

Impulsivity and home-cage activity are decreased by lentivirus-mediated silencing of serotonin transporter in the rat hippocampus



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HIGHLIGHTS

- Lenti-SERT vectors were designed to suppress SERT gene expression *in vivo*.
- We evaluated a rat model of ADHD through hippocampal inoculation of Lenti-SERT.
- Lenti-SERT rats exhibited less pronounced peaks of circadian activity than controls.
- Lenti-SERT rats displayed a transient decrease in cognitive impulsivity.
- Such phenotype is consistent both with 5-HT manipulations and hippocampal lesions.

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ABSTRACT

Brain serotonin (5-HT) systems modulate emotional, motivational and cognitive processes. Mutations in the serotonin transporter (SERT) gene have been associated with susceptibility towards the development of several psychiatric disorders, both in humans and animal models. Present approach exploited a bilateral intra-hippocampus stereotaxic inoculation of lentiviruses, for enduring *in vivo* silencing of SERT. Control rats were bilaterally inoculated with heat-inactivated lentiviruses. These Lenti-SERT vectors were intended to eventually manipulate the neurotransmitter reuptake at synaptic level, thus enhancing tonic 5-HT transmission. We investigated whether such manipulation could induce behavioural alterations relevant to the modelling of ADHD, in particular symptoms of hyperactivity and impulsivity. Wistar rats were monitored for spontaneous home-cage locomotor activity and studied for impulsivity (Intolerance-to-Delay task). Results show that rats inoculated with Lenti-SERT vectors exhibited less pronounced circadian peaks of activity than controls. Moreover, Lenti-SERT compared to control rats exhibited a transient increase in choice for a delayed-larger reward over an immediate-small reward. This suggests that enhanced hippocampal serotonergic transmission produced a profile of restfulness and a decrease in cognitive impulsivity. This phenotype is consistent with available data both on 5-HT manipulations and hippocampal lesions. In conclusion, present findings may possibly disclose novel avenues towards the development of innovative therapeutical approaches for behavioural symptoms relevant to ADHD.

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1. Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a heterogeneous syndrome, affecting 2–5% of infants and adolescents as well as about 2% of adults worldwide [59]. Together with inattention and motor hyperactivity, one key symptom is impulsivity, a multifaceted concept implicated in many disorders characterized by inappropriate inhibitory control [16]. It has been suggested that

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impulsivity is not a unitary construct, but describes a range of behaviours and encompasses a variety of related phenomena that may differ in their biological basis [12,60].

The serotonergic system is well known for modulation of emotional, cognitive and motivational processes [11]. Dysfunctions in this system play a crucial role in many psychiatric disorders, including affective and impulse-control disorders [39,50]. By mediating the interplay between limbic and cognitive loops, forebrain serotonin (5-HT) has a key role in the top-down inhibitory control over behavioural initiation and execution, which is important for withholding of instinctive reactions and for an appropriate feedback regulation of behaviour [11,22]. As such, it is central to psychomotor control over subcortical processing of reward and reinforcement [47].

Permanent *in vivo* interference with gene and protein expression and function is nowadays possible using lentiviral vectors [42]. We have recently used this approach exploiting brain inoculation of lentiviruses targeting the dopamine transporter (DAT): its overexpression in rats' nucleus accumbens leads to an impulsive and risk-prone phenotype [1,2]. Similarly, we prepared a novel vector designed to suppress the serotonin transporter (SERT) gene expression. Indeed, mutations in the SERT gene influence the rate of 5-HT reuptake and have been associated with susceptibility towards the development of several psychiatric disorders [10,53]. Specifically, a common polymorphism (5-HTTLPR), localised in the promoter of the SERT-encoding gene, gives rise to two major allelic variants [31] that significantly change the probability of being affected by ADHD [18,40,54]. In particular, the hippocampus was selected as the inoculation site, since its lesions are known to cause hyperactivity and impulsive choice in rats (e.g. [9,27,46]).

Experimental studies with different serotonergic manipulations have demonstrated an inverse relationship between 5-HT levels and impulsivity, with a reduction in the neurotransmitter causing an increase in impulsivity and vice versa (e.g. [8,55]). However, in humans, an increase in impulsivity appears to be associated with the short (s) allelic variant of 5-HTTLPR, leading to reduced SERT gene transcription [41,51,57]. Thus, we aimed to determine whether a partial silencing of the SERT-encoding gene within the hippocampus could induce alterations relevant to the modelling of ADHD, in particular symptoms of hyperactivity and impulsivity. Specifically, for measuring behavioural impulsivity, the Intolerance-to-Delay (ID) task was used, in which impulsive subjects are detected by their intolerance to periods of forced waiting before the delivery of a large reward [17]. Indeed, delay-discounting paradigms in general are among the most successfully utilised tools for measurement of impulsive choice [60].

2. Materials and methods

2.1. Lenti-SERT vectors

2.1.1. Construction of pTK431-SERT-siRNAs

To silence SERT expression *in vivo*, three targets were designed according to the SERT mRNA sequence. The following targets were selected, based on Hannon's design criterion: 1st target: bp64–29, 2nd target: bp2667–2629, 3rd target: bp1805–1829. To each oligo, a *XhoI* restriction site was added at 3' and a U6-3'-specific 10mer at 5'. Using the pSilencer 1.0-U6 (Ambion, UK) as a template and a U6 promoter-specific forward primer containing *BamHI* restriction site (5'-CGC CGC CGC GGA TCC CGC TCT AGA ACT AGT GC-3'), each siRNA target was added to the mouse U6 promoter by PCR, using the following program: 120 s at 94 °C (initial denaturation) followed by 35 cycles (45 s at 94 °C, 45 s at 64 °C and 45 s at 72 °C) in 4% dimethyl sulfoxide (Sigma, Switzerland). The PCR product

was digested with *BamHI* and *XhoI*, cloned into similar sites into pTK431, and sequenced to verify the integrity of each construct.

2.1.2. Lentivirus production

The vector plasmids (pTK431-U6-siSERT1, pTK431-U6-siSERT2, pTK431-U6-siSERT3 and pTK433-GFP), together with the packaging construct plasmid p891 and the envelope plasmid PMD2-G, were co-transfected into HEK293T cells to produce the viral particles [6,7]. Once harvested and concentrated, these viral particles were tested *in vitro*. The experiment of infection and transfection was run with 3, 10 and 25 μ l of each lentivirus stock (LV-siSERT1, LV-siSERT2, LV-siSERT3) and 20 μ l of a mix of the three siLVs. HEK293T cells were plated and infected with LV-siSERTs and then transfected with a plasmid that expresses SERT. The siLVs were added with Polybrene (Sigma, Switzerland, at 10 μ g/ml final concentration) to the cells. After 7 h, cells were transfected with pcDNA3-rat-SERT (2 μ g/well). The next day, cells were subjected to total RNA isolation, reverse transcription and real-time PCR, to quantify SERT expression/silencing (see Supplementary Data).

2.2. Subjects

Seventeen adult male Wistar rats (400 g; for housing conditions, see Supplementary Data) were randomly assigned to experimental groups: one group received bilateral inoculation of Lenti-SERT vectors (1 μ l of a mix of the three LV-siSERTs) intended to abolish the genetic expression of SERT. Inoculations were made bilaterally at coordinates AP – 3.3, ML \pm 2.2, DV – 4.0 from bregma [45]. The other group (controls) received a bilateral inoculation of heat-inactivated lentiviruses (1 μ l) at the same coordinates (see Supplementary Data). After surgery, rats were single-housed and left undisturbed for at least one month prior to behavioural experiments.

2.3. Circadian cycle

Rats were continuously monitored for spontaneous home-cage locomotor activity [3,4] by means of an automatic device equipped with small passive infrared sensors placed on a standard rack over the top of each home-cage (ActiScope system; TechnoSmart, Rome, Italy). These sensors (20 Hz) detected any movement of rats: scores were automatically divided into 60-min intervals. The spontaneous home-cage activity was continuously measured in Lenti-SERT and control subjects, starting from day 50 after inoculation and over 15 days. From this period, we extracted the central 5-days interval, from which a mean day was calculated.

2.4. ID task for impulsivity

Two months later, rats were daily tested (between 10:30 and 15:30) exploiting classical Skinner-boxes (for apparatus and food-restriction schedule, see Supplementary Data). Nose-poking in one hole (termed "Small & Soon", SS) resulted in the immediate delivery of one pellet in one magazine, whereas nose-poking in the other hole (termed "Large & Late", LL) resulted in the delivery of five pellets in the other magazine. After nose-poking and before food delivery, the chamber light corresponding to the nose-poked hole was switched on for 1 s. Following food delivery, the corresponding magazine light was turned on for 30 s, to signal the length of time-out (TO) during which additional nose-poking was recorded but had no scheduled consequences (i.e. inadequate, see Supplementary Data) [49,50]. The testing phase was preceded by three training sessions at delay 0 s, which allowed subjects to reach a significant preference for LL. During the testing phase (eight daily sessions, 40 min each), a delay was inserted between nose-poking in the LL hole and delivery of the 5-pellet reward. The chamber light over the

LL hole was kept on to signal the entire length of this delay, which was fixed for each daily session and was progressively increased across subsequent days, from 0 s to 90 s. The small-reward delivery was unchanged.

2.5. Data analysis

Data were analyzed using repeated-measures ANOVA. The general model was: 24-level time (hour; circadian cycle study) or 8-level session (delay; ID task) \times 2-level group (treatment). Statistical analysis was performed using Statview II (Abacus Concepts, USA). Data are expressed as mean \pm SEM. Level of significance was set at $P < 0.05$. Multiple post hoc comparisons were performed with the Tukey's HSD test.

3. Results

3.1. Quantification of SERT silencing

Compared to SERT expression level in transfected HEK293T cells (100%), infection with the three siLVs generated the following averaged mRNA expression levels: 78% for LV-siSERT1, 64% for LV-siSERT2, 91% for LV-siSERT3 and 78% for their mix.

3.2. Circadian cycle

Data collected from the automated ActiScope system showed that circadian activity patterns differed depending on treatment (Fig. 1A). Lenti-SERT animals were significantly less active than controls at specific time intervals throughout the day. Specifically, the ANOVA yielded significance for group, time and their interaction (treatment: $F_{(1,15)} = 9.11$, $P = 0.0087$; time: $F_{(23,345)} = 58.81$, $P < 0.0001$; time \times treatment: $F_{(23,345)} = 2.46$, $P = 0.0003$). Lenti-SERT animals showed no differences from controls in the expected diurnal rest pattern, as well as in the well-known peak observed at light switch off. As for nocturnal activity pattern, the progressive increase towards the major level of activity (between 10:00 and 15:00) was significantly less marked in the Lenti-SERT when compared to the control group. A second peak was observed in both groups between 17:00 and 20:00, and again the Lenti-SERT rats were significantly less active than controls.

3.3. ID task for impulsivity: preference (%) for LL reward

As expected, all animals showed a clear-cut shift in preference towards the SS reward as the delay length increased (group: $F_{(1,14)} = 1.52$, $P = 0.2372$; session: $F_{(7,98)} = 52.43$, $P < 0.0001$; session \times group: $F_{(7,98)} = 0.57$, $P = 0.7745$). However, animals belonging to the Lenti-SERT group seemed to display such a shift later (at delay of 45 s) than did control animals (at delay of 30 s). Post hoc comparisons, drawn at this latter value of delay, revealed that LL hole was still significantly preferred by Lenti-SERT rats, whereas a delay-induced shift was already pronounced in the corresponding controls (Fig. 1B). Therefore, we may conclude that Lenti-SERT subjects showed a transiently less impulsive choice than controls.

4. Discussion

Presently, a new approach exploited a lentivirus-mediated silencing of SERT within the hippocampus (HPC), with the aim of generating an animal model for impulsivity and hyperactivity, which in turn are symptoms relevant to ADHD. Such model is to some extent similar to a local knockout of the SERT gene [23], with

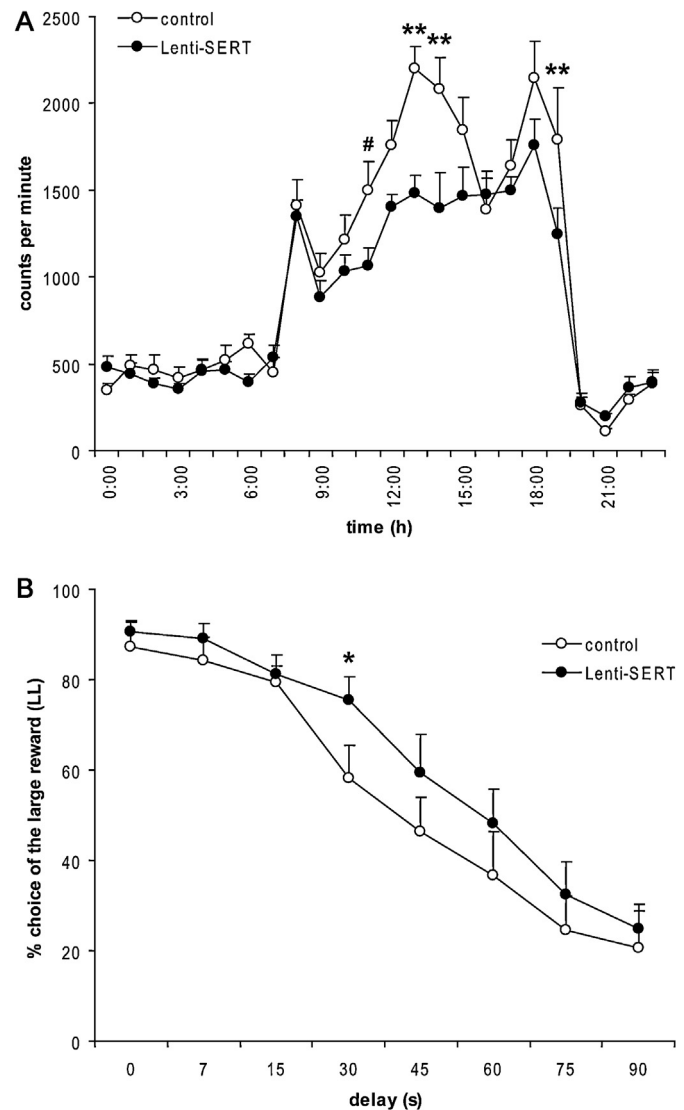


Fig. 1. (A) Circadian activity cycle. Circadian activity (mean \pm SEM) in Lenti-SERT ($n = 11$) and control ($n = 6$) rats, left undisturbed inside their home-cages (counts per minute, summed up to 1-h points). The 24-h profile was obtained by averaging five days of continuous registration. Marked differences were found in the nocturnal activity pattern: activity levels were significantly lower in Lenti-SERT rats compared to controls. $**P < 0.01$; $*0.05 < P < 0.1$ in post hoc test. **(B) Impulsivity in the ID task.** Mean (\pm SEM) choice (%) of the large reward (LL), as a function of absolute delay duration (s), shown by Lenti-SERT ($n = 10$) and control ($n = 6$) animals. Lenti-SERT rats experienced a clear-cut intolerance at a longer delay than did the corresponding controls. $*P < 0.05$ in post hoc test.

the advantages that silencing is performed in the HPC of already adult animals; therefore, this approach may imply less compensatory mechanisms compared to breeding knockout models. We emphasize that our aim was to induce a partial (not a total) silencing of the SERT-encoding gene, thus mimicking to some extent the short (s) allelic variant of 5-HTTLPR. Our results show that rats inoculated with Lenti-SERT exhibited decreased spontaneous locomotor activity and decreased cognitive impulsivity.

SERT, which selectively removes 5-HT out of the synaptic cleft, is a major determinant of serotonergic signalling efficiency, related to the neurotransmitter concentration (e.g. [52]). Studies *in vitro* showed that the s-variant of the 5-HTTLPR was associated with reduced SERT gene transcription efficiency, resulting in reduced SERT levels and reduced 5-HT uptake. Consequently, the extracellular levels of 5-HT were higher compared to the l-variant (e.g. [20,31]). In the present study, the silencing of SERT gene in the

Lenti-SERT rats was intended to produce an improvement of serotonergic tone, which we hypothesized to mimic, to a certain extent, the condition of human subjects carrying the *s*-variant. Similarly, homozygous SERT^{-/-} rodents, which are widely considered to resemble the effects of the *s/s* genotype in humans [25], show alterations in multiple neurobehavioral domains, including behavioural inhibition and decision making [28].

4.1. Circadian rhythm of activity

By analyzing the circadian pattern, the hypolocomotor effect in Lenti-SERT rats appears to be “intrinsic” to animals’ physiology, since it was observed in home-cage conditions and it was stable across days. It was specifically localized around two out of the three daily peaks of activity (in the middle of the dark phase and before the start of the lit period).

5-HT is known to have complex actions with respect to control of activity levels [36], although not all forms of locomotion are equally dependent on central 5-HT transmission [14]. For the purposes of the present paper, it is worthwhile to report that SERT^{-/-} mice show decreased home-cage activity [21]. Increases in home-cage activity have been reported following depletion of brain 5-HT. For example, both PCPA (parachlorophenylalanine, an inhibitor of the 5-HT-synthesizing enzyme) administration and lesions of the median raphe induce an enhancement of locomotor activity in familiar environments [15,19,26]. Conversely, chronic treatment with selective serotonin reuptake inhibitors (SSRIs; that, by inhibiting the reuptake, increase 5-HT extracellular level) caused rats to run significantly less on a running-wheel in their home-cages [5]. We conclude that decreased spontaneous activity exhibited by Lenti-SERT rats is consistent with available data on 5-HT manipulations in rodents. Moreover, HPC lesions are known to increase (nocturnal) home-cage activity (e.g. [27]). Thus, the decrease in home-cage activity observed in present Lenti-SERT rats is also consistent with a possibly improved HPC function, due in turn to higher amount of extracellular 5-HT in this brain area.

4.2. Cognitive impulsivity

Lenti-SERT rats preferred a delayed-larger reward over an immediate-small reward when the waiting interval was 30 s, suggesting that an enhanced HPC serotonergic transmission rendered these animals slightly less impulsive. Central 5-HT manipulations are reported to affect the ability to wait for reinforcement in studies performed with paradigms for either impulsive-choice (e.g. [8,38]) or impulsive-action (e.g. [13,15]). For example, lesions of 5-HT pathways [8,15,38] as well as PCPA administration [8,13] resulted in increased impulsivity. Moreover, SERT^{-/-} rats showed increased correct response latency and decreased premature responding in the 5-choice serial reaction time task (5-CSRTT; [24]). Based on available data, it has been speculated that SERT^{-/-} rats would also show improved “waiting” when reward is delayed [28]. Consistently, acute increase in 5-HT levels, through SSRIs treatment, resulted in decreased impulsivity in a reward-delay task [8].

Impulsive behaviour has been associated not only with orbitofrontal cortex (OFC) lesions (e.g. [29,37]) but also with HPC lesions (e.g. [9,46]). McHugh and colleagues [35] have shown, using a spatial task involving a choice between a delayed-larger vs. an immediate-small rewards, that both OFC- or HPC-lesioned rats showed an increase in impulsive choice. Interestingly, the same group [34] recently reported that, using a non-spatial version of the same task, HPC-lesioned rats exhibited impulsive choice whilst OFC-lesioned rats did not. Thus, the role of OFC in choice with delayed rewards could be more limited [61], and that of the HPC more extensive, than classically thought; this is probably related to HPC role in temporal information processing [34,61]. We may

conclude that the transient increase in choice for a delayed reward, exhibited by Lenti-SERT, supports the hypothesis of an inverse relationship between 5-HT and impulsivity [55], being consistent with available data both on 5-HT manipulations (including pathway lesions, SSRI administration and SERT knockout) and HPC lesions.

4.3. Clinical implications

Studies on the association between impulsivity and the polymorphic variants of 5HTTLPR in humans found rather controversial results [30]: many studies describe a significant association between impulsivity and the *s*-allele of 5-HTTLPR [41,51,57] but about as many studies do not find such a relationship [44,48,58]. As a whole, it may be not surprising that the *s*-allele could be associated with more impulsive performance in clinical research, given the anatomical complexity of the serotonergic projections, the variety of receptor families and subtypes, and the consequential breadth of its functions.

Moreover, although 5-HTTLPR studies in human lymphoblasts and platelets have shown that the *s*-variant is associated with reduced SERT expression and function (e.g. [20,31]), no definitive data exist showing that the *s/s* genotype is associated *in vivo* with decreased SERT expression in human CNS. Indeed, post-mortem studies on brain SERT mRNA levels (e.g. [32,33]) and PET studies on central SERT binding (e.g. [43,56]) have not always confirmed that the 5-HTTLPR variants have different effects on SERT transcription.

5. Conclusions

In summary, by allowing a selective SERT silencing in rats’ hippocampus, the present approach will hopefully contribute to a further understanding of the biological mechanisms underlying psychiatric disorders. The same approach is worth to be explored for modelling other pathologies, above all mood disorders (e.g. depression, anxiety), which are also related to an alteration of SERT function. Noteworthy, rats transfected with a SERT silencer are more restful in home-cage conditions and display more “patience” when facing a reward delay. This may possibly disclose novel avenues towards the development of innovative therapeutical approaches for behavioural symptoms relevant to ADHD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.neulet.2013.05.076>.

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