Organic cation transporter 3: Keeping the brake on extracellular serotonin in serotonin-transporter-deficient mice

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Mood disorders cause much suffering and are the single greatest cause of lost productivity worldwide. Although multiple medications, along with behavioral therapies, have proven effective for some individuals, millions of people lack an effective therapeutic option. A common serotonin (5-HT) transporter (5-HTT/SERT, *SLC6A4*) polymorphism is believed to confer lower 5-HTT expression in vivo and elevates risk for multiple mood disorders including anxiety, alcoholism, and major depression. Importantly, this variant is also associated with reduced responsiveness to selective 5-HT reuptake inhibitor antidepressants. We hypothesized that a reduced antidepressant response in individuals with a constitutive reduction in 5-HTT expression could arise because of the compensatory expression of other genes that inactivate 5-HT in the brain. A functionally upregulated alternate transporter for 5-HT may prevent extracellular 5-HT from rising to levels sufficiently high enough to trigger the adaptive neurochemical events necessary for therapeutic benefit. Here we demonstrate that expression of the organic cation transporter type 3 (OCT3, *SLC22A3*), which also transports 5-HT, is upregulated in the brains of mice with constitutively reduced 5-HTT expression. Moreover, the OCT blocker decynium-22 diminishes 5-HT clearance and exerts antidepressant-like effects in these mice but not in WT animals. OCT3 may be an important transporter mediating serotonergic signaling when 5-HTT expression or function is compromised.

5HTTLPR | antidepressant | polymorphism | hippocampus | chronamperometry

Dysfunction of the serotonergic system is strongly linked to many psychiatric illnesses, ranging from affective disorders to drug abuse and alcoholism. Serotonin (5-HT) neurotransmission is tightly regulated by high-affinity uptake of released 5-HT by the 5-HT transporter (5-HTT/SERT) (1). The 5-HTT is also a major site of action for many psychotherapeutic and addictive drugs. A common deletional polymorphism in the promoter region (5-HTTLPR) of the human 5-HTT gene confers reduced promoter activity, which a majority of studies show results in lower 5-HTT expression (2–5). Carriers of this short gene variant (*s*) are more prone to psychiatric disorders than noncarriers and are often more resistant to conventional treatment with selective 5-HT reuptake inhibitors (SSRIs) than individuals homozygous for the long allele (*l*) (2–5). The mechanistic basis for this relationship has come under close scrutiny in recent years (6), but remains to be fully elucidated. Here we tested the hypothesis that upregulation of an alternate 5-HT uptake mechanism might contribute to reduced clinical efficacy of SSRIs in carriers of the *s* allele.

Related to this hypothesis, we recently reported that ethanol increases extracellular 5-HT in the CA3 region of the hippocampus by inhibiting 5-HT clearance (7). Surprisingly, however, ethanol inhibition of 5-HT clearance was most pronounced in mice lacking the 5-HTT or with reduced 5-HTT expression. These results unmasked a mechanism for 5-HT clearance and, most significantly, are consistent with the idea that novel transporters for 5-HT may undergo adaptive upregulation when 5-HTT expression or function is constitutively compromised. If proven, these findings may also provide a neurochemical basis for resistance to treatment with SSRIs. Specifically, the buffering imposed by a functionally upregulated alternate transporter for 5-HT may prevent extracellular 5-HT from rising to levels sufficiently high enough to trigger the adaptive neurochemical events necessary for therapeutic benefit.

The identity of such a transporter is yet to be determined. Some efforts have focused on the closely related norepinephrine transporter (NET) and dopamine transporter (DAT), which can both transport 5-HT (8–14). However, although there is evidence that DAT is a compensatory alternative for 5-HT uptake in the ventral tegmental area, substantia nigra, and striatum of mice that lack the 5-HTT (11, 14) there is no evidence supporting such a role for the NET, at least in the CA3 region of hippocampus (15). Organic cation transporters (OCTs) also have a high capacity for 5-HT uptake, albeit with a lower affinity than the 5-HTT (16, 17) and, although yet to be determined, could be a putative site of action for ethanol based on our earlier findings (7). Until very recently, a role for OCTs in regulating central serotonergic neurotransmission has remained unexplored. We now know that OCTs are widely expressed in the brain (18, 19) that they are corticosterone- (20), methamphetamine- (21), and methylenedioxymethamphetamine (MDMA)-sensitive (19) and that their blockade robustly increases extracellular 5-HT in the brain (22). Of particular note is evidence that expression of at least some OCT subtypes is increased in 5-HTT KO mice (23, 24). These converging lines of evidence led us to test the hypothesis that OCTs are functionally upregulated in 5-HTT heterozygote and KO mice. Here we report that OCT3 expression and OCT-mediated clearance of 5-HT are increased in a manner inversely proportional to 5-HTT expression and that OCT blockade has antidepressant-like activity in 5-HTT mutant mice.

Results and Discussion

OCT3 Expression Is Increased in 5-HTT-Deficient Mice. The hippocampus is an important brain structure mediating the therapeutic

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Table 1. OCT1 and OCT3 mRNA expression in hippocampus

	5-HTT$^{+/+}$	5-HTT$^{+/-}$	5-HTT$^{-/-}$
OCT1	1.00 ± 0.06 (7)	1.08 ± 0.09 (7)	1.12 ± 0.04 (7)
OCT3	1.00 ± 0.07 (7)	1.34 ± 0.10 (6)**	1.28 ± 0.06 (4)*

Values expressed as mean ± SEM mRNA/18 SmRNA-relative units. *, $P = 0.025$; **, $P = 0.0158$, number of mice per group shown in parentheses.

response to treatment with antidepressants and is a region where effects of antidepressant drugs have been extensively studied (see ref. 25 for review). Of 3 OCT subtypes (OCT 1, 2, and 3), OCT 1 and 3 are located in neurons and glia in the hippocampi of mice (26) and rats (18–19). OCT3 expression is greater than that of OCT1 in the hippocampus. OCT2 expression is confined to the subventricular regions of the brain and is very low elsewhere (17, 27, 28) and so this subtype was not studied here. As a first step toward understanding the relationship between OCT and 5-HTT expression, we quantified levels of mRNA by using quantitative RT-PCR. As expected, 5-HTT gene expression was dependent on the 5-HTT genotype. In the hippocampus of 5-HTT$^{+/-}$ mice, 5-HTT mRNA levels were approximately half (43 ± 10% $n = 5$; $t_6 = 2.882$; $P = 0.028$) that of 5-HTT$^{+/+}$ mice and were undetectable in 5-HTT KO mice (data not shown). Consistent with published studies (24), we found that OCT1 gene expression in the hippocampus did not differ among genotypes, although there was a trend for higher levels of mRNA in 5-HTT$^{-/-}$ compared with 5-HTT$^{+/+}$ mice (Table 1). Also consistent with the study of Schmitt and coworkers (24), we found that mRNA for OCT3 was increased (+28%) in the hippocampi of 5-HTT$^{-/-}$ mice compared with those of 5-HTT$^{+/+}$ mice ($t_9 = 2.686$, $P = 0.025$). We extended these findings to show that OCT3 mRNA levels were also significantly elevated (+34%) in 5-HTT$^{+/-}$ mice compared with those of WT mice ($t_{11} = 2.85$, $P = 0.0158$) (Table 1).

Next, Western blot analysis was performed to determine whether increased levels of mRNA in 5-HTT mutant mice were accompanied by corresponding increases in protein expression. Fig. 1*A* shows that OCT3 protein is increased in 5-HTT$^{+/-}$ (+27%) and 5-HTT$^{-/-}$ (+36%) mice relative to 5-HTT$^{+/+}$ mice (5-HTT$^{+/-}$ $P = 0.0276$; 5-HTT$^{-/-}$ $P = 0.0063$, versus 5-HTT$^{+/+}$, 1-way ANOVA with Tukey's post hoc test, $n = 9$/genotype). Hippocampal OCT1 protein expression did not vary among 5-HTT genotypes (OCT1/β-actin expression ratios were 0.834 ± 0.037, 0.847 ± 0.045 and 0.799 ± 0.059 for 5-HTT$^{+/+}$, 5-HTT$^{+/-}$, and 5-HTT$^{-/-}$ mice, respectively; $n = 6$ per genotype). These data led us to focus on OCT3 as a primary candidate for regulation of 5-HT clearance in mice lacking 5-HTT or with constitutively reduced 5-HTT expression. Fig. 1*B* illustrates the reciprocal relationship between 5-HTT and OCT3 expression in the hippocampi of 5-HTT mutant mice, providing a rationale for pursuing the hypothesis that OCT3 function may be increased in response to reduced 5-HTT expression.

Before assessing the function of OCT3 we first determined its distribution in the hippocampus to confirm its expression in regions where SSRIs have potent actions to inhibit 5-HT clearance (7, 13) and to aid in the selection of stereotaxic coordinates for placement of carbon fiber electrodes used for high-speed chronoamperometric recordings of 5-HT in vivo. Fig. 1*C* shows OCT3 immunoreactive staining in a coronal section of a mouse (5-HTT$^{+/+}$) hippocampus. OCT3 was located most densely in the pyramidal and granule cell layers of the hippocampus and appeared to be contained primarily within the neuronal cell bodies (24, 26, 29). Importantly, like others (24) we did not detect differences in the distribution of OCT3 immunostaining in the hippocampus among 5-HTT genotypes, indicating that the cellular distribution of OCT3 does not change as a function of

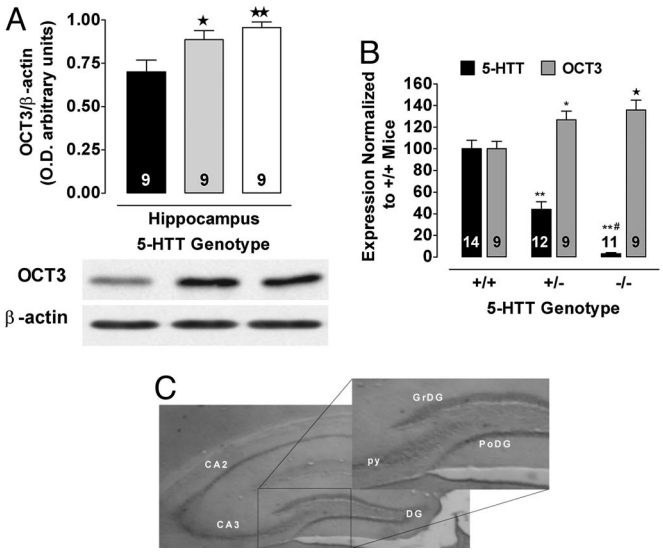

Fig. 1. OCT3 protein expression is increased in the hippocampi of 5-HTT$^{+/-}$ and 5-HTT$^{-/-}$ mice compared with those of 5-HTT$^{+/+}$ mice. (*A*) (*Upper*) OCT3 expression is increased by 27% and 36% in 5-HTT$^{+/-}$ (★, $P = 0.0276$) and 5-HTT$^{-/-}$ (★★, $P = 0.0063$) mice compared with their WT counterpart (1-way ANOVA with Tukey's post hoc comparison). (*Lower*) Representative Western blot showing OCT band at 70 kDa using an OCT3-specific antibody and β-actin loading control at 40 kDa. (*B*) Reciprocal expression of 5-HTT and OCT3 in 5-HTT mutant mice. Expression for each transporter was normalized to the expression level in WT mice. The values used to calculate 5-HTT expression were derived from our published [^{3}H]cyanoimipramine binding to the 5-HTT (14). Values for OCT3 were derived from the data shown in *A*. (**, $P < 0.001$, ★, $P < 0.01$, *, $P < 0.05$ compared with 5-HTT$^{+/+}$; #, $P < 0.01$ compared with 5-HTT$^{+/-}$, 1-way ANOVA with Tukey's post hoc comparison). (*C*) OCT3 immunostaining in hippocampus using the same OCT3-specific antibody as for Western blots. OCT3 was detected in the CA1, CA3, and dentate gyrus regions of the hippocampus. Staining was most pronounced in the CA3 region of the hippocampus, as well as in the polymorph region of the dentate gyrus, the granular layer of the dentate gyrus, and the pyramidal cell layer. A section of the CA3 region is enlarged to illustrate specific details of these regions.

5-HTT genotype. In WT and 5-HTT$^{+/-}$ mice, 5-HTTs are also expressed in these hippocampal layers (15, 30, 31). Thus, OCT3 is also positioned in the proximity of 5-HT terminals and therefore OCT3 could increase its contribution to 5-HT clearance when 5-HTT expression and/or function are compromised.

OCT3 Function Is Increased in 5-HTT-Deficient Mice. Having established that OCT3 expression is increased in the hippocampi of 5-HTT$^{+/-}$ and 5-HTT$^{-/-}$ mice, we reasoned that the potency of decynium-22 (D-22), a blocker of OCT3 (32), to inhibit 5-HT clearance would be greater in these mutants than in 5-HTT$^{+/+}$ mice. We used high-speed chronoamperometry to measure the clearance of 5-HT locally applied into the CA3 region of the hippocampi of anesthetized mice before and after intrahippocampal application of D-22. The CA3 region was selected based on our finding that OCT3 is expressed in this region and because the norepinephrine and dopamine transporters do not contribute significantly to 5-HT clearance under conditions used here [but see refs. 13, 33, and 34 and supporting information (SI) *Text*]. Before use in vivo, we assessed the effect of D-22 on the electrochemical properties of the Nafion-coated carbon fiber electrodes used for chronoamperometric recordings. D-22 itself did not produce an oxidation current. However, when applied at concentrations exceeding 1.0 μM the sensitivity of the carbon fiber to detect 5-HT was reduced, likely because of adherence of D-22 to the surface of the electrode. Because of this circumstance, we limited our dose–response analysis to 1.4 pmol

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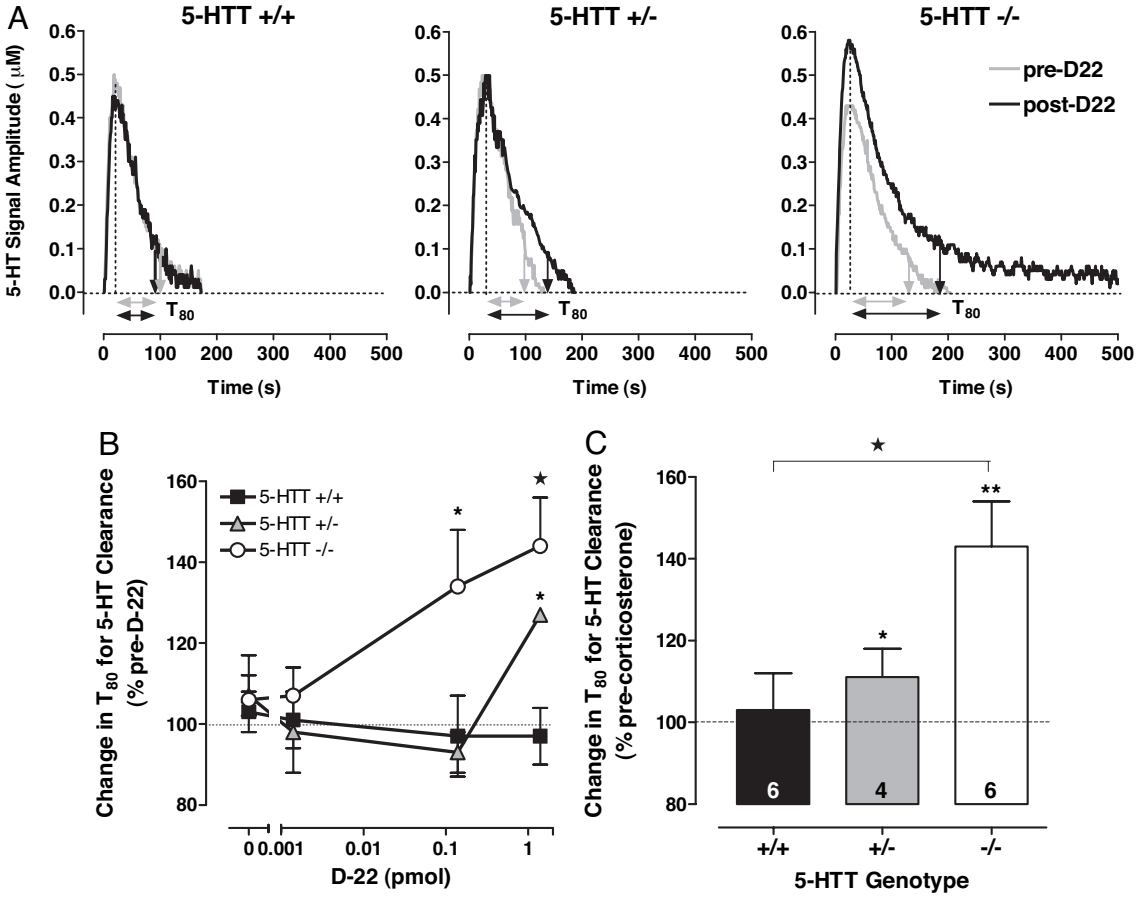

Fig. 2. OCT blockade inhibits 5-HT clearance in the hippocampi of 5-HTT mutant mice but not WT mice. (*A*) Representative oxidation currents (converted to micromolar values) produced by pressure ejection of 5-HT into the CA3 region of hippocampi before (gray line) and 2 min after (black line) intrahippocampal administration of D-22 (1.4 pmol) in 5-HTT$^{+/+}$, 5-HTT$^{+/-}$, and 5-HTT$^{-/-}$ mice, respectively. Raw tracings are superimposed for ease of comparison. Note the marked increase in T_{80} after D-22 in 5-HTT mutant mice compared with 5-HTT$^{+/+}$ mice. (*B*) Serotonin-clearance-inhibiting effects of hippocampally applied D-22 are evident only in 5-HTT mutant mice. Increasing concentrations of D-22 caused a significant increase in T_{80} values (expressed as a percent of baseline values) in hippocampi of 5-HTT$^{-/-}$ and 5-HTT$^{+/-}$ mice but was without effect in 5-HTT$^{+/+}$ mice. ★, $P < 0.001$; ∗, $P < 0.05$ vs. 5-HTT$^{+/+}$. Vehicle ejection did not influence T_{80} values. The percent change from prevehicle baseline was 103 ± 5 ($n = 8$), 107 ± 5 ($n = 8$) and 107 ± 14 ($n = 4$) for 5-HTT$^{+/+}$, 5-HTT$^{+/-}$, and 5-HTT$^{-/-}$ mice, respectively. (*C*) Corticosterone inhibits 5-HT clearance in the hippocampi of 5-HTT mutant mice. The OCT antagonist, corticosterone (55 pmol), inhibited 5-HT clearance in hippocampi of 5-HTT$^{+/-}$ (∗, $P < 0.05$) and 5-HTT$^{-/-}$ (∗∗, $P < 0.001$) mice, compared with predrug baseline (paired *t* test). Compared to 5-HTT$^{+/+}$ mice, corticosterone inhibited 5-HT clearance in hippocampi of 5-HTT$^{-/-}$ mice. ★, $P < 0.01$, 1-way ANOVA with Tukey's post hoc comparisons. Data are expressed as mean and SEM.

(pressure-ejection of 140 nL of 10 μM D-22). The concentration of the drug reaching the recording electrode is estimated to be between 10- and 200-fold more dilute than the concentration in the micropipette (33, 35). Thus, by not exceeding a barrel concentration of 10 μM, the sensitivity of the carbon fiber electrode for 5-HT was preserved. Highlighted in Fig. 2*A* and *B* is the 5-HTT-genotype dependency of the effect of D-22 to inhibit 5-HT clearance. Serotonin was pressure-ejected into the CA3 region to generate reproducible signals of ≈0.5 μM (peak amplitude) and then D-22 or an equivalent volume of vehicle PBS was applied directly into the same region via a glass multibarrel pipette. Representative traces are shown in Fig. 2*A*. D-22 slowed 5-HT clearance in a concentration-dependent ($F3,58 = 11.93$; $P = 0.0116$) and genotype-dependent ($F2,58 = 11.00$; $P = 0.0062$) manner. As evident in Fig. 2*B*, there was also a shift to the left in the dose–effect relationship in 5-HTT$^{-/-}$ mice, relative to 5-HTT$^{+/-}$ and 5-HTT$^{+/+}$ mice. Clearance of 5-HT in 5-HTT$^{-/-}$ mice was significantly inhibited by D-22 at both 0.14 and 1.4 pmol amounts ($P < 0.001$), whereas only the highest dose (1.4 pmol) of D-22 produced significant inhibition in 5-HTT$^{+/-}$ mice ($P < 0.05$). In 5-HTT$^{+/+}$ mice, D-22 did not affect 5-HT clearance at any of the pmol amounts tested. Vehicle was without effect on 5-HT clearance in all genotypes. Consistent with our published findings (7, 15), baseline clearance times (T_{80}, the time for the 5-HT signal to decline by 80% of the peak amplitude) were longer in 5-HTT$^{-/-}$ mice (110 ± 5 s, $n = 5$), compared with 5-HTT$^{+/-}$ (83 ± 14 s, $n = 6$) and 5-HTT$^{+/+}$ mice (75 ± 8 s, $n = 8$) and were not different between 5-HTT$^{+/+}$ and 5-HTT$^{+/-}$ mice (7, 15). Two minutes after intrahippocampal D-22 (1.4 pmol), T_{80} values increased further in 5-HTT$^{-/-}$ (156 ± 29 s) and 5-HT$^{+/-}$ (103 ± 13 s) mice but not in 5-HTT$^{+/+}$ (74 ± 5 s) mice (Fig. 2*B*). In 5-HTT$^{+/-}$ and 5-HTT$^{-/-}$ mice, the 5-HT signal returned to predrug values within 30 min after application of D-22. Signal amplitude was not significantly different among genotypes before drug (0.51 ± 0.09 μM, 0.53 ± 0.05 μM, and 0.54 ± 0.05 μM for 5-HTT$^{+/+}$, 5-HTT$^{+/-}$, and 5-HTT$^{-/-}$ mice, respectively). D-22 did not significantly affect signal amplitude in any genotype, although there was a trend for the amplitude to be greater in 5-HTT$^{-/-}$ mice 2 min after D-22 (0.60 ± 0.04 μM) when compared with baseline signal ampli-

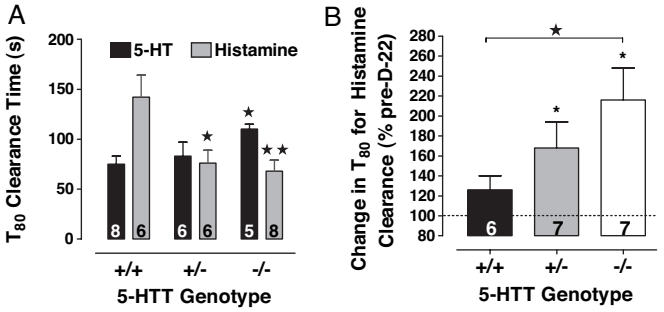

Fig. 3. Histamine clearance is faster and inhibited more profoundly by OCT blockade in 5-HTT mutant mice than in WT mice. (*A*) Basal histamine T_{80} values were significantly faster in 5-HTT$^{-/-}$ and 5-HTT$^{+/-}$ mice than in 5-HTT$^{+/+}$ mice. Shown is the reciprocal relationship between basal histamine and 5-HT T_{80} values, where 5-HT clearance is significantly longer in 5-HTT$^{-/-}$ mice than 5-HTT$^{+/+}$ or 5-HTT$^{+/-}$ mice. ★, $P < 0.05$ and ★★, $P < 0.01$ vs. 5-HTT$^{+/+}$ (1-way ANOVA with Tukey's post hoc comparisons). (*B*) Intrahippocampally applied D-22 (1.4 pmol) caused a robust percent increase in T_{80} values for histamine clearance over pre-D-22 baseline values in hippocampi of 5-HTT$^{-/-}$ and 5-HTT$^{+/-}$ mice but was without effect in 5-HTT$^{+/+}$ mice. Data shown are mean and SEM for 2 min after injection of D-22. *, $P < 0.05$ compared to predrug baseline value (paired *t* test); ★, $P < 0.05$ compared with 5-HTT$^{+/+}$ mice (1-way ANOVA with Tukey's post hoc comparisons).

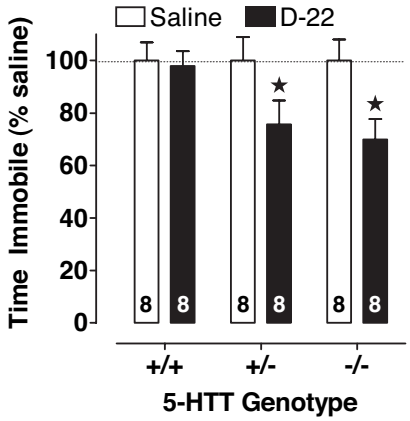

Fig. 4. Blockade of OCT3 elicits antidepressant-like effects in the tail suspension test in 5-HTT mutant mice but not in WT mice. Data shown are for the TST carried out 60 min after injection of D-22 or saline solution. Data are expressed as a percent of immobility time in saline-treated mice within genotype. All data are expressed as mean and SEM. ★, $P < 0.05$ vs. 5-HTT$^{+/+}$, ANOVA with Tukey's post hoc.

tude. Consistent with the idea that a low-affinity (but high-capacity) transporter for 5-HT is involved in mediating this inhibitory action of D-22 on 5-HT clearance, these effects were more pronounced when the extracellular concentration of 5-HT achieved at the recording electrode, after its local application, produced signal amplitudes exceeding 2.0 μM (see Fig. S1).

Together, the present data indicate that OCTs are capable of taking up 5-HT from extracellular fluid, but this is revealed only when 5-HTT expression is compromised. However, in addition to its action at the OCT, D-22 has an appreciable affinity for the plasma membrane monoamine transporter (PMAT), which can also transport 5-HT (36). To distinguish OCT-mediated 5-HT uptake from that mediated by PMAT, we examined 5-HT clearance in the hippocampus in response to locally applied corticosterone (55 pmol), which blocks OCT3 but not PMAT (20, 36). As shown in Fig. 2*C*, there was again a significant effect of genotype. Whereas corticosterone had no effect on 5-HT clearance in 5-HTT$^{+/+}$ mice, the magnitude of inhibition of 5-HT clearance increased in a fashion inversely proportional to the density of 5-HTTs, being greatest in 5-HTT$^{-/-}$ mice ($F2,15 = 8.609$; $P = 0.0032$). These data strongly suggest that the effect of D-22 and corticosterone to inhibit 5-HT clearance is mediated by OCT and not PMAT.

Although 5-HT is the major substrate for the 5-HTT, it is also a substrate for OCT and other transporters, including NET and DAT (12–14). Histamine is a substrate for OCT3 but not other biogenic amine transporters (20). Therefore, to isolate OCT-mediated uptake from that mediated by 5-HTT and other transporters, we measured the clearance of histamine from the CA3 region of the hippocampus. Consistent with our evidence that OCT3 expression and function are increased in 5-HTT mutants, histamine clearance was faster in 5-HTT$^{+/-}$ (76 ± 13 s, $n = 6$) and 5-HTT$^{-/-}$ mice (68 ± 11 s, $n = 8$) than in 5-HTT$^{+/+}$ mice (142 ± 22 s, $n = 6$) (F2,17 = 6.814; $P = 0.0067$) (Fig. 3*A*). Likewise, the effect of D-22 to inhibit histamine clearance was significantly greater in 5-HTT mutant mice than WT mice (F2,17 = 4.146; $P = 0.0342$) (Fig. 3*B*). Peak signal amplitudes produced by intrahippocampally applied histamine did not differ among genotypes and were 0.50 ± 0.06, 0.44 ± 0.04 and 0.45 ± 0.06 for 5-HTT$^{+/+}$, 5-HTT$^{+/-}$, and 5-HTT$^{-/-}$ mice, respectively. Amplitude was not significantly altered after application of D-22.

OCT Blockade Has an Antidepressant-Like Effect. One way that antidepressant drugs are thought to initiate therapeutic efficacy is by increasing extracellular 5-HT. The robust inhibitory effect of D-22 on 5-HT clearance in the CA3 region of the hippocampus in 5-HTT mutant mice, led us to test the hypothesis that D-22 might have antidepressant efficacy in these mice, an idea consistent with that proposed by Schildkraut and Mooney (37). To test this hypothesis, we measured the time that mice spent immobile in the tail suspension test (TST), a commonly used mouse model to test drugs for antidepressant-like activity (see ref. 38 for review). We reasoned that because 5-HT clearance was slowed after application of D-22 in 5-HTT$^{+/-}$ and 5-HTT$^{-/-}$ mice (and therefore extracellular 5-HT levels were elevated from the basal, regardless of what the level might be), we might expect an antidepressant-like effect of D-22 in 5-HTT mutant mice. In contrast, because D-22 did not change 5-HT clearance in 5-HTT$^{+/+}$ mice, we hypothesized that D-22 would have little, if any, antidepressant-like effect in this 5-HTT genotype. This is indeed what we found. Time the mice spent immobile was significantly decreased 30 min after application of D-22 (1.0 μg/kg, i.p.) compared with saline-treated mice ($F1,42 = 10.05$, $P = 0.0034$, data not shown). However, the decrease in immobility time only reached statistical significance in 5-HTT$^{-/-}$ mice ($P < 0.05$, Tukey's post hoc). In a second TST 60 min after injection, immobility time remained less in D-22-treated 5-HTT mutant mice compared with saline-treated control mice ($F1,42 = 12.76$, $P = 0.0121$). The antidepressant-like effect of D-22 persisted in 5-HTT$^{-/-}$ mice ($P < 0.05$) and became significant in 5-HTT$^{+/-}$ mice ($P < 0.05$, Tukey's post hoc). Again, D-22 treatment did not result in any significant change in immobility time in 5-HTT$^{+/+}$ mice. These data were mirrored by the time course for systemically administered D-22 to exert its inhibitory effect on 5-HT clearance (see Fig. S2). Fig. 4 illustrates results for the TST carried out 60 min after injection of D-22 or saline solution where data for each genotype are presented as a percent of immobility time of saline-treated mice. The antidepressant-like effects of D-22 were apparent in 5-HTT mutant mice but absent in WT mice (main effect treatment: $F1,42 = 15.01$, $P = 0.0052$). It is important to note that the baseline phenotypes of these mice

in the TST are consistent with those of Holmes *et al.* (39) and show no difference among 5-HTT genotypes (bred on a C57BL/6 background as we used) in the amount of time spent immobile. All genotypes spent approximately half of the 6 min test period immobile. For example, time spent immobile in the test 60 min after the injection of saline solution was 167 ± 13 s, 164 ± 17 s, and 179 ± 16 s for 5-HTT$^{+/+}$, 5-HTT$^{+/-}$, and 5-HTT$^{-/-}$ mice, respectively (n = 8 per group). By comparison, after D-22 the time spent immobile was 164 ± 16 s, 125 ± 27 s, and 125 ± 16 s in 5-HTT$^{+/+}$, 5-HTT$^{+/-}$, and 5-HTT$^{-/-}$ mice, respectively (n = 8 per group).

An interesting aspect of these data in comparison with those of Holmes *et al.* (39), is that Holmes *et al.* found that the tricyclic antidepressant and NET blocker desipramine (DMI) decreased the time 5-HTT$^{-/-}$ spent immobile in the TST to a greater extent than 5-HTT$^{+/+}$ and 5-HTT$^{+/-}$ mice. Although the most parsimonious explanation might be that NET upregulates in 5-HTT$^{-/-}$ mice, there is currently no evidence to support this idea (15), but interestingly DMI has appreciable affinity for OCTs (IC_{50} values ranging from 5–14 μM) (17). Taken together with the present data, OCTs might well contribute to this greater antidepressant-like effect of DMI in 5-HTT$^{-/-}$ mice. Along these lines, it is noteworthy that mice subjected to acute footshock 2–4 min before the TST spend less time immobile than control mice (40). The footshock resulted in a dramatic increase in plasma corticosterone. Thus, the antidepressant-like effect of acute stress might also be explained by acute blockade of OCT3 by corticosterone. This supposition is supported further by a recent study reporting that exogenous administration of corticosterone to mice produced greater reduction in immobility time than DMI administered via the same route and dose (41). Currently desipramine, imipramine, and citalopram are the only antidepressants for which affinity at the human OCT3 have been reported, with IC_{50} values of 14, 42, and 158 μM (17). Thus, whether OCT3 contributes to the antidepressant action of currently prescribed medications remains unknown.

The dose of D-22, 1.0 μg/kg, used in our study was chosen based on pilot studies to identify a dose that did not produce directly observable effects that may interfere with TST performance. At a higher dose of 1.0 mg/kg, D-22 decreased locomotion. To our knowledge, there is only 1 published report documenting the behavioral effects of D-22. In that study, D-22 was administered directly into the medial hypothalami of rats via a microdialysis probe (22). Those authors reported an increase in the amount of time rats spent grooming, but no other overt behavioral effects were observed. Over a range of doses tested in pilot studies we selected 1.0 μg/kg D-22 for our studies using the TST, because this dose induced a modest amount of grooming compared with saline-injected mice and thereby demonstrated that D-22 was likely acting centrally. Importantly, locomotor activity in mice measured after injection of D-22 was not different from those mice given saline solution (see Fig. S3). It is unlikely therefore, that increased general motor activity accounts for the decreased immobility time of 5-HTT$^{+/-}$ and 5-HTT$^{-/-}$ mice in the TST after D-22 treatment. Together these data raise the possibility that blockers of OCT3 may be effective antidepressants. To be clinically useful, antidepressant effects should persist after chronic treatment. Our preliminary data suggest that D-22 continues to produce antidepressant-like effects in the TST after repeated treatment. For example, 5-HTT$^{-/-}$ mice given D-22 daily (1.0 μg/kg, i.p.) for 4 days and then administered saline or D-22 on the fifth day 60 min before the TST, spent 163 ± 28 s (saline, n = 3) and 108 ± 16 s (D-22, n = 5) immobile (see *SI Text*).

Clinical Implications. Our data show that OCT3 contributes to 5-HT clearance when 5-HTT expression is low or absent. This finding has potentially far-reaching implications. Already reports have emerged showing an important role for OCTs in central nervous system regulation of dietary salt intake (26) and the stress axis (20), as well as their regulation by chronic treatment with the psychostimulant methamphetamine (21). Related to this finding, a polymorphism of the gene encoding OCT3 was recently linked to methamphetamine-use disorder (42). Interestingly, OCT3 KO mice, although normal in a number of behavioral and neurochemical measures, have increased sensitivity to psychostimulants and increased levels of anxiety (43). These findings, together with data presented here, suggest that OCT3 may be important in the homeostatic regulation of serotonin neurotransmission, particularly in the face of constitutively reduced 5-HTT expression or function. Given the important role for 5-HT during critical periods of development, the level of OCT3 expression and function over these periods could have major implications (44). For example, autism is linked to abnormal development of the 5-HT system and to variants in the 5-HTT gene (44, 45).

Our data support the idea that, in mice whose genetic makeup imposes reduced 5-HTT expression, OCT3 expression and function increase as a compensatory alternative for 5-HT uptake. There is evidence that humans carrying the *s* allele of the 5-HTTLPR, who presumably have lower 5-HTT expression, are more prone to psychiatric disorders and are often resistant to treatment with SSRIs compared with individuals homozygous for the *l* allele (6, 46). Our data, together with that of others (47), suggest that OCT3 may serve to buffer the increase in 5-HT that follows antidepressant treatment. This in turn may prevent 5-HT levels from climbing sufficiently high enough to trigger the cascade of events which ultimately lead to therapeutic efficacy and may, in part, account for treatment resistance in some patients, including those for whom antidepressants lose their therapeutic utility over time. For example, because it is known that chronic treatment with SSRIs downregulates 5-HTT expression in mice (48, 49), as well as in cells transfected with human 5-HTT (50, 51), it will be interesting in future studies to investigate the possibility that chronic pharmacological inactivation of 5-HTT also leads to compensatory upregulation of OCT3. Further characterization of OCT3 function in the brain will be an important line of investigation that may help us to better understand many serotonin-related disorders.

Methods Summary.

We generated 5-HTT mutant mice, bred on a C57BL/6 background described previously (52). Male 5-HTT$^{+/+}$, 5-HTT$^{+/-}$, or 5-HTT$^{-/-}$ mice, weighing 25–30 g, were used for all experiments. RT-PCR was used to quantify OCT1 and OCT3 mRNA according to published methods (23). A Western blot analysis of OCT1 and OCT3 protein expression was performed with commercial antibodies to OCT1 and OCT3 (Alpha Diagnostics International) according to the method of Sata *et al.* (53). Tissue was assayed for protein content by the method of Bradford (54). Immunocytochemical staining for OCT3 was performed by using the same antibody as was used for Western blot analyses (see *SI Text* for details). High-speed chronoamperometry was used according to established protocols (*SI Text*) (33) to measure clearance of 5-HT and histamine from extracellular fluid of the hippocampi of anesthetized mice in vivo as well as the effect of locally applied D-22 on the clearance of these transmitters. All solutions were applied intrahippocampally by pressure ejection, and electrode placement was verified histologically at the conclusion of each experiment (55). The TST was based on the method of Steru *et al.* (56). Mice were injected i.p. with either D-22 (1.0 μg/kg) or saline solution and then placed in an observation chamber for 30 min. Immediately after this period they were securely fastened by the distal end of the tail to a flat metallic surface and suspended in a visually isolated area (40 × 40 × 40 cm white box). The amount of time spent immobile, defined as the absence of limb movement, was recorded over a 6-min test session by a trained observer who remained blind to genotype and treatment. The mice underwent a second TST 60 min after injection of D-22 or saline solution. The effects of genotype and drugs were analyzed with ANOVA followed by Tukey's post hoc tests. Data are presented as the mean and SEM.

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