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A Causative Link Between Inner Ear Defects and Long-term Striatal Dysfunction

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Abstract

There is a high prevalence of behavioral disorders that feature hyperactivity in individuals with severe inner ear dysfunction. What remains unknown is whether inner ear dysfunction can alter the brain to promote pathological behavior. Using molecular and behavioral assessments of mice that carry null or tissue-specific mutations of *Slc12a2*, we found that inner ear dysfunction causes motor hyperactivity by increasing in the nucleus accumbens the levels of pCREB and pERK, key mediators of neurotransmitter signaling and plasticity. Hyperactivity was remedied by local administration of the pERK inhibitor SL327. These findings reveal that a sensory impairment, such as inner ear dysfunction, can induce specific molecular changes in the brain that cause maladaptive behaviors, such as hyperactivity, that have been traditionally considered exclusively of cerebral origin.

The inner ear contains the cochlea, devoted to hearing, and the vestibular end organs, devoted to balance. In 20-95% of children with severe hearing loss, auditory and vestibular dysfunction co-occur (1, 2). In such cases, there is a high incidence of behavioral disorders that feature hyperactivity as a core diagnostic symptom (3-5). Although socio-environmental variables have been proposed as risk-factors (6), it is unclear whether sensory impairments such as inner ear defects can directly induce specific changes in the brain that lead to maladaptive behavior. In non-human vertebrates including rodents and frogs, surgical or pharmacological lesions to the vestibulo-auditory system are also linked to long-term changes in locomotor activity, although to date the associations between ear dysfunction and behavior remain unexplained (7-9). Genetic mouse models of inner ear dysfunction can exhibit increased levels of locomotor hyperactivity (10), but because the gene is mutated in the brain as well as the inner ear, the causal neural underpinnings of this behavior remain unknown.

Slc12a2 (also known as *Nkcc1*) is a gene that encodes a sodium-potassium-chloride co-transporter broadly expressed in tissues including the inner ear and central nervous system (CNS) (11, 12). The *Slc12a2* mutant mice used in this study arose spontaneously in our mouse colony and exhibit increased levels of motor hyperactivity, including locomotion,

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circling, and head tossing (Fig. 1A,B, movie S1, fig. S1A), as with previously characterized *Slc12a2* mutants (13-16). The spontaneous mutation was identified as an A to T mutation in exon 17 that changes codon 842 of *Slc12a2* from encoding a lysine (K) to a stop codon (*) (hereafter referred to as $Slc12a2^{K842*}$), resulting in no detectable SLC12A2 protein using antibodies raised to either the N- or C-terminus (fig. S1B-E). The inner ear of $Slc12a2^{K842*/K842*}$ mice revealed a collapse of Reissner's membrane and the membranes of the vestibular compartments (fig. S1F). These morphological defects were associated with profound deafness and balance deficits (fig. S1G, S2; ref 15).

An increase in locomotor activity is not readily explained by dysfunction of the inner ear but rather points to a disruption of brain functions that regulate movement. Consistent with this notion, the dopaminergic antagonist haloperidol, which acts in the brain to alleviate increased motor behavior in humans (17), normalized the open-field locomotor behavior of $Slc12a2^{K842*/K842*}$ mice (Fig. 1A,B). The same dose of haloperidol did not decrease the locomotor activity in littermate controls, indicating that the dose used was not sedating. Haloperidol did not affect grooming in either mutants or controls, nor did it ameliorate performance of mutants on a rotarod test, which requires an intact inner ear, indicating a specificity in the behaviors modified by haloperidol (Fig. 1B, fig. S2).

To test whether loss of *Slc12a2* expression in the brain leads to increased locomotor activity, we deleted a floxed *Slc12a2* allele ($Slc12a2^{fx}$) specifically from individual brain areas that control movement using $Emx1^{Cre}$ for the cortex, *Dlx5/6-Cre* for the striatum, $En1^{Cre}$ for the cerebellum, and *Nestin-Cre* for the entire CNS. With these lines, recombination occurs prior to neurogenesis and expression of *Slc12a2* (18-21). Western blot analyses confirmed the loss of *Slc12a2* specifically in the expected tissues (fig. S3A,C). While mice with a germline deletion of the $Slc12a2^{fx}$ allele, which lack expression in all tissues, are behaviorally indistinguishable from $Slc12a2^{K842*/K842*}$ mice, mice with deletions in the cortex, striatum, cerebellum, or entire CNS are behaviorally normal (Fig. 1C,D, fig. S3B), indicating that loss of *Slc12a2* from any single or combination of brain areas does not cause the behavior. Notably, inner ear morphology and *Slc12a2* expression were normal in *Nestin-Cre;*$Slc12a2^{fx/fx}$ mutants (fig. S3C).

To determine whether loss of *Slc12a2* expression in the inner ear causes the behavioral phenotype, the $Foxg1^{Cre}$ line was used to delete *Slc12a2* from the embryonic precursors of the inner ear (Fig. 2A). However, because $Foxg1^{Cre}$ also targets the telencephalon (neocortex, hippocampus, and striatum; Fig. 2B), *Slc12a2* was deleted from the inner ear using a combination of *Pax2-Cre* and $Tbx1^{Cre}$, which together sufficiently deleted *Slc12a2* to result in the anatomical ear defects indicative of dysfunction (fig. S4). In *Pax2-Cre;*$Tbx1^{Cre}$-driven mutants, *Slc12a2* deletion also occurred in the mid-hindbrain area, but not the telencephalon. Both the *Pax2-Cre;*$Tbx1^{Cre}$ and $Foxg1^{Cre}$-driven mutants recapitulated all the hyperactive features of $Slc12a2^{K842*/K842*}$ mice (Fig. 2C, movie S2, fig. S4). Together with the absence of the behavioral phenotype when *Slc12a2* is deleted from the entire CNS, these results demonstrate that inner ear dysfunction caused the abnormal behavior of these mice.

Because increased locomotor activity and a responsiveness to haloperidol are indicative of brain rather than ear dysfunction, we reasoned that inner ear defects may cause abnormal functioning of the striatum, a central brain area regulating motor output levels. Striatal levels of 26 candidate proteins involved in neurotransmitter signaling were examined by Western blot (Fig. 3A,B; fig. S5). Initially, *Slc12a2*$^{K842*/K842*}$ mutants were examined and proteins showing significant differences in expression levels compared with controls were then also examined in *Nestin-Cre;Slc12a2*$^{fx/fx}$ and *Foxg1*$^{Cre/+}$*;Slc12a2*$^{fx/fx}$ striatums to identify the relevant changes that correlate with the presence of the ear defects and behavioral phenotype. Amongst the proteins examined, significant increases were observed only in the levels of phosphorylated extracellular signal-regulated kinase (pERK), a key component of dopamine and glutamate neurotransmission in the striatum, and its common downstream target phospho-cAMP response-element binding protein (pCREB-Ser133; refs. 22-24) (Fig. 3A,B). Although phosphorylated forms were elevated, total ERK and CREB were unaffected. Increased pERK and pCREB were specific to the striatum and not observed in other forebrain regions (fig. S5D). Immunohistochemical analysis of *Slc12a2*$^{K842*/K842*}$ mutants revealed that the number of pERK+ cells was upregulated specifically in the nucleus accumbens, the ventral part of the striatum (Fig. 3C). All pERK+ cells were NeuN+, indicating that they were neurons. Of these, most were DARPP32+ medium-sized spiny neurons (MSNs) and a few were somatostatin+ interneurons (Fig. 3D), but not calretinin+, parvalbumin+, or ChAT+ interneurons. The proportions of pERK+ cell types in the mutants were nevertheless similar to controls. In addition, pCREB-positive cells were pERK-positive (Fig. 3D).

Robust increases in pERK occur in MSNs of the nucleus accumbens in response to psychostimulants and other drugs of abuse and are considered critical for enabling their long-lasting behavioral changes (22 ,25, 26). The induction of such behaviors is inhibited by the local or systemic application of MEK or ERK kinase inhibitors such as SL327 (22, 27-29). To determine whether striatal ERK phosphorylation was necessary for the abnormal increase in locomotor activity, *Slc12a2*$^{K842*/K842*}$ mice were given an intraperitoneal injection or a local injection of SL327 to the nucleus accumbens. In both sets of experiments, SL327 administration restored locomotor activity to normal levels without affecting the levels of activity in controls (Fig. 4). Mutant mice treated with local SL327 returned to their baseline, pre-surgery locomotor levels of activity by day 3, suggesting that the injection did not cause permanent damage. SL327 administration did not affect grooming, suggesting that increased striatal pERK selectively elevates locomotor activity levels and not general activity.

This study demonstrates that inner ear dysfunction can induce molecular changes in the striatum that promote increased motor hyperactivity**.** The neural circuits linking inner ear defects to abnormal striatal function are likely transmitted by the normal auditory and vestibular input pathways, primarily via the thalamus and neocortex (30), but this remains to be demonstrated. Our results also suggest that a neurobiological cause, rather than simply socio-environmental factors, contributes to the high incidence of behavioral disorders associated with inner ear dysfunction in children and adolescents. Moreover, disruption of the ERK pathway in the striatum provides a potential target for intervention. Finally, it is

intriguing to ponder whether sensory impairments other than those associated with inner ear defects could also cause or contribute to psychiatric or motor disorders that have traditionally been considered exclusively of cerebral origin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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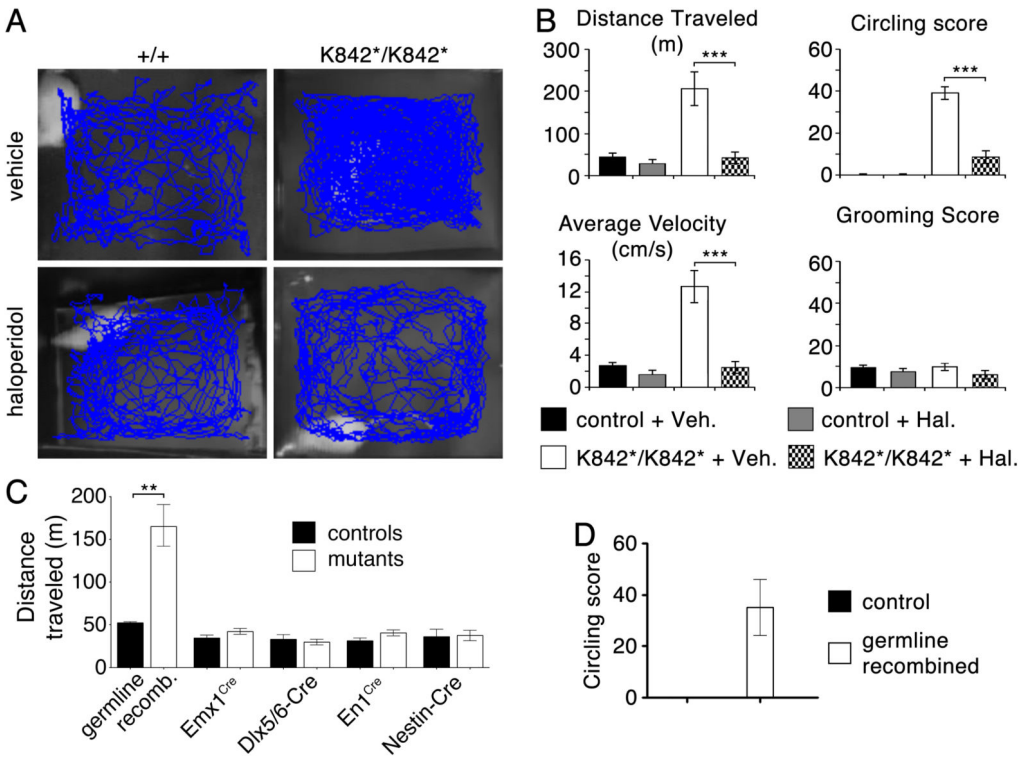

Fig. 1. *Slc12a2*$^{K842*/K842*}$ mutants display a dopamine receptor-mediated increase in locomotor activity that cannot be explained by disruption of *Slc12a2* in the brain

(**A**) Traces and (**B**) quantification of mouse locomotion in an open field showing that haloperidol alleviates locomotor activity and circling in *Slc12a2*$^{K842*/K842*}$ mice without affecting grooming (*** $p < 0.0001$; repeated measures ANOVA with Bonferroni post-hoc comparison). (**C**) Germline recombination of *Slc12a2*$^{fx/fx}$ mice recapitulates the increased locomotion of the *Slc12a2*$^{K842*/K842*}$ mutant ($p = 0.0032$, unpaired two-tailed test). Mice lacking *Slc12a2* in the neocortex and hippocampus (*Emx1*$^{Cre/+}$;*Slc12a2*$^{fx/rec}$), striatum (*Dlx5/6-Cre;Slc12a2*$^{fx/fx}$), cerebellum (*En1*$^{Cre/+}$;*Slc12a2*$^{fx/fx}$), and CNS (*Nestin-Cre;Slc12a2*$^{fx/fx}$) display normal levels of motor activity (unpaired two-tailed test). (**D**) Germline recombination of *Slc12a2*$^{fx/fx}$ also recapitulates the circling behavior of the *Slc12a2*$^{K842*/K842*}$ mutants. N = 4-11 mice per genotype. All data are illustrated as mean ± s.e.m.

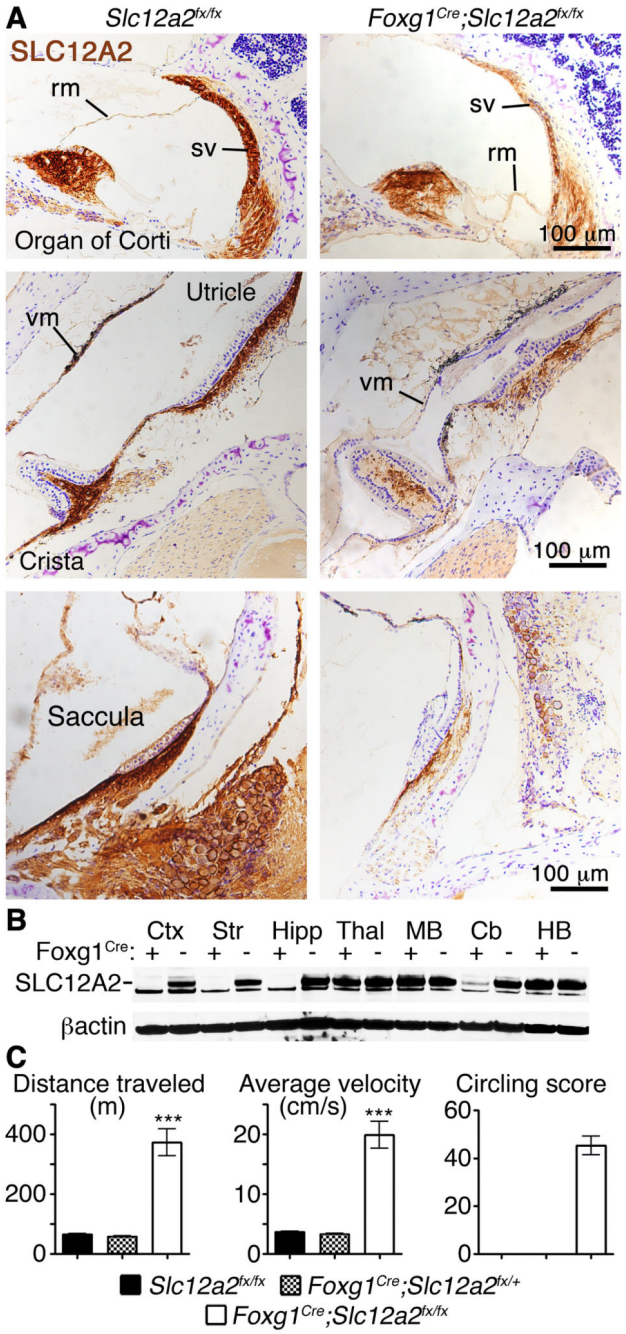

Fig. 2. Increased locomotor activity of *Slc12a2* mutants depends on inner ear dysfunction

(**A**) The inner ear defects of *Foxg1*$^{Cre/+}$;*Slc12a2*$^{fx/fx}$ mice are identical to those of the *Slc12a2*$^{K842*/K842*}$ mice: vestibular (vm) and Reissner's (rm) membrane collapse and degeneration of the vestibular end organs (sacculae, utricles, and cristae). In *Foxg1*$^{Cre/+}$;*Slc12a2*$^{fx/fx}$ mice, SLC12A2 immunostaining (brown) is reduced in the endolymph secreting stria vascularis (sv) of the cochlea and in the vestibular end organs. Purple: Nissl counterstain. (**B**) Western Blot analysis shows loss of SLC12A2 in the neocortex (Ctx), hippocampus (Hipp), and striatum (Str) in *Foxg1*$^{Cre/+}$;*Slc12a2*$^{fx/fx}$ mice,

but not in the thalamus (Thal), midbrain (MB), cerebellum (Cb), or hindbrain (HB). (**C**) *Foxg1*$^{Cre/+}$;*Slc12a2*$^{fx/fx}$ mice recapitulate the abnormal behavior of *Slc12a2*$^{K842*/K842*}$ mice (n = 7-9 mice per genotype, *** $p < 0.0001$, unpaired two-tailed test, mean ± s.e.m.).

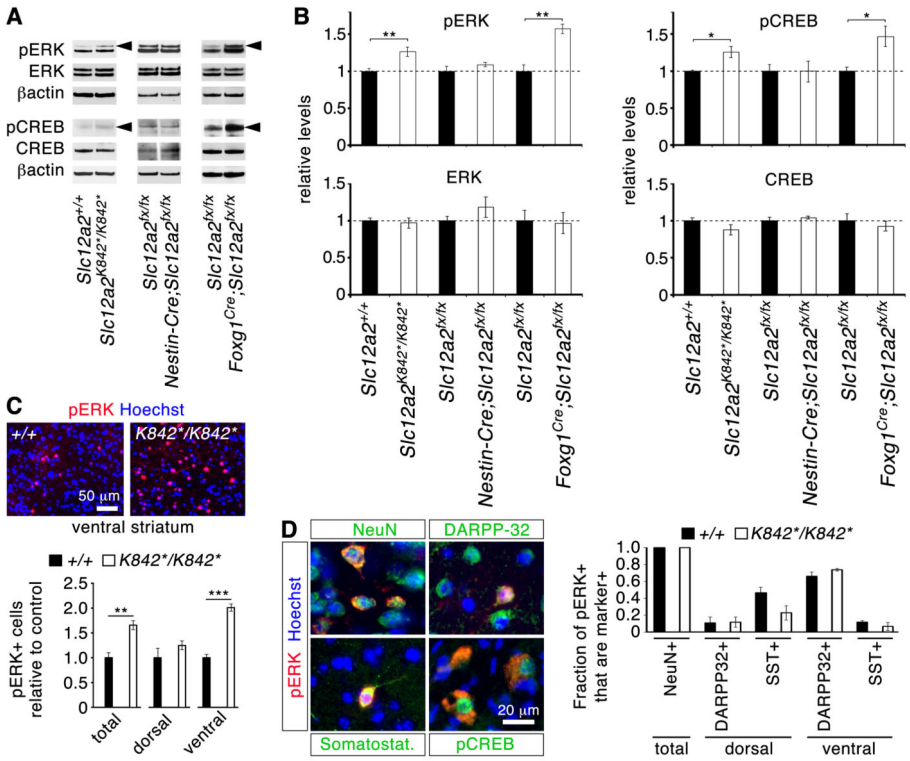

Fig. 3. Inner ear dysfunction contributes to the up-regulation of pERK1 in striatal neurons

(**A**) Western blot analyses reveal increased (arrowheads) pERK1 (upper band of doublet) and its common target pCREB in mice with inner ear defects and increased motor activity (*Slc12a2*$^{K842*/K842*}$ and *Foxg1*$^{Cre/+}$;*Slc12a2*$^{fx/fx}$ mice), but not in phenotypically normal mice. (**B**) Quantification of Western analyses (pERK: p = 0.009 for *Slc12a2*$^{K842*/K842*}$; p = 0.006 for *Foxg1*$^{Cre/+}$;*Slc12a2*$^{fx/fx}$; pCREB: p = 0.028 for *Slc12a2*$^{K842*/K842*}$; p = 0.033 for *Foxg1*$^{Cre/+}$;*Slc12a2*$^{fx/fx}$; n = 3-6 mice per genotype; unpaired two-tailed t-test). (**C**) Immunohistochemical analysis showed an increase in the number of pERK+ cells in the ventral striatum of mutants (n = 4 mice per genotype, ** p = 0.0066, *** p = 0.00046, unpaired two-tailed t-test). (**D**) In both controls and mutants, pERK+ cells in the ventral striatum are primarily DARPP32+, whereas in the dorsal striatum they are somatostatin+ (SST+) interneurons (n = 3 per genotype) (mean ± s.e.m.).

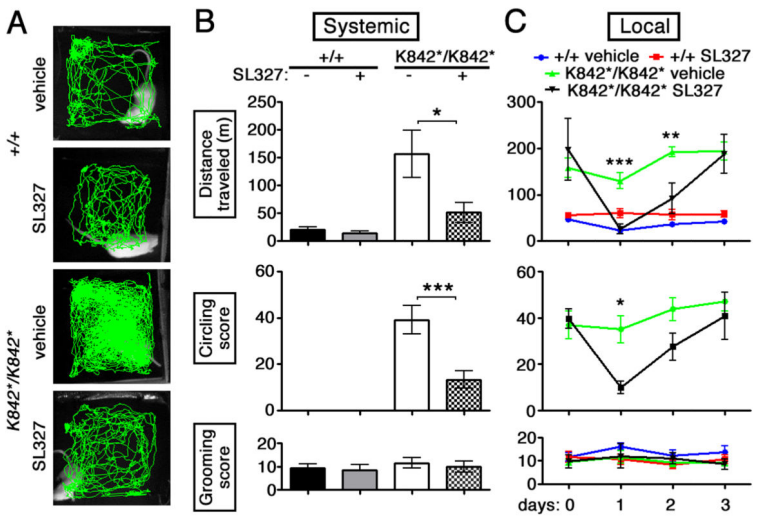

Fig. 4. The increased striatal pERK levels induced by inner ear defects promote increased locomotor activity

(**A**) Traces and (**B**) quantification of motor activity in an open-field one hour after systemic SL327 administration, which ameliorates both locomotion and circling in the *Slc12a2*$^{K842*/K842*}$ mutants while having little effect on grooming (n = 6 mice per genotype, * p = 0.015, *** p = 0.0002). (**C**) Local SL327 administration to the nucleus accumbens of *Slc12a2*$^{K842*/K842*}$ mice restored open-field activity and circling to control levels, which were unaffected by treatment, for 2-days post-injection, without affecting grooming (n = 5-7 mice per genotype and per treatment condition, *** p = 0.0025, ** p = 0.0082, *** p = 0.018; mean± s.e.m; two-way repeated measures ANOVA with Bonferroni post-hoc comparison).