

## ORIGINAL RESEARCH ARTICLE

## siRNA-mediated knockdown of the serotonin transporter in the adult mouse brain

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Selective serotonin reuptake inhibitors (SSRIs) are widely used antidepressant drugs that increase the extracellular levels of serotonin by blocking the reuptake activity of the serotonin transporter (SERT). Although SSRIs elevate brain serotonergic neurotransmission acutely, their full therapeutic effects involve neurochemical adaptations that emerge following chronic drug administration. The adaptive downregulation of SERT has recently been implicated in the therapeutic response of SSRIs. Interestingly, studies using *SERT*-knockout mice reveal somewhat paradoxical depression-related effects, probably specific to the downregulation of SERT during early development. However, the behavioral significance of SSRI-mediated downregulation of SERT during adulthood is still unknown. We investigated whether somatic gene manipulation, triggered by infusing short interfering RNA (siRNA) into the ventricular system, would enable the downregulation of SERT in the adult mouse brain. Infusing the SERT-targeting siRNA, for 2 weeks, significantly reduced the mRNA levels of SERT in raphe nuclei. Further, a significant, specific and widespread downregulation of SERT-binding sites was achieved in the brain. In contrast, 2-week infusion of the SSRI, citalopram, produced a widespread downregulation of SERT-binding sites, independent of any alterations at the mRNA level. Irrespective of their mechanisms for downregulating SERT in the brain, infusions of SERT-siRNA or citalopram elicited a similar antidepressant-related behavioral response in the forced swim test. These results signify a role for the downregulation of SERT in mediating the antidepressant action of SSRIs in adults. Further, these data demonstrate that siRNA-induced widespread knockdown of gene expression serves as a powerful tool for assessing the function of endogenous genes in the adult brain.

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The serotonin transporter (SERT) controls the temporal and spatial activity of extracellular serotonin by facilitating a rapid and high-affinity reuptake of this neurotransmitter into presynaptic terminals.<sup>1</sup> SERT is also a primary molecular target for the most widely prescribed antidepressant drugs, the selective serotonin reuptake inhibitors (SSRIs).<sup>2</sup> SSRIs readily inhibit SERT activity and elevate the serotonergic tone in the brain. However, full therapeutic benefits ensue only after chronic use of SSRIs, requiring the manifestation of long-term adaptations, secondary to the blockade of serotonin reuptake.<sup>3,4</sup>

Emerging studies reveal downregulation of SERT as an adaptive consequence of sustained and high occupancy by chronically administered SSRIs.<sup>5–8</sup> The

downregulation of SERT favorably potentiates the SSRI-mediated increase in the brain serotonergic neurotransmission.<sup>6–7</sup> Further, the lag time required by SSRIs to produce a substantial downregulation of SERT correlates well with the time needed for eliciting a full therapeutic response. Therefore, downregulation of SERT has been implicated in the antidepressant action of SSRIs.<sup>7</sup>

Recently, *SERT*-deficient mice were used to ascertain the role of SERT in the etiology of depression vs the antidepressant action of SSRIs.<sup>9,10</sup> Consequently, the depression-related behavior illustrated by these mice has been attributed to the developmental changes that result from early-life absence of SERT.<sup>10–12</sup> Indeed, an elevated propensity toward abnormal emotional behavior is noted in adult animals chronically treated with antidepressants during early development,<sup>12–14</sup> or in individuals genetically expressing low levels of *SERT*.<sup>15–18</sup> Therefore, although *SERT*-knockout mice represent a useful model for investigating disorders involving genetic alterations in SERT during early life,<sup>11,19</sup> they are less appropriate

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for studying the partial SERT downregulation observed following chronic use of SSRIs. Moreover, these mice fail to establish the functional relevance of downregulating SERT during adulthood.

In an effort to obtain a specific downregulation of *SERT* in the adult mouse brain, we employed nonviral RNA interference (RNAi) methodology, which we have recently validated *in vivo*.<sup>20</sup> RNAi is a cellular surveillance mechanism that destroys the endogenous mRNAs containing sequences complementary to a double-stranded RNA (dsRNA) trigger.<sup>21</sup> Short interfering RNAs (siRNAs) mimic the cleavage products of dsRNA and produce sequence-specific gene-knockdown in mammalian cells, without eliciting the lethal dsRNA-interferon response.<sup>22,23</sup> Recently, we demonstrated that intracerebroventricularly (i.c.v.) infused siRNAs can efficiently produce a widespread downregulation of gene expression in the adult mouse brain.<sup>20</sup> Here, we compare the neurochemical as well as the behavioral consequences of infusing a SERT-targeting siRNA vs the SSRI, citalopram, in the brain.

## Materials and methods

### Animals

Male BALB/c mice (19–29 g; Iffa Crédo, France) were housed two/cage for at least 1 week before surgery, and thereafter one/cage, in a temperature- and humidity-controlled room with 12–12 h light–dark cycle (lights on at 0600 A. M.). Food pellets and tap water were available *ad libitum*, except during behavioral testing. Animal handling and experimentation were performed during the light cycle, in accordance with the Veterinary Authority of Basel-Stadt, Switzerland.

### Drug/siRNA administration in mouse brain

The sequences of SERT-targeting siRNAs were checked for theoretical specificity against the mouse transcriptome using the Smith Watermann algorithm. In all, 12 different siRNA sequences were accepted, with at least two nonconsecutive and nonterminal mismatches against any other transcript. siRNAs were screened for their *in vitro* knockdown efficiency in a reporter assay, consisting of the cotransfection of siRNAs with a plasmid coding for a SERT-YFP fusion mRNA, as previously described for antisense oligonucleotides.<sup>24</sup> The target sequence for each siRNA (GenBank accession no. NM\_010484), and its efficiency in reducing the mRNA levels from 100% to the value in parentheses, along with the corresponding coefficient of variation, is indicated as follows: nt 429–449 (27%, 4.4), 528–548 (43%, 7.3), 643–663 (20.8%, 13.8), 648–668 (45.7%, 4.7), 801–821 (14.3%, 8.9), 1230–1250 (10.4%, 4.8), 1233–1253 (16.5%, 7.5), 1239–1259 (19.8%, 4.3), 1455–1475 (23.6%, 1.7), 1459–1479 (24.8%, 6.5), 1725–1745 (25.2%, 4.4) and 2061–2081 (12.4%, 12.1). Subsequently, the siRNA, targeting nt 1230–1250, that revealed a consistently maximal knockdown of ~90% was chosen for all *in vivo* experiments. A

corresponding 3-nt-mismatch siRNA (mmRNA; guide, 5'-UUGUGGAACGUGUGGUAGCdTdA-3'; complement, 5'-GCÜACCACACGÜUCCACAAdTdT-3') served as a control. The guide and complement strands of each siRNA were annealed in an isotonic RNAi buffer as described previously.<sup>20,25</sup> Citalopram.HBr was also dissolved in the same RNAi vehicle. Osmotic minipumps (Alzet model 1002, Durect Corporation, Cupertino, CA, USA) were filled in order to infuse vehicle (6 µl/day), citalopram (20 µg/day), SERT-siRNA or mmRNA (0.4 mg/day) for 2 weeks. This duration of infusion was chosen based on our previous studies showing that a maximally effective RNAi response requires 2 weeks of siRNA infusion.<sup>20</sup> A maximally effective dose of siRNA was used that was well tolerated with no signs of neurotoxicity (hind-limb paralysis, vocalization, food intake or neuroanatomical damage) following i.c.v. application for 2 weeks. Implantation of the cannula for infusion into the dorsal third ventricle (AP: -0.5 mm; ML: 0 mm, DV: -3 mm, relative to bregma),<sup>26</sup> from the subcutaneously implanted minipump, was performed as described previously.<sup>20</sup>

### Behavioral assessment

**Forced swim test** was conducted as described previously,<sup>27,28</sup> 14 days after implanting the cannula–minipump assembly. Briefly, mice were individually placed into plexiglas cylinders (24 cm high × 21 cm internal diameter) filled with water (25.4 ± 0.7°C) to a depth of 15 cm. Test sessions were recorded by a video camera positioned directly above the cylinders. A well-trained observer, blinded to the treatment groups, scored these videotapes for the duration of mouse immobility during the last 4 min of the 6 min test period. A mouse was judged to be immobile when making only those movements necessary to keep its head above water. We chose the forced swim test, which is the most widely used paradigm for assessing depression- or antidepressant-related behavior in mice,<sup>29,30</sup> and also because it was sensitive to the partial knockdown of SERT in *SERT*<sup>+/-</sup> mice.<sup>9</sup>

**Locomotor activity** was assessed in a novel environment as described previously,<sup>28</sup> for 60 min between 08:00 and 11:30, ~24 h after conducting the forced swim test.

### Processing of the brain for mRNA and protein analysis

Mice were decapitated immediately after their locomotor activity trial. Brains were removed and serial coronal sections of 10 µm thickness were obtained at the following AP coordinates, in mm, relative to bregma:<sup>26</sup> 2.46 (prefrontal cortex), 1.18 (caudate putamen, nucleus accumbens, olfactory tubercle, lateral and medial septum), -0.22 (globus pallidus), -0.46 (to confirm the site of injection), -1.58 (cerebral cortex, hippocampus, thalamus, hypothalamus, amygdala), -3.08 (substantia nigra, ventral tegmental area), -4.36 (superior colliculus, dorsal and median raphe), -5.34 (locus coeruleus) and -7.2 (brainstem). Sections were thaw-mounted onto poly-L-lysine-

coated slides, coded for a blind analysis of mRNA and protein, and stored at  $-80^{\circ}\text{C}$  until use.

*In situ* hybridization was performed as described,<sup>31</sup> using [ $^{35}\text{S}$ ]-labeled antisense riboprobes, in order to detect the SERT or neuron-specific enolase (NSE) mRNA on adjacent brain sections. Hybridizing brain sections with the corresponding [ $^{35}\text{S}$ ]-labeled sense riboprobes did not reveal any mRNA signal and served as a control for each probe. The DNA templates, for riboprobe synthesis, were generated from cDNA fragments of mouse SERT (nt 187–637; GenBank accession no. NM\_010484) or NSE (nt 2089–2290; GenBank accession no. X52380). At the end of the *in situ* hybridization procedure, slides were dipped in liquid nuclear emulsion and the optical density (OD) of silver grains, positive for the probed mRNA, was quantified as described previously.<sup>31</sup>

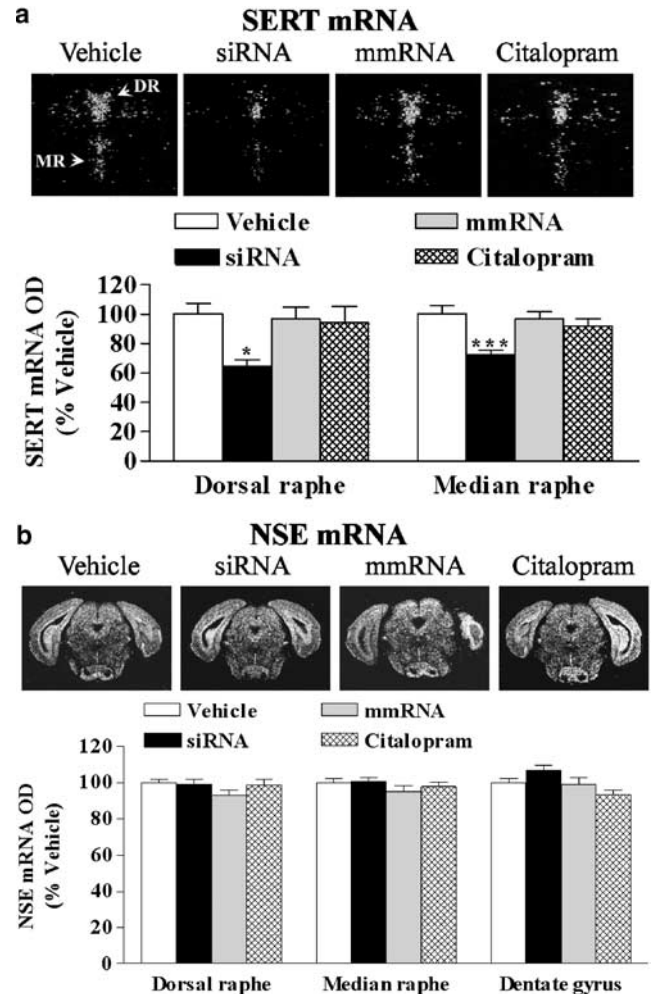
*Quantitative autoradiography* was performed on brain sections as described previously,<sup>20,32</sup> with minor modifications, in order to determine changes in densities of SERT- or dopamine transporter (DAT)-binding sites following various treatments. Briefly, sections were subjected to two washes in an aqueous buffer (120 mM NaCl, 0.1 M sucrose, 10 mM sodium phosphate) for 1 h followed by incubation with a nonsaturating concentration of [ $^{125}\text{I}$ ]RTI-55 (10 pM, 2200 Ci/mmol, Perkin-Elmer, Boston, MA, USA) in fresh buffer for 2 h. [ $^{125}\text{I}$ ]RTI-55 selectively labeled SERT, provided the binding to DAT was occluded with 10  $\mu\text{M}$  GBR-12909. The same radioligand was used to label DAT upon inhibition of SERT binding with 50 nM citalopram. By using tissue-calibrated data from the coexposed radioactive standards, OD values of autoradiograms were transformed to levels of radioactivity bound (nCi/mg tissue protein) to specific brain regions in tissue sections. Nonspecific binding was determined in adjacent brain sections by using 50 nM citalopram and 10  $\mu\text{M}$  GBR-12909, in addition to the radioligand, and was equivalent to the autoradiographic film background.

## Results

### *siRNA-induced specific knockdown of SERT mRNA and protein in the brain*

We recently demonstrated the ability of siRNAs, infused into the dorsal third ventricle, to knockdown genes expressed in caudal brain regions as far as the substantia nigra and ventral tegmental area.<sup>20</sup> Here, we tested if a similar protocol could be used to target *SERT* expressed further away from the site of infusion, specifically in the serotonergic neurons of the dorsal and median raphe nuclei.<sup>1</sup> A maximally effective RNAi response requires 2 weeks of siRNA infusion, irrespective of the half-life of the target protein.<sup>20</sup> The same infusion period is also required by SSRIs in order to produce a substantial downregulation of *SERT*.<sup>6,7</sup> Therefore, mice were infused with the *SERT*-targeting siRNA, a corresponding mmRNA, citalopram or vehicle for 2 weeks into the ventricular

system. *In situ* hybridization analysis revealed a significant downregulation of *SERT* mRNA in the dorsal as well as median raphe of mice receiving siRNA (Figure 1a). *SERT* mRNA levels were unaltered

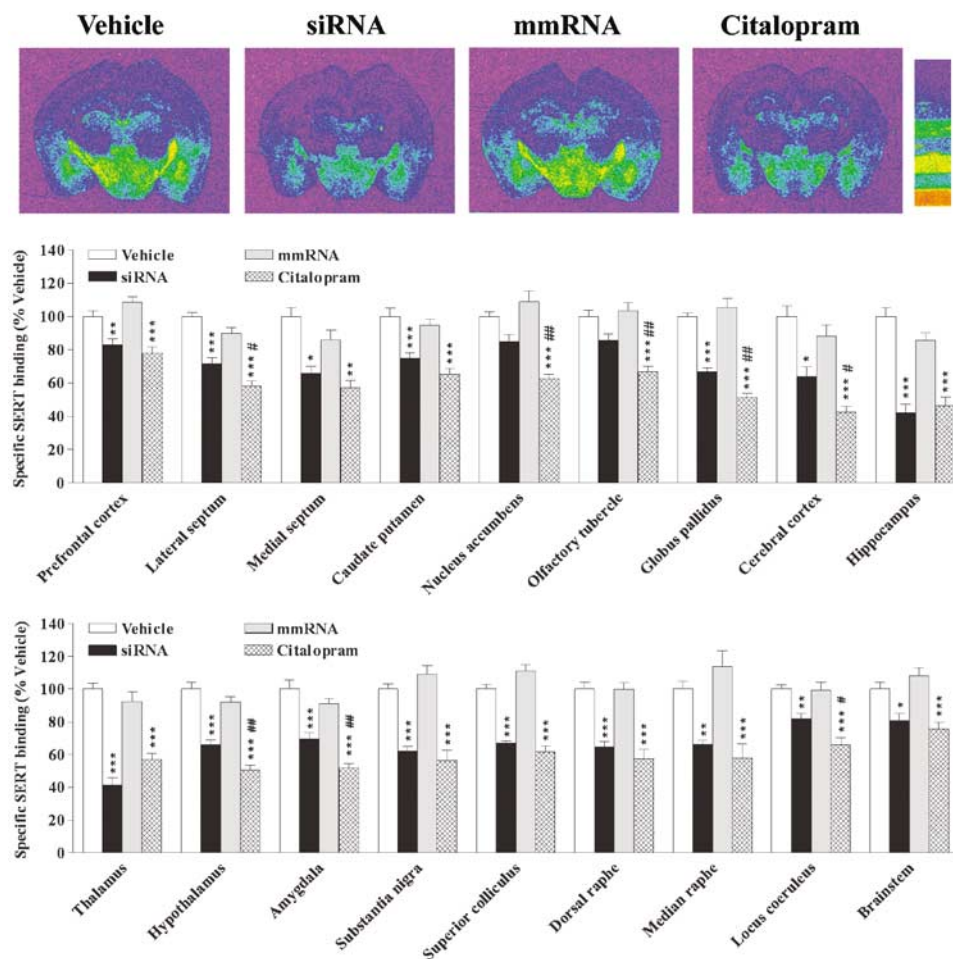


**Figure 1** siRNA-induced specific knockdown of *SERT* mRNA in the dorsal and median raphe nuclei. Mice received a 2-week infusion of vehicle, *SERT*-targeting siRNA, mmRNA or citalopram into the dorsal third ventricle. (a) siRNA infusion significantly reduced the mRNA levels of *SERT* specifically expressed in the dorsal and median raphe nuclei, DR and MR, respectively. (b) *NSE* mRNA levels in the same regions, and the dentate gyrus, were unaffected by any treatment. At the top of each panel are low magnification, dark-field photomicrographs illustrating representatives of *in situ* hybridization with *SERT* (a) or *NSE* (b) riboprobes in adjacent, coronal brain sections followed with emulsion-dipping of slides. The extent of siRNA-induced knockdown is revealed by expressing the densitometric quantification of mRNA positive grains as % OD values of those in corresponding regions from vehicle-treated mice. Bars represent means  $\pm$  SEM of 12–26 observations (2–4 observations/animal, depending on the brain region, and 6–7 animals/group). \* $P < 0.05$ , \*\*\* $P < 0.001$ , significantly different from all other treatments; raw OD values were analyzed using one-way ANOVA followed by Tukey's *post hoc* test.

with infusion of mmRNA or citalopram as compared to those in vehicle-infused mice (Figure 1a). mRNA levels of the neuronal marker, NSE, were quantified in the same regions of adjacent brain sections. None of the treatments, including siRNA, produced a significant change in raphe levels of NSE mRNA (Figure 1b). The dentate gyrus is the most susceptible region to the gene-knockdown effect of i.c.v. siRNA.<sup>20</sup> However, SERT-siRNA or any other treatment had no effect on NSE mRNA levels in this region, indicative of a SERT-specific action of siRNA (Figure 1b).

SERT protein is localized not only in the soma and dendrites of neurons originating in the raphe nuclei but also is transported down the axons to serotonergic projection areas widespread in the brain.<sup>1</sup> [<sup>125</sup>I]RTI-55

autoradiography was performed for quantification of SERT-binding sites in 18 regions along the rostro-caudal axis of the brain. Infusing the SSRI, citalopram, for 2 weeks produced a significant decrease, by ~40–50%, in the density of SERT-binding sites in almost all brain regions analyzed (Figure 2). Infusion of siRNA, but not mmRNA, also led to a significant downregulation of SERT in all these brain regions, except the nucleus accumbens and olfactory tubercle (Figure 2). The extent of siRNA-mediated SERT downregulation varied across brain regions: a maximal 60% effect observed in the hippocampus and thalamus, while only 18% decrease noted in the prefrontal cortex, locus coeruleus and brainstem (Figure 2). All other brain regions exhibited a



**Figure 2** Widespread down regulation of SERT-binding sites following infusion of siRNA or citalopram in the brain. Infusion of the SERT-targeting siRNA or citalopram led to an extensive downregulation of SERT in the brain as compared with infusions of vehicle or mmRNA. (Top) Representative autoradiograms of [<sup>125</sup>I]RTI-55 binding to SERT in sections showing maximum number of brain regions with siRNA- and citalopram-induced downregulation of SERT. The microscale bars from top to bottom represent binding values of 0.74, 1.26 and 2.52 nCi/mg. (Bottom) Densitometric analysis of specific SERT binding is presented as % binding in the corresponding region of vehicle-infused mice, in order to illustrate the extent of siRNA- or citalopram-induced SERT downregulation in each region. Bars represent mean  $\pm$  SEM of 12–52 observations (2–8 observations/animal, varying with the brain regions analyzed, and 6–7 animals/group). Raw values were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , significantly different from vehicle- or mmRNA-treated mice in each brain region; # $P < 0.05$ , ## $P < 0.01$ , significantly different from siRNA treatment in the same region.

moderate decline of ~33% in the density of SERT-binding sites following siRNA treatment (Figure 2). Citalopram-induced downregulation of SERT was marginally, yet significantly, greater than that induced by siRNA in some regions, including the lateral septum, nucleus accumbens, olfactory tubercle, globus pallidus, cerebral cortex, hypothalamus, amygdala and locus coeruleus (Figure 2). Differences in basal levels of SERT binding may possibly account for differences observed among brain regions in siRNA-induced downregulation of SERT. However, this possibility was ruled out due to a lack of any significant correlation ( $r=0.02$ ,  $P=0.93$ ) illustrated between these factors. We also assessed changes in the binding levels of DAT, a protein closely related to SERT, following infusions of vehicle, SERT-siRNA, mmRNA or citalopram. DAT-binding sites throughout the brain were unaffected by any treatment, again, suggesting a SERT-specific effect of siRNA as well as of citalopram (Figure 3).

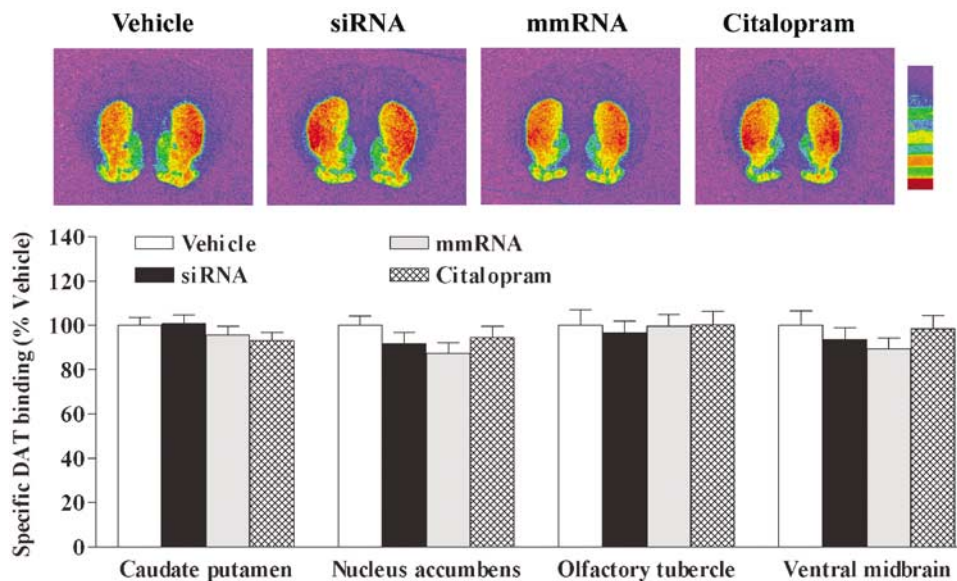
#### Antidepressant-like response in mice receiving SERT-targeting siRNA

The forced swim test is the best characterized and most widely used model for assessing alterations in depression- or antidepressant-related behavior in mice following genetic or drug-induced manipulations.<sup>29,30</sup> The behavioral readout is the duration for which the mouse remains immobile following initial escape-intended movements when subjected to this test. In this case, immobility is thought to reflect a behavioral despair that disengages the mouse from

actively coping with stressful stimuli, also known as 'entrapment' in clinical depression.<sup>30</sup> This model was employed to test if mice displayed a depression- or antidepressant-like phenotype following 2-week infusions of vehicle, SERT-siRNA mmRNA or citalopram. One-way ANOVA revealed a significant effect of treatment on the duration of immobility in the forced swim test ( $F_{3, 36}=4.08$ ,  $P=0.014$ ). Consistent with the antidepressant action of SSRIs, mice infused with citalopram demonstrated a significant decrease in immobility as compared to those receiving vehicle or mmRNA (Figure 4a). Interestingly, the duration of immobility was also significantly reduced to the same extent for siRNA-treated mice (Figure 4a). Further, we tested if the antidepressant-like phenotype, exhibited by citalopram- or siRNA-infused mice in the forced swim test, was confounded by a possible hyperactivity in these mice. A two-way repeated measures ANOVA failed to reveal any significant effect of treatment on locomotor activity ( $F_{3, 396}=1.425$ ,  $P=0.252$ , Figure 4b). A significant effect of time ( $F_{11, 396}=28.188$ ,  $P<0.001$ ) was noted, however, with no significant treatment  $\times$  time interaction ( $F_{33, 396}=0.712$ ,  $P=0.882$ ), indicating that all animals equally habituated to the behavioral assessment, irrespective of their treatments.

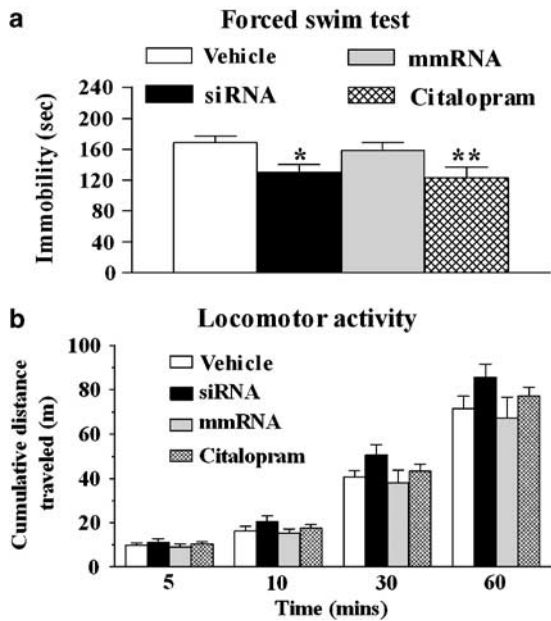
#### Discussion

In the present study, we describe a nonviral, *in vivo* RNAi method for downregulating *SERT* in the adult mouse brain. Recently, we demonstrated that siRNA



**Figure 3** DAT binding levels in the brain are unaltered with SERT-siRNA or citalopram treatment. (Top) Representative autoradiograms of [<sup>125</sup>I]RTI-55 binding to DAT in the caudate putamen, nucleus accumbens and olfactory tubercle. Microscale bars from top to bottom represent binding values of 0.74, 1.26, 2.52 and 5.36 nCi/mg. (Bottom) Densitometric analysis of specific DAT binding presented as % binding in the corresponding region of vehicle-infused mice. Infusing the vehicle, SERT-targeting siRNA, mmRNA or citalopram for 2 weeks had no significant effect on DAT binding in any brain region. Bars represent mean  $\pm$  SEM of 22–26 observations (3–4 observations/animal and 6–7 animals/group).





**Figure 4** Antidepressant-like response in mice treated with *SERT*-targeting siRNA or the SSRI, citalopram. (a) Mice infused with citalopram demonstrated a significantly decreased immobility in the forced swim test as compared to mice infused with vehicle or mmRNA. A similar antidepressant response was noted for siRNA-infused mice. (b) Locomotor activity of these mice was assessed, ~24 h later, as the distance traveled (m) in 5 min intervals upto 60 min. None of the treatments had a significant effect on mouse locomotor activity as shown with the cumulative distance traveled in a 5, 10, 30 or 60 min period. Bars represent mean  $\pm$  SEM values from 10 animals/group. \* $P < 0.05$ , \*\* $P < 0.01$ , significantly different from vehicle, using one-way (a) or two-way repeated measures (b) ANOVA followed by Fisher's *post hoc* test.

infusion into the dorsal third ventricle can knock-down gene expression in regions as far rostral as the prefrontal cortex and as caudal as the substantia nigra.<sup>20</sup> Here, we efficiently target *SERT* expression in the dorsal and median raphe nuclei, ~1 mm caudal to the substantia nigra (~5 mm from the infusion site), which further extends the potential utility of our RNAi protocol to downregulate genes in a broader expanse of the brain.

We compared the neurochemical consequences of infusing a *SERT*-targeting siRNA vs the SSRI, citalopram. Infusion of citalopram i.c.v., for 2 weeks, produced a significant downregulation of *SERT*, by a magnitude equivalent to that previously reported with subcutaneous infusions of SSRIs.<sup>6,7</sup> Further, citalopram-induced downregulation of *SERT* was independent of any changes at the mRNA level, consistent with previous studies using other SSRIs.<sup>5,6</sup> In contrast, 2-week infusion of *SERT*-siRNA specifically reduced the mRNA levels of *SERT* in the dorsal and median raphe nuclei. It may be argued that this effect on *SERT* mRNA is rather modest, and a longer

infusion period is required in order to obtain a substantial knockdown at the mRNA level. However, siRNA-induced specific knockdown of *SERT* was also noted at the protein level to an extent comparable to that produced by citalopram in most of the brain regions analyzed. Further, the possibility of achieving a greater magnitude of *SERT* knockdown, with extended siRNA infusions, seems unlikely considering the half-life of *SERT* is ~3 days.<sup>33,34</sup>

The behavioral consequence of downregulating *SERT*, following infusion of siRNA, was an antidepressant-related response in the forced swim paradigm, the most widely used model for assessing antidepressant-related behavior in rodents.<sup>30</sup> The siRNA-induced antidepressant response was similar to that elicited by the SSRI, citalopram, in this test. Further, this antidepressant response was not due to any hyperactivity *per se*, as the locomotor activity of these mice was unaltered when placed in a novel environment. In marked contrast to the antidepressant-related response of mice infused with either *SERT*-siRNA or citalopram, a depression-related behavior has been illustrated by the *SERT* heterozygous mice in addition to full knockout mice, using the same forced swim paradigm.<sup>9</sup> *SERT* heterozygous knockouts display reduced levels of *SERT* during early development as opposed to the downregulated *SERT* in adult mice obtained using *SERT*-siRNA or citalopram in this study. Therefore, a depression-like behavior of the heterozygous knockouts implies that downregulation of *SERT* during early developmental stages may have a negative influence on emotional behavior. Previous studies have reported an abnormal emotional behavior in animals subjected to an early-life regimen of SSRIs or other less-SSRIs.<sup>12–14</sup> More recently, an increased risk of abnormal emotional behavior was also documented in pediatric patients taking SSRIs.<sup>35</sup> Therefore, it remains to be verified if such deleterious effects result from downregulation of *SERT* following early-life exposure to SSRIs. Interestingly, a downregulation of *SERT* has been reported in the brains of depressed patients.<sup>36–38</sup> Therefore, on the surface these findings are incongruent with our current data and that of Frazer and colleagues.<sup>5–8</sup> However, genetically-mediated downregulation of *SERT* during early life could also explain for the apparent discrepancy between the antidepressant-related outcome of siRNA- or SSRI-induced downregulation of *SERT* and the downregulation reported in the brains of depressed patients.<sup>36–38</sup> Whether these depressed patients were, indeed, genetically predisposed to expressing low levels of *SERT* remains unknown. Clearly, a more comprehensive analyses of an association between the downregulation of *SERT* with polymorphisms in the *SERT* gene and the vulnerability to affective disorders is warranted.

In conclusion, we demonstrate the efficiency of our nonviral RNAi protocol to produce a specific and widespread downregulation of *SERT* in the adult mouse brain. Using this technique, we highlight the functional significance of *SERT* downregulation in

mediating the antidepressant response of SSRIs during adulthood. With an emerging knowledge of mechanisms underlying the regulation of SERT levels at presynaptic nerve terminals,<sup>39</sup> we anticipate that targeting the mechanisms that facilitate a down-regulation of SERT would not only advance our understanding of how SSRI antidepressants work but also potentially lead to the development of more rapid-acting antidepressant agents.

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