism within side structures of Str (despite silencers if present). Apparently, the enhanced DAT mRNA (although not translated into protein) may trigger some accumbal compensatory feedback which, independently from synaptic DA uptake, may disinhibit the Str.

MRS data suggest a specific re-arrangement of the energy capacity in two functionally related areas, the dorsal and the ventral striatum (i.e. Str *vs.* NAc). Final behavioural phenotypes, observed in the four rat groups, may be explained as a linear combination of these two aspects. In the Sil rats, lower metabolic energetics within NAc may support increased sub-cortical aversion for uncertainty, when waiting requirement becomes excessive as successful deliveries are rare. Indeed, ventro-striatal (NAc) function is needed to overcome spontaneous aversion, classically triggered in rats by probabilistic and/or delayed gratification (Cardinal & Cheung, 2005; Cardinal & Howes, 2005). On the other hand, due to functional

Str up-regulation, DAT+ animals may be more prone to elaborate novel and/or alternative behavioural strategies (Adriani *et al.* 2007). These procedural-learning abilities may have been recruited by DAT+ rats, which are eventually able to show extinction of such habit. Indeed, a control-like curve was shown when they faced the task under DAT-enhancement switch-off (second replication), and that 'normal' choice strategy was then maintained when DAT enhancement was turned on again (third replication). Such a flexible change of strategy was supported by a fully functional NAc (Cardinal *et al.* 2004; Christakou *et al.* 2004; Ragozzino, 2007; Yin *et al.* 2004, 2005), as indicated by MRS.

The DAT+Sil group was the most interesting, since it was characterized by both increased Str function and decreased NAc energetics, as revealed by MRS. When faced with a binge-reward rarefaction, exacerbated gambling-like drives emerged within this group. Not only were these rats more convincingly tempted by a 'rare-but-bigger reward' opportunity than were the DAT+ rats, but they also failed to learn the lesson despite experiencing the same task three times. Consistently, such excessive risk-proneness may emerge as a consequence of: (1) increased habit formation, which originates in the hyper-functional Str, and (2) decreased affective-feedback activity in the NAc, which would be required for a flexible modulation of strategies and for choice adaptation towards a better outcome (Cardinal *et al.* 2004; Christakou *et al.* 2004; Ragozzino, 2007; Yin *et al.* 2004, 2005). With such an unbalanced DAergic energetics (i.e. reduced in the NAc and increased in the Str), rats seem entirely unable to evaluate an outcome as 'adverse' or a reward feature as 'negative', with no feedback onto the motivational value assigned to that goal. Rather, they seem to express a fixed pattern of choice, focused on the bigger prize, despite its uncertainty and rarity. Intriguingly, due to functional NAc silencing and to dominating Str habits, these rats never flexibly adapted their behaviour and their 'gambling-like' habit reappeared during the third replication.

Conclusion

DA, released by meso-limbic pathways onto accumbal targets, subserves the incentive salience (or 'wanting') of reinforcing stimuli and facilitates feedback adaptation for a flexible approach response (Berridge, 2007; Berridge & Robinson, 1998; Cardinal *et al.* 2004; Christakou *et al.* 2004; Salamone & Correa, 2002; Salamone *et al.* 2003). In contrast, ability to display, store and maintain a new pattern, by acquisition

and expression of instrumental strategies and habits, rather involves the nigro-striatal DA pathways (Ikemoto & Panksepp 1999) and their dorsal striatum targets (Ragozzino, 2003, 2007; Ragozzino *et al.* 2002; Yin *et al.* 2004, 2005). In humans, activation of the putamen is associated with decision patterns leading to actual monetary reward (Hollander *et al.* 2005b), while accumbal activation is proportional to the motivation for anticipated gain magnitude (Knutson *et al.* 2005; Schultz *et al.* 1997). Any dysfunction in this hedonic reward/habit-forming cascade may lead to multiple sensation-seeking and impulse-control disorders (Blum *et al.* 2000; Bowirrat & Oscar-Berman 2005; Zuckerman & Kuhlman, 2000).

Presently, DAT silencing in the NAc led to major neurochemical effects, yet behavioural consequences only consisted of increased anxiety, under both environmental and social novelty, together with very poor, risk-averse performance in the operant Probabilistic-delivery task. These data add new insights on the previous literature about DAT knockout mice, which show an inflexible, stereotyped and perseverative pattern of social response, together with novelty-avoidant features (Pogorelov *et al.* 2005; Rodriguiz *et al.* 2004). Conversely, the highest levels of social withdrawal and persistent risk-prone phenotypes were specific of the DAT+Sil group, although evident for DAT+ rats but to a much lesser extent. Concomitantly, the energetic potential was decreased for NAc and enhanced for Str, as clearly revealed by MRS in DAT+Sil rats. We propose that these animals could serve as a model for symptoms observed in cases of comorbidity between ADHD, PG and social avoidance. Further studies on these rats will explore directly the effects of DAT-targeting drugs currently used in clinics, such as methylphenidate. The present findings have implications for a number of neuropsychiatric symptoms, such as those found in ADHD, PG, as well as in other affective-instability and socio-pathological disorders.

Note

Supplementary material accompanies this paper on the Journal's website (<http://journals.cambridge.org/pnp>).

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Statement of Interest

None.

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