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The synaptic protein encoded by the gene Slc10A4 suppresses epileptiform activity and regulates sensitivity to cholinergic chemoconvulsants

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ABSTRACT

The expanding number of disease-causing dysfunctions of synaptic proteins illustrates the importance of investigating newly discovered proteins involved in neuronal transmission. The gene *Slc10A4* encodes a recently described carrier protein present in pre-synaptic terminals of cholinergic and monoaminergic neurons. The biological significance of this recently described transporter protein is currently unknown. We here investigated whether absence of the Slc10a4 protein has any impact on function of the cholinergic system. We first investigated the sensitivity of Slc10a4 null mice to cholinergic stimulus in vitro. In contrast to wild type mice, gamma oscillations occurred spontaneously in hippocampal slices from Slc10a4 null mice. Furthermore, moderate treatment of Slc10a4 null slices with the cholinergic agonist carbachol induced epileptiform activity. In vivo, 3-channel EEG measurements in freely behaving mice revealed that Slc10a4 null mice had frequent epileptiform spike-activity before treatment, and developed epileptic seizures, detected by EEG and accompanied by observable behavioral components, more rapidly after injection of the cholinergic agonist pilocarpine. Similar results were obtained on non-operated mice, as evaluated by behavioral seizures and post mortem c-Fos immunohistochemistry. Importantly, Slc10a4 null mice and wild type control mice were equally sensitive to the glutamatergic chemoconvulsant kainic acid, demonstrating that absence of Slc10a4 led to a selective cholinergic hypersensitivity. In summary, we report that absence of the recently discovered synaptic vesicle protein Slc10a4 results in increased sensitivity to cholinergic stimulation.

Introduction

Synapses are specialized junctions between nerve cells and in recent years, remarkable progress has been made in our understanding of synaptic function. Detailed knowledge on a vast range of synaptic processes, from synaptogenesis to neurotransmitter packaging and release, has drawn increasing attention to the pathogenic potential of abnormalities in synaptic proteins. Genetic studies and animal models have revealed that defects in synaptic proteins can result in a wide range of neurological conditions. For instance, mice with reduced expression of proteins involved in vesicular packaging of neurotransmitters have altered seizure thresholds (Guidine et al., 2008; Leggio et al., 2011; Schallier et al., 2009), making synaptic vesicular transporter proteins highly relevant in epilepsy research. These findings also illustrate the short step from dysfunction of synaptic proteins to clinical relevance and the importance of thorough analysis of newly discovered proteins that can impact neuronal transmission.

The gene *Slc10A4* encodes a carrier protein that is expressed in cholinergic and monoaminergic neurons, where it colocalizes with markers for pre-synaptic terminals such as VAChT (Burger et al., 2011; Geyer et al., 2008). The Slc10a4 protein (henceforth referred to as Slc10a4) is highly expressed in the cholinergic system, but the biological significance of Slc10a4 regarding cholinergic function has not been investigated. The expression pattern has led to proposals that Slc10a4 may be involved in neurotransmitter sequestering, vesicle sorting, and/or exocytosis (Burger et al., 2011).

Dysfunction of cholinergic synapses has been demonstrated to be of pathological significance. For instance, mice with reduced expression of VAChT, another vesicular protein in cholinergic synapses, display an augmented response to the cholinergic chemoconvulsant pilocarpine, indicating that presynaptic dysfunction of cholinergic synapses might impact epileptic vulnerability (Guidine et al., 2008). Newly discovered synaptic proteins in cholinergic synapses, such as Slc10a4, therefore merit closer study.

We set out to study the biological significance of Slc10a4 in the cholinergic system. We hypothesized that absence of Slc10a4 would result in an altered sensitivity to cholinergic stimulus. Our findings presented here suggest that absence of Slc10a4 results in an augmented response to cholinergic stimulus. In addition, we report that absence of Slc10a4 results in altered signaling pattern in CNS networks, indicative of epileptic vulnerability, and that Slc10a4 null

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mice are more sensitive to a cholinergic model of status epilepticus. We therefore suggest that Slc10a4 should be included in future studies on genes that contribute to epilepsy. These findings demonstrate a biologically significant role for a recently discovered protein in cholinergic synapses.

Materials and methods

Mice

The *Slc10A4* mouse line (strain 129/SvEvBrd) was obtained from Texas A&M Institute for Genomic Medicine (College Station, TX, USA). Heterozygote breedings were used to generate adult male and female homozygous mice and their wild type littermate controls. In total, 64 mice were used. All mice were adults (older than 7 weeks) and the experimental groups were age and gender matched. All animal experiments were approved by the relevant animal ethics committee in Uppsala, approval numbers C79/9, C268/10 and 144/10.

EEG

Electroencephalography (EEG) was recorded using the cable-tethered PAL-8200 system (Pinnaclet Technology Inc., Lawrence, KS, USA). The mice were anesthetized with a mixture of fentanyl and midazolam and after local application of bupivacain, six epidural electrodes were placed in needle-generated holes in the skull according to the instructions of the manufacturer (Pinnaclet Technology Inc.). Subcutaneous injection of saline and buprenorphin provided fluid resuscitation and analgesia. The mice were allowed to recover for 1 week prior to EEG-recordings and then connected to the recording system, with a preamplifier setting of 100× and the signal filtered at 40 Hz (manufacturer default settings for mouse). Some seizure recordings were also inspected at higher filter settings (100 Hz), to verify that seizure activity was not a misinterpreted filtering artifact. Mice displaying seizure activity on EEG also displayed typical seizure behavior with low-grade seizure manifestations being intermittently interrupted by high-grade seizures, seizures graded as in Borges et al. (2003). The three channels recorded were ipsilateral left frontal (EEG1), cross cortical frontoparietal (EEG2), and cross-cortical occipital (EEG3), and are illustrated in Fig. 2. Recordings were inspected for interictal spikes, defined as high amplitude events with an amplitude of more than 2× background activity, or seizures, defined as a high frequency repetitive pattern with an amplitude of 2× background activity (Fig. 2C). Pre-treatment-EEG was recorded from 12 untreated animals of each genotype. EEG parameters were assessed with the genotype of the animal blinded to the experimenter. Some mice, including mice that had suffered seizures, were dissected post mortem and the brain surface inspected for signs of damage from the electrodes. No such damage could be seen, and gross histopathological microscopic examination of brain sections also failed to reveal any damage induced by the electrodes.

Electrophysiology

Electrophysiological in vitro experiments were performed in hippocampal slices of Slc10a4 null and wild type mice (postnatal day 30–60 days). All procedures followed Uppsala University guidelines for the care and use of laboratory animals. Mice were decapitated and the brains were placed in ice-cold artificial sucrose ACSF, containing the following (in mM): 2.49 KCl, 1.43 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 25 sucrose, 1 $CaCl_2$, 4 $MgCl_2$. After hemisection, the brains were sliced and 400 μm-thick hippocampal slices were collected. Slices were kept in interface holding chamber containing ACSF with (in mM): 124 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 1.5 $MgCl_2$, 1.5 $CaCl_2$, 30 $NaHCO_3$, 10 glucose) and constantly bubbled with 95% O2 and 5% CO_2 in a closed interface chamber at room temperature for 1 h. Afterwards, slices were transferred for recording to interface recording chamber kept at 36 °C. Data were digitized using custom acquisition software developed in MATLAB Mathworks (Leao et al., 2009). Extracellular field recordings were made in stratum pyramidale of the hippocampal area CA3 and the signal low-pass filtered at 3 kHz. Events with the duration of 200 ms or shorter were defined as spikes and events larger than 500 ms were defined as epileptic activity. Glass pipettes with tip resistances between 9 and 11 MΩ filled with ACSF were used. Carbachol and kainic acid were purchased from Sigma. All recordings were performed 10 min after drug application for each drug dose and recorded for the next 20–30 min. Spontaneous oscillations observed in the KOs were recorded for at least 10 min.

Pilocarpine or kainic acid treatment

Mice were intraperitoneally injected with 325 mg/kg pilocarpine (P6503, Sigma-Aldrich, Stockholm, Sweden) or 25 mg/kg kainic acid (K0250, Sigma-Aldrich), connected to the recording system, and EEG was recorded for 90 min. Pilocarpine injection was preceded by injection of 1 mg/kg methylscopolamine (S2250, Sigma-Aldrich) to prevent peripheral cholinergic side effects of the pilocarpine. Evaluated EEG parameters were latency to first spike, latency to first seizure, latency to first seizure activity >30 min (status epilepticus), and time with seizure activity. Six mice of each genotype were evaluated in each of the pilocarpine and kainic acid experiments. If animals died during status epilepticus, these mice were considered to have seizure activity for the remainder of the 90 min.

Behavioral observations

Nine animals of each genotype were injected with pilocarpine as described above without prior surgery. Following injection, the animals were observed for 90 min and scored for behavioral seizures involving jumping or loss of postural control, also known as grade 5 or 6 seizures (Borges et al., 2003).

Immunohistochemistry

c-Fos expression levels were determined at 90–120 min after pilocarpine injection (n = 5 per genotype). As controls, two mice of each genotype were injected with saline solution. The mice were age and gender matched in pairs, anesthetized with isoflurane and perfused with phosphor buffered saline (PBS) and 4% formaldehyde in phosphate buffer by cardiac puncture via the left ventricle. After perfusion, the brains were removed from the skull and post fixed in the same fixative for 12–18 h. After rinsing in PBS, the brains were cryoprotected in a 30% sucrose solution for 12–18 h, embedded in Tissue-Tek O.C.T. (Sakura Finetek Europe, The Netherlands) and frozen on dry ice. Coronal sections of 14 μm at the level of the hippocampus were made using a cryostat (Microme Hm 560). Sections were collected on glass slides (Thermo Scientific, Superfrost plus, USA) and stored at −20 °C until use.

Immunohistochemistry for c-Fos protein

Sections were rinsed in PBS and incubated in a blocking solution containing 0.2% Tween-20 (Sigma-Aldrich), 5% BSA (Sigma-Aldrich), 10% normal donkey serum (Sigma-Aldrich) in PBS for 1 h. c-Fos antiserum (sc-52-G; Santa Cruz Biotechnology, CA, USA, 1:100) in PBS containing 0.2% Tween-20, 5% BSA and 5% normal donkey serum and incubated for 12–18 h at 4 °C. Sections were rinsed 4× at 10 min each time in PBS and incubated with a donkey-anti-goat-488 secondary antibody (Jackson Immunoresearch, PA, USA) diluted 1:500 in PBS containing 0.2% Tween-20. The sections were rinsed 3× at 10 min each time in PBS and the nuclei stained with DAPI (Sigma-Aldrich). The sections were mounted in Mowiol (Sigma-Aldrich). Images were taken using an Olympus BX61W1 microscope, a camera (Q-Imaging

Retiga-4000RV) and Volocity software (Improvision, UK). The percentage of pixels positive for both c-Fos and DAPI in the piriform cortex was measured using the levels feature of ImageJ® software (NIH, USA). For pilocarpine treated mice, measurements were performed on three sections from each animal and the average value for that animal was used for statistical analysis. For saline treated controls, one section from each animal was analyzed. All settings for compared images were identical.

In situ hybridization

Expression pattern of mRNA encoding several vesicular transporters (*Slc10a4*, *Vglut-2*, *Vacht*, *Cht1*, *Dat* and *Viaat*) was examined with in situ hybridization. The probes were obtained by in vitro transcription of commercialized plasmids (Invitrogen, Stockholm, Sweden). For tissue preparation, mice were processed as described above, but after rinsing in PBS the brains were embedded in 4% agarose. Sections were cut in PBS at 70 μm using a Vibratome (Leica, VT1000S) and dehydrated by successive washes in 25%, 50% and 75% methanol in PBT (PBS with 0.1% Tween-20) and stored in 100% methanol at −20 °C. Sections were rehydrated by reverse washes in methanol/PBT and washed in 100% PBT. Subsequently, the sections were bleached in 6% hydrogen peroxide in PBT, washed in PBT and treated with 0.5% Triton X-100 (Sigma-Aldrich). Sections were digested with 1.5 μg/ml proteinase K (Invitrogen) for 6 min and the reaction was stopped by several washes in PBT. After 20 min postfixation in 4% formaldehyde the sections were pre-hybridized in hybridization buffer (50% formamide, 5× SSC, pH 4.5, 1% sodium dodecyl sulfate [SDS], 50 μg/ml tRNA [Sigma-Aldrich], 50 μg/ml heparin [Sigma-Aldrich] in PBT). Then 0.3 μg probe/ml was heat-denatured for 5 min at 80 °C in hybridization buffer followed by 5 min incubation on ice. After pre-warming to 57 °C the hybridization solution was added and incubated overnight (14–18 h) at 57 °C. Unbound probe was removed by sequential washes with washing buffers (50% formamide, 5× SSC, pH 4.5 and 1% SDS in PBT) and (50% formamide, 2× SSC, pH 4.5, and 0.1% Tween-20 in PBT) at 57 °C. The sections were further washed in Tris-buffered saline + 0.1% Tween-20 (TBST) followed by incubation in 1% blocking reagent (Roche Diagnostics) and overnight-incubation with 1:5000 diluted anti-digoxigenin alkaline phosphatase-conjugated antibody (Roche Diagnostics) at 4 °C. After unbound antibody was removed by sequential washes with TBST and NTMT (100 mM NaCl, 10 mM Tris–HCl, pH 9.5, 50 mM $MgCl_2$ and 0.1% Tween-20) sections were developed in BM purple AP substrate (Roche Diagnostics) until appropriate staining was achieved. Developed sections were imaged with a Leica MZ16F dissection microscope with DFC490 camera.

RT-qPCR

Striatal and hippocampal RNA was isolated by Tri-reagent (Life Technologies Inc., Paisley, UK) from Slc10a4 null mice (n = 7) and controls (n = 7) as per the manufacturer's instructions. Following DNase I treatment RNA samples were further purified with Tri-reagent, and 1 μg total RNA was subjected to cDNA synthesis using M-MLV RT-PCR kit (Invitrogen). Quantitative PCR was performed on cDNAs in duplicates using KAPA™ SyBr® FAST qPCR Kit (KAPA Biosystems Inc., Woburn, USA) and Bio-Rad iCycler™ (Bio-Rad). Primer efficiency between samples was checked using free software based on linear regression method (LinRegPCR) and mean efficiency was obtained from Grubb's test (http://www.graphpad.com/quickcalcs/Grubbs1.cfm). Five housekeeping genes were chosen based on their level of expression in the striatum and hippocampus by using the BioGPS site as on 25th of November 2011 (http://biogps.org/#goto=welcome). Stability of housekeeping genes' expression was calculated by geNorm algorithm. CT values corrected using the mean efficiencies were normalized to the geometric mean of the two most stable housekeeping genes, and relative expression was plotted using GraphPad Prism© (GraphPad Software, La Jolla, USA).

Data analysis

Data values are reported as means ± SEM. Unpaired *t*-test, Mann–Whitney test and one-way ANOVA with Tukey's post-test were used for statistical analysis and comparison of groups, depending on normal distribution. Chi-square test was used for comparison of proportions. Specific tests are stated in the result section and figure legends. Fast Fourier transformation for power spectra was computed for 120-s-long data traces that were previously low pass filtered at the stop band frequency of 100 Hz. Repeated measures ANOVA was used for comparison of series in the electrophysiological experiments. All statistical calculations were performed with GraphPad Prism©.

Results

Increased gamma-oscillation and decreased seizure threshold to cholinergic stimuli in Slc10a4 null hippocampal slices

We hypothesized that absence of Slc10a4 would alter the response to cholinergic stimulus. Cholinergic stimulation regulates oscillatory activity in the hippocampus (Fisahn et al., 1998), and in our initial experiment, we set out to investigate the threshold for appearance of gamma oscillations after cholinergic stimulation. In this model, application of increasing concentrations of the cholinergic agonist carbachol results in occurrence of gamma oscillations as analyzed by field potential measurements in the CA3 area of the hippocampus. To our surprise, already without treatment, field potentials in slices from Slc10a4 null mice displayed activities in the gamma range (57 ± 17.3 Hz, n = 14, $p<0.01$), which, as expected, were not observed in slices from control animals (Figs. 1A, B). Following administration of a low dose of carbachol, similar oscillations were induced in wild type hippocampal slices (48 ± 7.6 Hz, n = 14, $p<0.01$, Fig. 1A). However, the power of frequencies in gamma range evoked by carbachol application was ~2× higher (2.8 ± 0.23 μV^2) in all of the observed wild type slices compared to spontaneous oscillations detected in Slc10a4 null mice (1.2 ± 0.14 0.23 μV^2, Figs. 1A, B, right panels). Application of 10 μM carbachol evoked a significant frequency increase in slices from Slc10a4 null mice (127 ± 23.57 Hz, n = 7, $p<0.01$) and a further increase of the concentration of carbachol resulted in a replacement of the gamma oscillations by epileptiform activity (Fig. 1C). To detect such epileptiform activity in wild type animals, a three times higher concentration of carbachol (90 μM) was required compared to Slc10a4 null mice. Moreover, at each given concentration, the number and duration (n = 8, $p<0.001$) of recorded epileptic events were significantly higher in slices from Slc10a4 null animals (Figs. 1D, E).

Slc10a4 null mice display spontaneous spike discharges on EEG

Our in vitro experiments indicated that absence of Slc10a4 results in an increased sensitivity to cholinergic stimulus. Furthermore, it seemed that the absence of Slc10a4 disturbed normal network activity, and that a modest cholinergic challenge to the system evoked epileptiform activity. Cholinergic stimulation has been extensively linked to epileptic activity, and in vivo application of cholinergic agonists can induce seizures or status epilepticus (Friedman et al., 2007). We therefore proceeded to test whether the epileptiform changes seen in vitro were also detectable in vivo, as measured by EEG on freely moving mice. To establish baseline levels of seizure activity in our experimental setup, we first performed EEG on untreated animals (Fig. 2). No spontaneous behavioral seizures were observed in either control or Slc10a4 null mice during handling of the mice and no seizure activity could be detected in 15–20 min EEG of untreated animals of either genotype (n = 12 per genotype). Recordings were also inspected for interictal spikes, defined as events with amplitude of more than twice the normal background, and for seizures. Spikes occurred fairly regularly in nine out of twelve Slc10a4 null animals, whereas such events were only

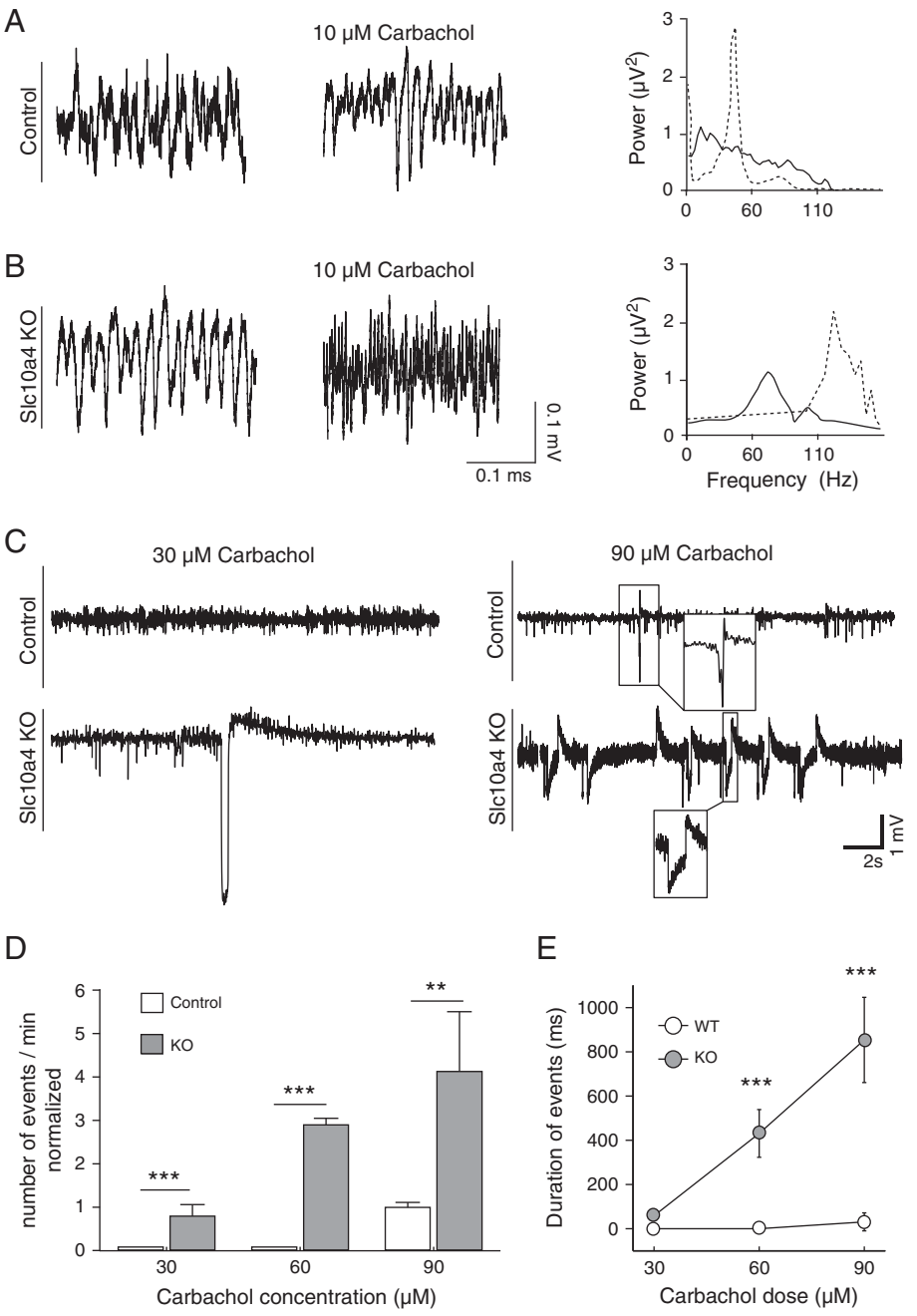

Fig 1. Slc10a4 null mice (Slc10a4 KO) display spontaneous oscillations and lower concentration of carbachol induces epileptic activity in Slc10a4 null animals. A. Wild type (Control) mice did not display any spontaneous oscillations (left), while gamma oscillation was evoked by 10 μM carbachol application (middle). B. Slc10a4 null mice displayed spontaneous oscillations (left) and frequency increase by 10 μM carbachol application (middle). Right panels of both A and B show respective power spectra (solid line control, dashed line 10 μM carbachol). C. 30 μM carbachol application was not sufficient to induce epileptic activity in wild type mice (upper panel), while at this dose epileptic activity was detected in Slc10a4 null mice (lower panel). By application of 90 μM carbachol, epileptic activity was evoked in the wild type mice (upper panel), while in Slc10a4 null mice both duration and frequency of this activity was significantly higher (lower panel). Insets show higher resolution traces. D–E. Quantification of normalized number of events/min and duration in wild type and Slc10a4 null animals. **Indicates $p<0.01$, ***Indicates $p<0.001$, repeated measures ANOVA.

rarely seen in wild type mice (Fig. 2D). The number of spikes observed was significantly higher in Slc10a4 null mice than in wild type mice, 12.67 ± 6.8 vs. 0.41 ± 0.19 spikes/10 min ($\pm$SEM, Mann–Whitney test, $p<0.01$, Fig. 3E).

Increased sensitivity of Slc10a4 null mice to pilocarpine-induced status epilepticus

We next studied if absence of Slc10a4 affects the seizure threshold in a cholinergic model of epilepsy in vivo, in analogy to the in vitro experiment with carbachol. We subjected the mice to pilocarpine-induced status epilepticus, a well established model used to induce acute epileptic seizures by cholinergic stimulation (Cavalheiro et al., 1996). In this model, intraperitoneal injection of the potent cholinergic agonist pilocarpine induces spiking activity on EEG, proceeding to high-frequency and high-amplitude seizure activity in responding animals. Mechanistically, pilocarpine is believed to initiate seizures by activating muscarinic M1 receptors, and the seizures are then maintained by NMDA receptor activation (Nagao et al., 1996; Smolders et al., 1997). We detected seizure activity by epidural recordings of

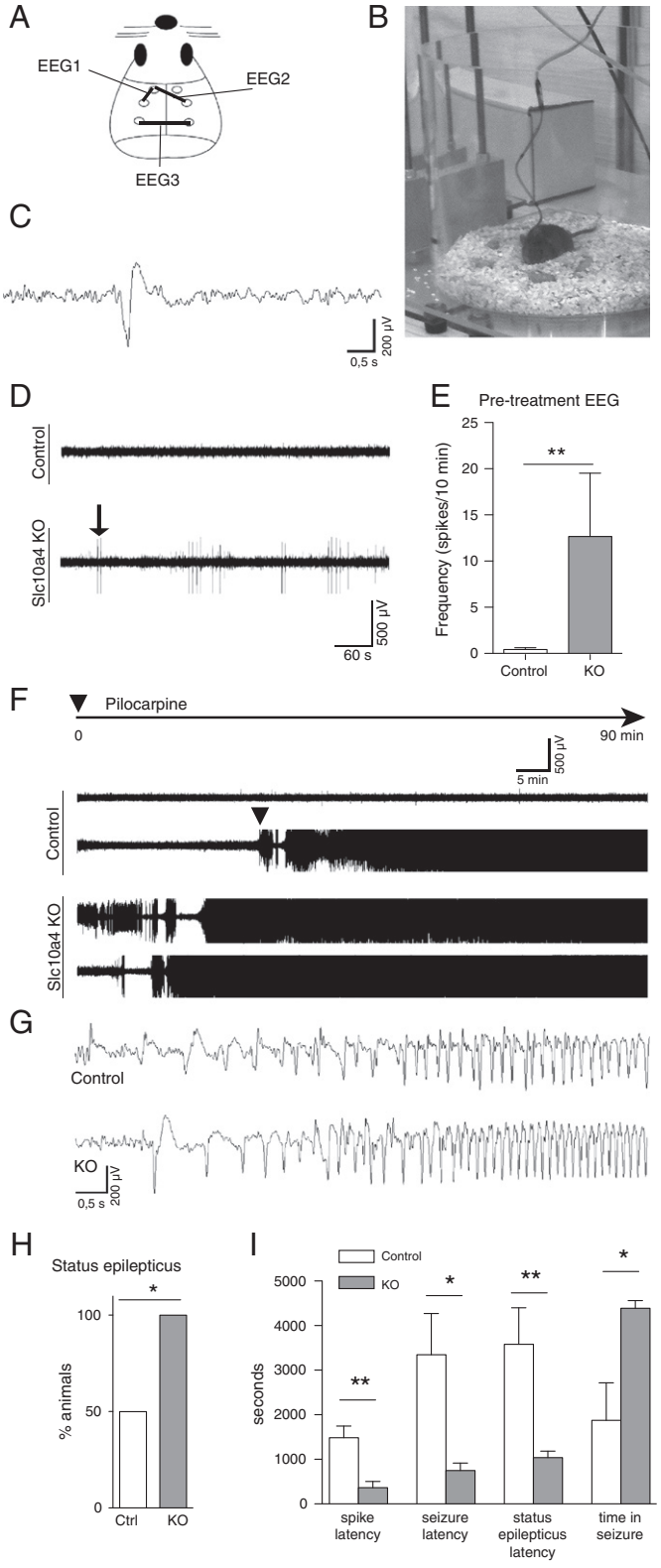

Fig 2. EEG-setup and epileptiform activity in untreated mice and after pilocarpine administration. A. Six electrodes were implanted and used to generate 3 EEG-channels, as indicated by the schematic drawing. B. EEG was recorded using a tethered system. C. EEG-activity was classified as normal, spikes or seizure. D. EEG-recordings from untreated animals. Spikes are more common in the Slc10a4 null mice trace (Slc10a4 KO, arrow). E. Quantification of spike frequency in wild type (Control) and Slc10a4 null animals. F. EEG-recordings from four animals after treatment with pilocarpine, the top two traces are wild type and the bottom two traces are Slc10a4 null mice. Onset of seizure activity is marked by arrowhead in trace 2. G. High-resolution EEG showing onset of a pilocarpine-induced seizure in wild type (top) and Slc10a4 null (bottom) mice. H. A single injection of pilocarpine resulted in status epilepticus in all Slc10a4 null animals, but only half of the wild type animals. I. Epileptiform activity, spike and seizure, started earlier in Slc10a4 null mice, as did persistent seizure activity (status epilepticus). Slc10a4 null mice also spent a larger time of the 90 min with seizure activity on EEG. $^{*}p<0.05$, $^{**}p<0.01$, Mann–Whitney (H) or Chi-square test (I), n = 6 per group.

3-channel EEG in moving mice. Injection of 325 mg/kg pilocarpine induced seizure activity in all Slc10a4 null mice, but only in half of the wild type mice ($n=6$ per group, Figs. 2F, H). Moreover, significantly shorter latencies to the first spike (365 ± 138 vs 1485 ± 260 s, Mann–Whitney, $p<0.001$) to the first seizure event (750 ± 166 vs 3350 ± 921 s, $p<0.05$), and to continuous seizure activity lasting to the end of the recording period (status epilepticus, 1042 ± 143 vs 3583 ± 819 s, $p<0.01$), were observed in Slc10a4 null animals (Fig. 2I).

Increased behavioral seizures and c-Fos expression in SLC10A4 null mice

Our EEG-experiment showed a clear increase in sensitivity to pilocarpine-induced status epilepticus in Slc10a4 null mice. However, intracranial surgery carries an inherent risk of inducing epilepsy. We therefore wanted to confirm that the observed difference was due to differences in pilocarpine sensitivity, and not due to an increased sensitivity to the surgical procedure in the Slc10a4 null animals (Fig. 3). For this purpose, we injected equal doses of pilocarpine into surgery naive mice. Pilocarpine injections induced observable seizures with jumping or loss of postural control, grade 5–6 seizures (Borges et al., 2003), in eight out of nine Slc10a4 null animals, but only in two out of eight wild type animals, a statistically significant difference ($p<0.01$, Chi-square test). The latency to the first high-grade seizure was 77 ± 8.5 min for wild type mice and 50 ± 8.5 min for Slc10a4 null mice ($p<0.05$, Mann–Whitney).

Pilocarpine injection induces c-Fos expression in several brain regions, and early expression of c-Fos is seen in the piriform cortex (Motte et al., 1998). As a further control experiment, we assessed whether induction of c-Fos after pilocarpine injection in non-operated animals differed between genotypes. We perfused five mice of each genotype at 90 min after injection of pilocarpine. A significantly larger increase in the staining of c-Fos positive cells was seen in the piriform cortex of Slc10a4 null animals 90 min after injection ($n=5$/genotype, $p<0.05$, ANOVA, Fig. 3).

No difference in sensitivity to kainic acid induced status epilepticus

Our initial experiments demonstrated abnormalities before the application of a cholinergic agonist, both in vitro and in vivo, in the form of gamma oscillations or epileptiform spikes, respectively. This suggests general network instability in the absence of Slc10a4, which could result in a subsequent increased sensitivity to all chemoconvulsants and not specifically to cholinergic ones. Therefore, we next evaluated the effect of the glutamatergic chemoconvulsant kainic acid, which is also used to induce epileptic seizures in mice (Mathis and Ungerer, 1992). Application of kainic acid in acute hippocampal slices revealed no differences between wild type and Slc10a4 null slices in evoked epileptiform events. Epileptic activity could be detected both in Slc10a4 null slices and wild type slices at concentrations of 400 nM kainic acid (Figs. 4B, C), or higher ($n=9$ per group), and no significant differences could be detected in the duration (81 ± 18.2 ms for wild type and 69 ± 19.4 ms for KO) or frequency (2.4 ± 0.1 events/min for wild type and 2.3 ± 0.28 events/min for KO) of epileptic events (Figs. 4A, B). In vivo, intraperitoneal injection of 25 mg/kg kainic acid induced electrographic seizure activity in all wild type and Slc10a4 null mice (Fig. 4D). In contrast to the pilocarpine-induced seizures, no significant differences were detected in any of the evaluated EEG-parameters, including spike latency, seizure latency, status epilepticus latency, or total time with seizure activity (Fig. 4F).

No difference in expression of synaptic proteins

To minimize the risk that lack of Slc10a4 in Slc10a4 null mice had resulted in confounding developmental compensatory expression changes of other synaptic proteins that may affect the seizure threshold, we performed an expression analysis of other synaptic proteins known to have an impact on seizures (Fig. 5). We first analyzed the mRNA expression pattern of synaptic transporters in the glutamatergic, GABAergic, cholinergic, and dopaminergic systems as well as ChAT by in situ hybridization. Slc10a4 null animals did not display any altered

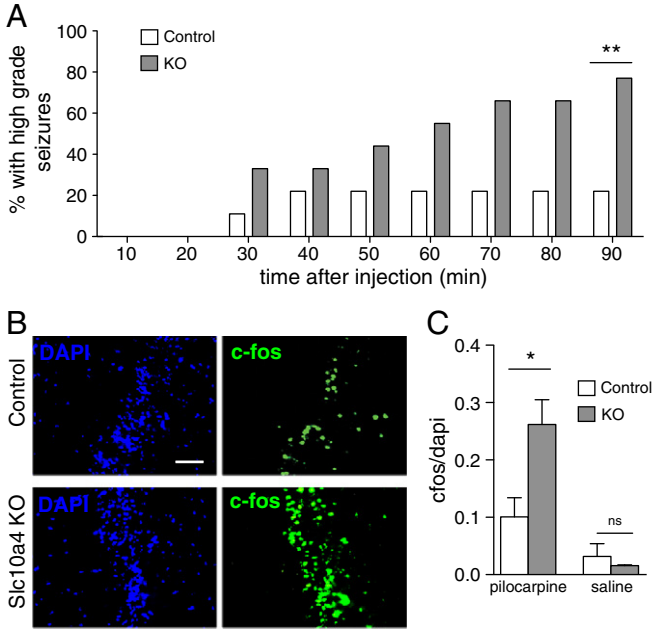

Fig 3. Seizures and c-Fos expression after injection of pilocarpine in wild type (Control) and Slc10a4 null (Slc10a4 KO) mice. A. Cumulative percentage of mice with high-grade seizures after injection of 325 mg/kg pilocarpine. The proportion of mice that had suffered seizures in the KO group was significantly larger than in the wild type group at the end of the period (Chi-square test). B. DAPI and c-Fos staining in the piriform cortex of wild type and Slc10a4 null animals 90 min after pilocarpine injection. Blue depicts DAPI, green depicts c-Fos. C. Semiquantitative measurements of immunoreactivity showed that a higher percentage of pixels were positive for c-Fos in Slc10a4 null animals 90 min after injection of pilocarpine. No difference could be detected between wild type mice or KO mice after saline treatment. One-way ANOVA with Tukey's post test. *Indicates $p<0.05$, **indicates $p<0.01$, ns indicates not significant.

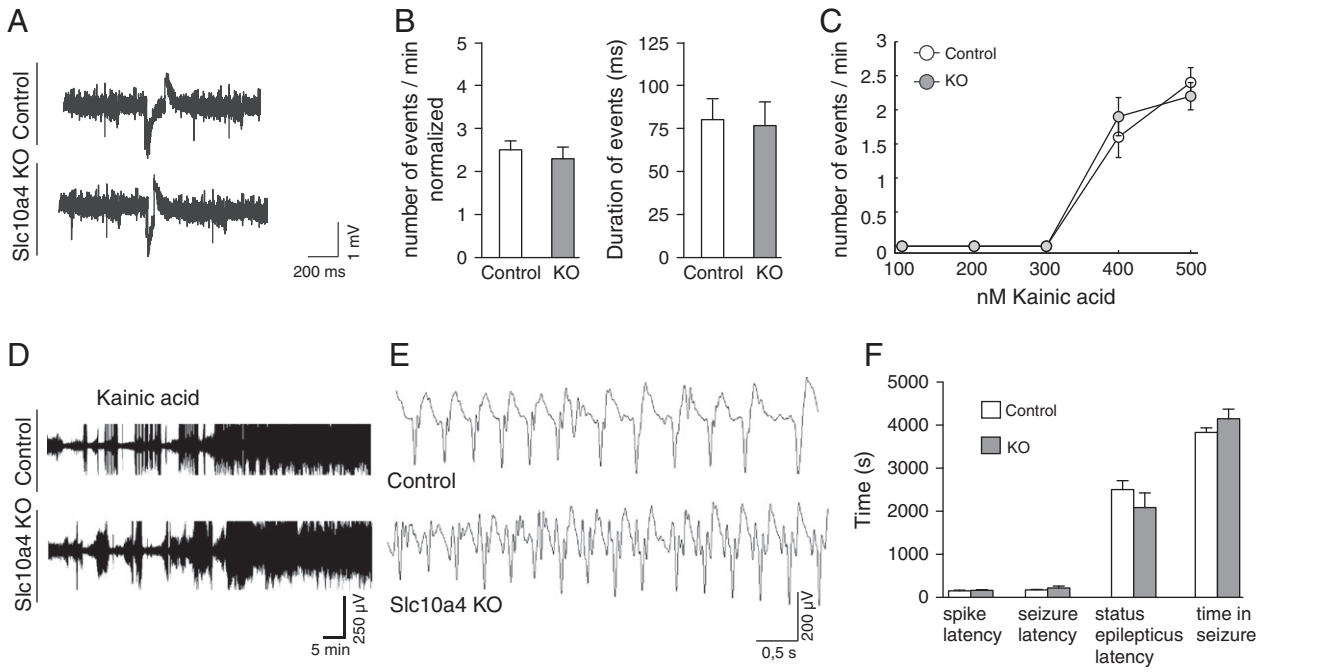

Fig 4. Epileptic activity after kainic acid application in wild type (Control) and Slc10a4 null (Slc10a4 KO) mice. A. Epileptic activity in response to application of 500 nM kainic acid in wild type and Slc10a4 null acute hippocampal slices. B. No significant differences in duration or frequency of epileptic events were observed at 400 nM (n = 9 per group). C. No significant differences could be detected at other concentrations of kainic acid in a subsequent dose titration experiment (separate experiment from B). D. EEG-recordings from two animals, one wild type and one Slc10a4 null mouse. Onset of trace at kainic acid injection. E. Sample high-resolution EEG traces showing kainic acid-induced seizure activity. F. No difference could be detected between the strains in any of the evaluated EEG parameters; spike or seizure latency, status epilepticus latency, or time in seizure (n = 6 per group).

gross brain anatomy, and no difference in the expression pattern of mRNA encoding VGLUT2, VIAAT, ChAT, VAChT, or DAT was found (Supplementary Table 1). In addition, we performed RT-qPCR for transporter mRNAs in the striatum, a region with substantial transporter expression, where no differences in expression were seen in Slc10a4 null mice compared to controls. To address the possibility that the increased sensitivity to cholinergic chemoconvulsants could be due to altered expression of postsynaptic cholinergic receptors in the hippocampus, we performed RT-qPCR for mRNA encoding postsynaptic cholinergic receptors in this region. With the exception of a very small change in the level of M3 mRNA, no differences were found between wild type and Slc10a4 null mice.

Discussion

We report that absence of the recently discovered synaptic protein Slc10a4 augments the response to cholinergic stimulus, as determined by our electrophysiological characterization. The vulnerability is manifested through an increased specific sensitivity to cholinergic activation in a common model for status epilepticus. Our findings, summarized in Table 1, show that a newly described synaptic protein affects the seizure threshold, and represent the first demonstration of a biologically significant role for Slc10a4 in the cholinergic system.

We set out to investigate our hypothesis that Slc10a4 is required for proper cholinergic function and that loss of Slc10a4 would result in an altered response to cholinergic stimulation. Somewhat surprisingly, we noted that in hippocampal slices, Slc10a4 null mice demonstrated an abnormal spontaneous activity in the gamma range. Such activity is frequently seen prior to seizures, but the precise role of gamma oscillations in ictogenesis remains poorly understood (Medvedev, 2002; Neu and Soltesz, 2005). It is however possible that the observed gamma range activity in vitro reflects network instability, resulting in the interictal spikes seen on EEG of untreated Slc10a4 null mice in vivo. Such interictal spikes might be an indicator of epileptic vulnerability, but can also be a normal phenomenon (White et al., 2010). Also, previous findings have suggested a modulatory role of the cholinergic system in epileptic seizures (de Freitas et al., 2004; Friedman et al., 2007).

We next detected an increased response to cholinergic stimuli in Slc10a4 null mice in vitro. Carbachol normally induces gamma oscillations in hippocampal slices, and as expected, this was seen in slices from wild type animals. In Slc10a4 null animals however, administration of carbachol resulted in the gamma oscillations being replaced by epileptiform activity. Gamma oscillations proceeded to epileptiform activity also in wild type animals, but only after exposure to 3 times higher concentrations of carbachol. Thus, our observations are well in line with the view that the cholinergic system has important modulatory roles in epileptic seizures (de Freitas et al., 2004; Friedman et al., 2007) and suggest that the presence of Slc10a4 suppresses epileptiform activity.

Because of the observed epileptiform activity, we next decided to evaluate the role of Slc10a4 in an epilepsy model in vivo. One of the most commonly used models for status epilepticus is injection of the cholinergic agonist pilocarpine (Scorza et al., 2009). This treatment induced status epilepticus earlier and more often in mice lacking Slc10a4. The difference was first noted electrographically on EEG, but was confirmed in non-operated animals. In this setting, Slc10a4 null mice displayed behavioral seizures earlier and more frequently than their wild type counterparts. In addition, immunohistochemical analysis of the piriform cortex showed that Slc10a4 null animals displayed an increased number of c-Fos positive cells compared to wild type mice after injection of pilocarpine, which is in line with earlier and/ or more severe seizures in Slc10a4 null mice. Taken together, both our in vitro and our in vivo experiments supports our initial hypothesis — that absence of SLC10A4 conveys an increased sensitivity to cholinergic stimulation.

From a clinical perspective, it is most interesting to note that absence of Slc10a4 seems to induce abnormalities in neuronal network signaling already in the absence of exogenous cholinergic stimulation,

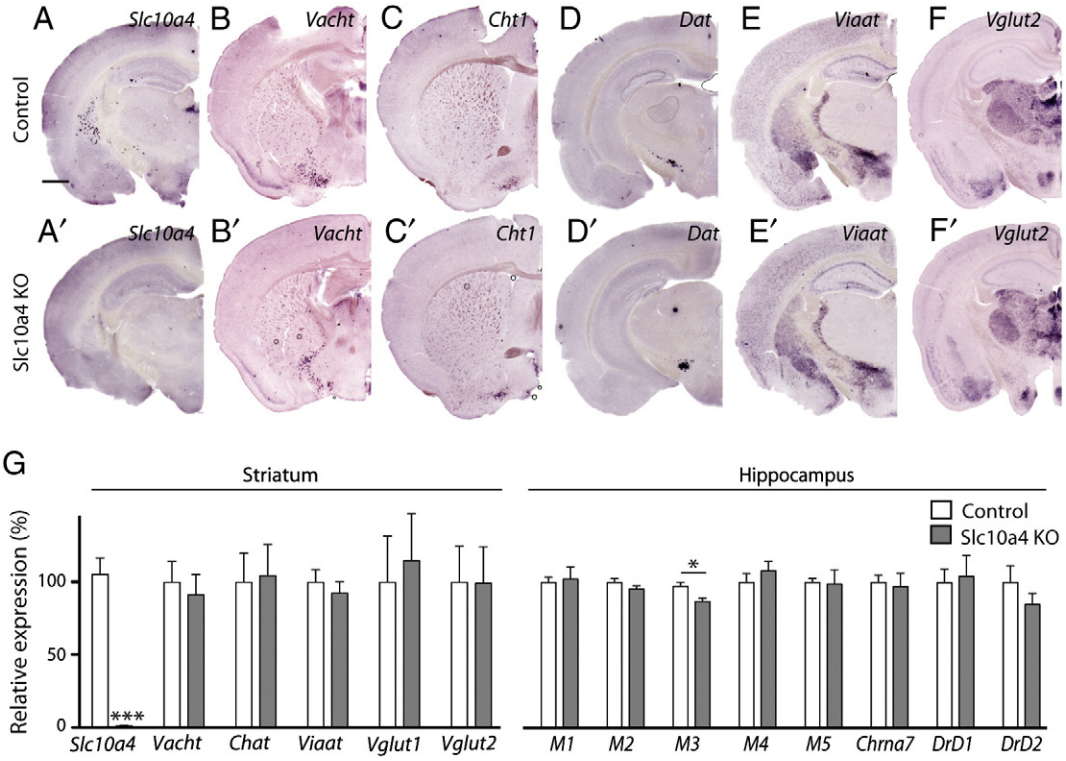

Fig 5. Expression analysis of other synaptic transporters in wild type (Control) and Slc10a4 null (Slc10a4 KO) mice. A–F′. In situ hybridization did not reveal any differences in the expression distribution of synaptic transporters *Vacht*, *Cht1*, *Dat*, *Viaat*, or *Vglut2*. A–F represent wild type mice and A′–F′ represent Slc10a4 null mice. G. RT-qPCR performed on striatal cDNA for genes *Vacht*, *Chat*, *Vglut1*, *Vglut2* and *Viaat*, and on hippocampal cDNA for genes muscarinic cholinergic receptors M1–M5, *ChrnA7*, *DrD1*, *DrD2* did not show any change in the level of mRNAs for these genes, except for a slight downregulation for M3. *Indicates $p<0.05$, ***indicates $p<0.001$, unpaired *t*-test.

manifested as gamma oscillations in the hippocampal slices and as spikes on EEG. Epilepsy affects 0.5–1% of the population and is a severe disabling neurological condition. About one-half to one-third of cases of epilepsy are of unknown origin (Forsgren et al., 2005) and many of these are presumed to be of multigenetic origin, which underlines the need to identify genes that affect the susceptibility to seizures. Our findings indicate that Slc10a4 might be of interest in this quest. However, while Slc10a4-deficient mice displayed spontaneous spike discharges, we have not detected spontaneous seizures and did not find a reduced seizure threshold in Slc10a4 null mice challenged with the glutamatergic agonist kainic acid, indicating that absence of Slc10a4 alone is not enough to induce epilepsy. The kainic acid experiment also indicates that the increased sensitivity to pilocarpine is due to a selective sensitivity to cholinergic stimulation, rather than a generally decreased seizure threshold. It should however be noted that our EEG-sampling periods on untreated mice is relatively short, and that infrequent behaviorally silent seizures remain a possibility. Further long-term experiments are needed to rule out epilepsy in Slc10a4 null mice.

Our findings indicate that more effort should be devoted to investigating possible roles for Slc10a4 in the cholinergic system. Our mRNA expression analysis of synaptic transporters and postsynaptic cholinergic receptors failed to identify clues to the origin of the cholinergic sensitivity and further experiments are required before conclusions can be drawn. One possibility is that absence of Slc10a4 results in a hypocholinergic nervous system, which in turn results in sensitization. Observations similar to ours have been made in mice with reduced expression of VAChT, which also display increased sensitivity to pilocarpine (Guidine et al., 2008). It is therefore possible that our results reflect that absence of Slc10a4 results in constitutively reduced acetylcholine release, in turn causing increased cholinergic sensitivity. An alternative explanation is that there are subtler changes in the postsynaptic receptor function than could be detected by our RT-PCR-screening method; for instance changes in postsynaptic receptor sensitivity or second messenger systems.

This in turn raises more questions. First, how can Slc10a4 impact the release of acetylcholine? Second, the molecule transported by Slc10a4 remains undiscovered. Third, Slc10a4 is expressed by cholinergic and dopaminergic neurons, and the differences between these types of neurons regarding neurotransmitter release and reuptake makes it difficult to envision a directly analog role for Slc10a4 in presynaptic function. For example, dopamine is subject to direct reuptake and vesicular packaging, whereas acetylcholine is synthesized in the presynaptic terminal and degraded in the synaptic cleft following release.

In summary, this paper demonstrates that the recently discovered synaptic protein Slc10a4 affects the sensitivity to cholinergic

Table 1
Summary of evoked epileptic activity in vivo and in vivo.

	Control	KO
In vitro		
Carbachol 90 μM		
Number of events (min)	1.08 ± 0.2	4.1 ± 1.18 ($p<0.01$)
Duration of events (ms)	49 ± 12	810 ± 93 ($p<0.01$)
Kainic acid 500 nM		
Number of events (min)	2.2 ± 0.12	2.1 ± 0.18 (ns)
Duration of events (ms)	82 ± 13	75 ± 14 (ns)
In vivo		
Pilocarpine 325 mg/kg		
Time in seizure (s)	1873 ± 845	4390 ± 170 ($p<0.05$)
Latency to status epilepticus (s)	3583 ± 819	1042 ± 143 ($p<0.01$)
Kainic acid 25 mg/kg		
Time in seizure (s)	3835 ± 105	4152 ± 219 (ns)
Latency to status epilepticus (s)	2505 ± 209	2088 ± 338 (ns)

chemoconvulsants and that absence of Slc10a4 seems to induce altered function of neural networks and possibly epileptic vulnerability. Future studies should be directed at determining the exact function of the protein, as well as pinpointing the synaptic disturbances resulting from its absence. Furthermore, our data indicate that Slc10a4 might be a candidate gene in human genetic studies on epilepsy, either epilepsy of a multigenetic origin or acquired epilepsy where monogenetically induced vulnerability might contribute. Thus, more studies on epileptogenesis in Slc10a4 null mice are warranted.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.expneurol.2012.09.006.

Conflict of interest

The authors declare that they have no conflict of interest.

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