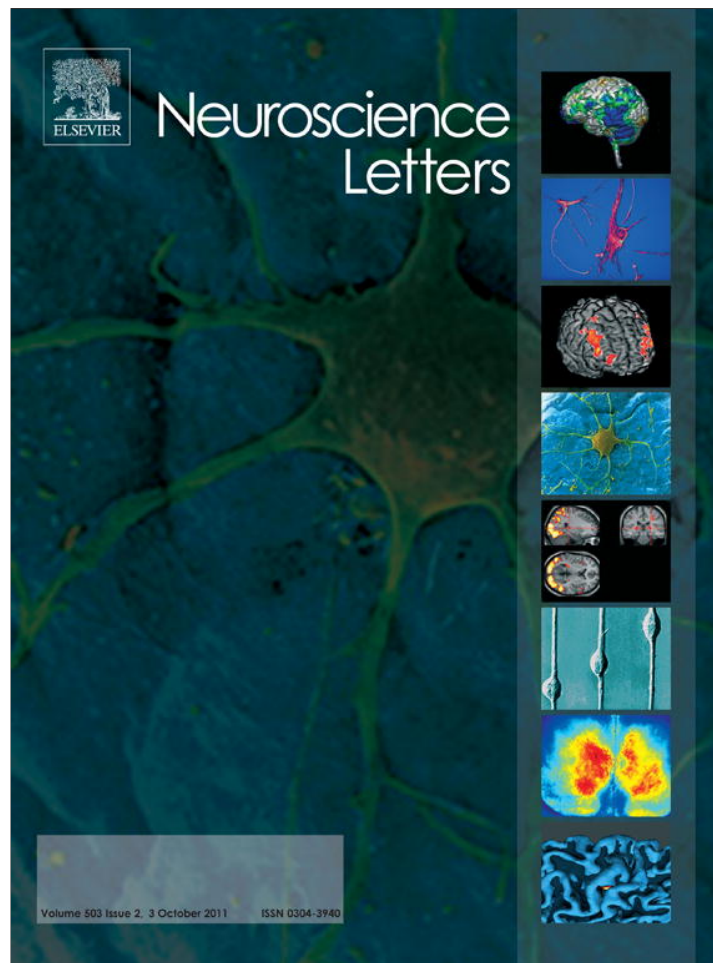


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5-HTTLPR genotype and gender, but not chronic fluoxetine administration, are associated with cortical TREK1 protein expression in rhesus macaques

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ABSTRACT

TREK1 is a widely expressed background potassium channel. Similar to mice treated with selective serotonin reuptake inhibitors (SSRIs), TREK1 knockout mice are resistant to depression-like behavior and have elevated serotonin levels leading to speculation that TREK1 inhibition may contribute to the therapeutic effects of SSRIs. This study examined how chronic fluoxetine administration and a common functional polymorphism in the serotonin-transporter-linked promoter region (5-HTTLPR) influence cortical TREK1 expression in 24 rhesus monkeys. The short rh5-HTTLPR allele as well as female gender were associated with reduced cortical TREK1 protein expression but chronic SSRI administration had no effect. These results suggest that serotonin may influence TREK1, but that chronic SSRI treatment does not result in long lasting changes in cortical TREK1 protein expression. TREK1 gender differences may be related to gender differences in serotonin and require further research.

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TREK1 is a background potassium channel expressed throughout the brain that contributes to overall neuronal excitability and shows promise as an antidepressant target [5,6,7,12,14,18]. Much like mice treated with selective serotonin reuptake inhibitors (SSRIs), TREK1 knockout mice are resistant to depression-like behavior and have elevated serotonin levels; moreover, they are insensitive to SSRI administration suggesting that the therapeutic effects of SSRIs may be related to TREK1 inhibition [6]. Consistent with this notion, SSRIs have been shown to inhibit TREK1 [5,6,9] and variation in the TREK1 gene (*KCNK2*) has been linked to depression as well as antidepressant response in humans [11,14]. However, few studies have examined the relationship between TREK1 and serotonin and there are no studies we are aware of that have evaluated how chronic SSRI administration (how SSRIs are often used in practice) affects TREK1.

This study examined associations between serotonin and TREK1 by evaluating how pharmacologically manipulated (via chronic fluoxetine administration) as well as genetically conferred (*SLC6A4*;

5-HTTLPR genotype), differences in serotonin transporter function are linked to cortical TREK1 protein expression in rhesus monkeys. SSRIs function by blocking the serotonin transporter (5-HTT) while the short allele of the 5-HTTLPR results in a less efficient transporter protein; both of these mechanisms result in elevated synaptic serotonin. Additionally, as a control we evaluated TRAAK protein expression. TRAAK is another background potassium channel with similar properties and localization to TREK1, but lacking in the sophisticated hormonal and neurotransmitter regulation; it was chosen as a control protein to demonstrate specificity of serotonin-related associations with the TREK1 background potassium channel [6].

We hypothesized that differences in synaptic serotonin levels, be it through pharmacologic (i.e., fluoxetine) or genetic (i.e., short rh5-HTTLPR allele carriers) means, would be associated with differences in TREK1, but not TRAAK, protein expression. Additionally, given evidence for gender differences in depression and 5-HT functioning [8], we explored the contribution of gender to cortical TREK1 protein expression.

Rhesus monkeys ($n = 24$; 50% female) were treated with fluoxetine or a vehicle-alone for 39 weeks. Fluoxetine-treated monkeys had reduced brain weight (mean/SD: 92.72 ± 11.59 g) relative to

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Table 1
Treatment group descriptors.

Treatment group	Age (years)	Gender	rh5-HTTLPR distribution (n)	Brain weight (g)	Body weight (kg)	Fluoxetine (ng/ml)	Norfluoxetine (ng/ml)
Vehicle alone (n = 12)	13.12 ± 1.64	50% Female	L/L=5, S/L=6, S/S=1	103.29 ± 9.72	9.54 ± 2.23	n/a	n/a
Fluoxetine (n = 12)	13.81 ± 2.51	50% Female	L/L=6, S/L=4, S/S=2	92.72 ± 11.60	8.19 ± 2.27	106.67 ± 44.19	313.25 ± 91.59

those treated with vehicle alone (103.29 ± 9.72 g), $P < 0.03$, but no significant differences in age, gender, body weight or group status emerged between treatment or genotype groups, all P s > 0.10 (Table 1).

Monkeys were housed in stainless steel cages in which water was continuously available. They were fed monkey chow (Teklad 25% Monkey Diet; Harlan/Teklad, Madison, WI) in amounts sufficient to maintain stable body weights and were given a chewable vitamin tablet 3 days per week. Environmental enrichment was provided daily.

Over two-three weeks monkeys were trained to be removed from cages and seated in a primate restraint chair (Primate Products, Inc., Immokalee, FL, USA) to allow for unanaesthetized blood draws. Next, monkeys were allowed to drink 50 ml of 4% Tang (Kraft Foods Company, Northfield, IL, USA) from a 500 ml bottle hung on the front of the cage at approximately 1:00 PM each day. Initially, bottles were left on the cages until all 50 ml were consumed. Gradually, Tang availability was shortened to 30 min/day. When all Tang was consumed in 30 min for at least three consecutive days, pre-drug baseline blood samples were drawn.

Fluoxetine HCl was added to Tang for the treated group while the control group continued to drink Tang only. The initial concentration of fluoxetine in Tang was 0.03 mg/kg. As long as intake was stable, fluoxetine concentration was increased by 0.5 log units (about three-fold) every three days until monkeys consumed 2.0 mg/kg fluoxetine each day. One-two weeks after this dosage was reached, monkeys were placed in chairs 23 h after consuming fluoxetine for measurement of trough blood levels. Since steady state blood levels of fluoxetine in humans are achieved after 3–4 weeks [2], two additional blood samples were taken at 3–4 week intervals. Blood levels of fluoxetine were low in this initial period compared to the reported clinical range of 19–199 ng/ml of fluoxetine and 45–244 ng/ml of the active metabolite, norfluoxetine [19]. Therefore, after approximately two months exposure to 2.0 mg/kg, we increased the dose to 3.0 mg/kg/day in the treated group. After approximately one month at 3.0 mg/kg/day, we drew blood at several time points after fluoxetine administration (1, 2, 4 and 23 h) on a monthly basis for three months. Based upon that information we concluded that peak fluoxetine levels, seen at 2 h after administration, approximated clinically relevant levels (52–168 ng/ml) [3].

Drug exposure continued for a total of 39 weeks, with the final 30 weeks at 3.0 mg/kg/day. Monkeys were euthanized 20–24 h after their last drug or vehicle exposure. The hormone levels of female monkeys were monitored throughout the study and they were sacrificed at trough hormone levels (serum estradiol < 100 pg/ml; progesterone < 0.2 ng/ml) during the early follicular phase following verified ovulation (or extended follicular phase for animals that never ovulated). For euthanasia, most were initially sedated with the combination of midazolam (Versed, 0.3 mg/kg, i.m.) and medetomidine (Domitor, 0.06 mg/kg, i.m.). Three monkeys (two treated, one control) required additional sedation with Isoflurane. They were then administered a lethal overdose of pentobarbital (75 mg/kg, i.v.) via the saphenous vein. Immediately following euthanasia the cranial dome covering the brain was lifted using an autopsy saw (810 Autopsy Saw from Stryker, 810-2-11-REV, Kalamazoo, MI, USA). The brain was bisected into hemispheres and each hemisphere dissected into coronal blocks. Blocks were immediately frozen in chilled isopentane and stored at -80°C .

Before beginning the experiment, monkeys were sedated with the combination of midazolam (Versed, 0.3 mg/kg, i.m.) and medetomidine (Domitor, 0.06 mg/kg, i.m.) and 3–5 ml of whole blood was collected from each animal for genotyping. Blood samples were stored at -70°C until required for PCR. Genomic DNA was extracted from whole blood using a Qiagen QIAmp DNA Blood Maxi Kit following manufacturer's instructions. Isolated genomic DNA was stored at -20°C until processed for PCR as previously described [10]. rh5-HTTLPR genotype was in Hardy–Weinberg equilibrium $P = 0.09$.

Immunolabelling of TREK1 and TRAAK were determined in the tissue punches of PFC (BA10) from cortical blocks as previously described [17]. Hippocampal and striatal regions were not available for this study. Equal volumes of protein samples containing mostly membrane and nuclear fraction (30 μg protein) were resolved on 12.5% sodium dodecyl sulfate–polyacrylamide gel and blotted on nitrocellulose membrane. Blots were incubated overnight at 4°C with rabbit anti-TREK1 polyclonal antibody (1:200; Alamone Laboratories, Jerusalem, Israel) or with rabbit anti-TRAAK polyclonal antibody (1:1,000; Aviva System Biology, San Diego, CA, USA). After overnight incubation and then washing, secondary polyclonal antibodies (rabbit HRP; 1:3000) were added. As a control for transfer and sample loading, anti-actin monoclonal antibody was used (primary: 1:10,000; secondary 1:5000; Chemicon (Millipore); Billerica, MA, USA). Each sample was immunoblotted in duplicate. The relationship between optical density values and the concentration of TREK1 and TRAAK immunoreactivity was determined by loading increasing concentrations of sample onto gels and immunoblotting with anti-TREK1 or anti-TRAAK antibody. Relative optical density values of immunoreactive bands were measured and presented as a function of protein concentration. The relationship between optical density and protein concentrations was linear. Relative optical density of TREK1 and TRAAK bands were analysed using imaging software (MCID Elite 7.0; Imaging Research, Canada) and normalized by the optical density of the corresponding β -actin band. The molecular weight for TREK1 was 65 kDa; for TRAAK it was 46 kDa.

Separate hierarchical regressions in which brain weight was entered first (because of differences between treatment groups), followed by treatment group (Fluoxetine or Vehicle-alone), the number of short rh5-HTTLPR alleles (0, 1, 2), and the interaction between rh5-HTTLPR genotype and treatment group predicted TREK1 and TRAAK protein expression. In exploratory analyses, gender was added as a 5th step; gender \times rh5-HTTLPR, gender \times treatment group, and gender \times rh5-HTTLPR \times treatment group interactions were entered in subsequent steps. All predictors were mean centered prior to the computation of interaction terms and regression analyses. Raw data are depicted in figures.

The hierarchical regression in which brain weight was entered first, followed by treatment group (Fluoxetine or Vehicle-alone), the number of short rh5-HTTLPR alleles (0, 1, 2), and the interaction between rh5-HTTLPR genotype and treatment group, produced a significant overall model, accounting for 37.9% of the variance in cortical TREK1 expression, $F(4,19) = 2.90$, $P < 0.05$. Only the number of short rh5-HTTLPR alleles significantly contributed to TREK1 levels, $\Delta F(1,20) = 8.08$, $\Delta R^2 = 0.27$, $P = 0.01$ (see Fig. 1A). In exploratory analyses, gender was added as a 5th step, which produced a significant overall model, predicting 65.4% of the variance in cortical TREK1 expression, $F(4,19) = 7.61$, $P < 0.01$, gender $\Delta F(1,18) = 14.31$,

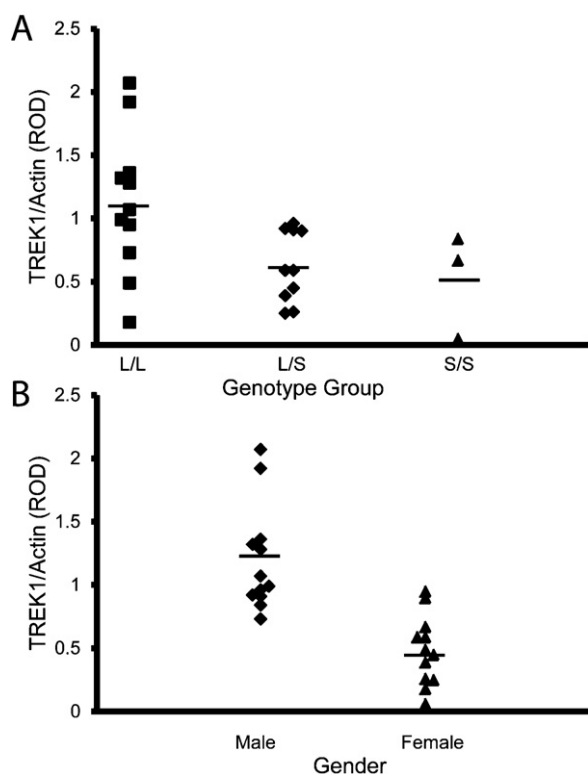


Fig. 1. Cortical TREK1 protein expression by (A) rh5-HTT genotype and (B) Gender. (A) The number of short alleles is negatively associated with cortical TREK1 expression, simple bivariate correlation, $r = -0.51$, $P < .02$. Genotype groups: squares = L/L, diamonds = L/S, triangles = S/S. (B) Females (triangles) were characterized by reduced cortical TREK1 protein expression relative to males (diamonds), simple bivariate correlation, $r = -0.72$, $P < .02$. ROD = relative optical density. Data points represent raw data values (mean-centered values were used in analyses). Lines represent mean values.

$\Delta R^2 = 0.28$, $P = 0.001$ (see Fig. 1B). Interactions involving gender entered in subsequent steps failed to significantly contribute to the model, all P s > 0.28 . Identical regressions for TRAAK produced no significant effects, all P s > 0.35 .

In the present study, the rh5-HTTLPR short allele and female gender, but not chronic SSRI administration, were associated with reduced cortical TREK1 protein expression. Given that the short allele confers elevated synaptic serotonin [1], and females have been shown to have reduced serotonin transporter function and elevated levels of serotonin [8], these findings suggest that elevated serotonin levels may reduce cortical TREK1 expression. The reason that chronic SSRI administration did not affect cortical TREK1 protein expression is unknown. Given that the therapeutic effects of SSRIs typically arise 2–6 weeks after treatment has begun, it may be that the effect of SSRIs on TREK1 occurs transiently during this therapeutic state, as opposed to following chronic (i.e., 39 weeks) administration. Additionally, the effects of SSRIs on TREK1 inhibition may be regionally-specific. Specifically, in light of evidence that genetic variation in the TREK1 gene (*KCNK2*) confers individual differences in not only antidepressant response [11,14], but also striatal reactivity to reward [4] in humans, it will be important for future research to evaluate the relationship between serotonin and TREK1 expression within the striatum. Moreover, recent evidence suggesting that fluoxetine induced reductions in TREK1 expression within the hippocampus may attenuate inhibitory neurogenesis by glucocorticoid hormones underscore the importance of examining TREK1 expression in the hippocampus and in the context of environmental challenge and stress [20].

The mechanism(s) through which elevated serotonin is associated with reduced TREK1 expression are unknown. Evidence suggests that SSRIs may directly inhibit TREK1 and/or that reductions in cAMP as a result of serotonin 1A receptor binding may do so [5]. Moreover, a recent report suggests that fluoxetine is associated with dissociation of the C-terminal domain of TREK1 from the membrane resulting in reduced TREK1 activity [16]. It remains to be seen whether this mechanism may play a role in genetically conferred elevations in serotonin. By better understanding the relationship between TREK1 and serotonin we may gain insights into the biological mechanisms of depression.

Lastly, while this study was designed to assess serotonin–TREK1 associations, it is entirely possible that the link between rh5-HTTLPR genotype and gender with cortical TREK1 protein expression are driven by an independent variable not measured. For example, both rh5-HTTLPR and gender are associated with differential hypothalamic–pituitary–adrenal axis function [13,15]. Provided that HPA-mediated mechanisms may impact TREK1 expression in limbic regions [20], it is entirely possible that these or other mechanisms may influence cortical TREK1 protein expression, as opposed to, or in addition to, hypothesized serotonin-mediated mechanisms.

The limitations of the present study must be acknowledged. First, while this study was composed of a large number of monkeys for a chronic pharmacologic challenge study, it is limited in size for a genetic study. As such, the associations between 5-HTTLPR genotype and gender with TREK1 protein expression await replication and extension across species. Moreover, in this study we evaluated TREK1 protein expression levels in the PFC of rhesus macaques; it will be important for future research to evaluate TREK1 mRNA and protein expression in additional brain regions, most notably the hippocampus and striatum as mentioned above and to extend this research across species. Lastly, future research should evaluate more complex relationships within the serotonin–TREK1 system, such as 5-HT1A receptor function. Limitations notwithstanding, the present findings link genetically conferred reductions in 5-HTT function and female gender with reduced cortical TREK1 expression. Such reduced expression may contribute to differences in depression.

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