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CHARACTERIZATION OF COGNITIVE IMPAIRMENTS AND NEUROTRANSMITTER CHANGES IN A NOVEL TRANSGENIC MOUSE LACKING *SLC10A4*

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Abstract—An orphan member of the solute carrier (SLC) family SLC10, SLC10A4 has been found to be enriched in midbrain and brainstem neurons and has been found to co-localize with and to affect dopamine (DA) homeostasis. We generated an SLC10A4 knockout mouse (*Slc10a4*$^{\Delta/\Delta}$) using Cre-targeted recombination, and characterized behavioral measures of motor and cognitive function as well as DA and acetylcholine (ACh) levels in midbrain and brainstem. In agreement with previous studies, *Slc10a4* mRNA was preferentially expressed in neurons in the brains of wild-type (*Slc10a4*$^{+/+}$) mice and was enriched in dopaminergic and cholinergic regions. *Slc10a4*$^{\Delta/\Delta}$ mice had no impairment in motor function or novelty-induced exploratory behaviors but performed significantly worse in measures of spatial memory and cognitive flexibility. *Slc10a4*$^{\Delta/\Delta}$ mice also did not differ from *Slc10a4*$^{+/+}$ in measures of anxiety. High-performance liquid chromatography (HPLC) measures on tissue punches taken from the dorsal and ventral striatum reveal a decrease in DA content and a corresponding increase in the metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), indicating an increase in DA turnover. Punches taken from the brainstem revealed a decrease in ACh as compared with *Slc10a4*$^{+/+}$ littermates. Together, these data indicate that loss of SLC10A4 protein results in neurotransmitter imbalance and cognitive impairment.

Key words: SLC10A4, dopamine, acetylcholine, cognitive impairment.

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Abbreviations: ACh, acetylcholine; ANOVA, analysis of variance; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HPLC, High-performance liquid chromatography; PD, Parkinson's disease; PBS, phosphate-buffered saline; qPCR, quantitative PCR; RM, repeated-measures; SLCs, solute carriers; SNC, substantia nigra pars compacta; VTA, ventral tegmental area.

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INTRODUCTION

During normal cell function, transport of proteins, compounds, and sugars across cell membranes is crucial to maintaining proper signaling. One of the primary mechanisms of transport across cell membranes are proteins that belong to the superfamily of solute carriers (SLCs), which comprises over 300 different proteins and vary greatly in their expression and function (Lin et al., 2015). Members of this family of transporters are the primary conveyance for neurotransmitters across cell membranes, resulting in properly functioning vesicles and maintenance of the synapse. While primary SLCs for the transport of major neurotransmitters like glutamate, GABA, dopamine (DA), serotonin, norepinephrine or acetylcholine (ACh) are well known and characterized, there are many "orphan" SLCs that are expressed in the brain, the function of which is still poorly understood. One SLC that has recently emerged as a potentially novel monoaminergic and cholinergic vesicular transporter is SLC10A4 (Larhammar et al., 2015).

The human *SLC10A4* gene was first discovered through a bioinformatics approach and a gene expression analysis confirmed expression of SLC10A4 in the human brain (Splinter et al., 2006). Robust expression in the brain tissue was then validated both in mouse by *in situ* hybridization (Lein et al., 2007) and rat brain by immunohistochemistry (Geyer et al., 2008). Although, Geyer and coworkers (2008) did not elucidate functional properties of SLC10A4, they demonstrated expression of SLC10A4 in cholinergic neurons. They later validated this finding and extended it, reporting co-expression of SLC10A4 with the vesicular monoamine transporter VMAT2 (Burger et al., 2011). At the same time, a gene-expression analysis of dopaminergic neurons in rats confirmed *Slc10a4* mRNA enrichment in the midbrain (Zhou et al., 2011).

Although homology analysis has shown that SLC10A4 belongs to the family sodium-bile acid cotransporters, experiments in rat and human cells either failed to confirm transport activity for bile acids or related molecules (Splinter et al., 2006; Geyer et al., 2008), or reported only low bile-acid transport activity for SLC10A4 (Bijsmans et al., 2012; Abe et al., 2013). The first evidence for a function of SLC10A4 in the brain was reported from the Kullander group (Zelano et al., 2013). They showed that mice lacking SLC10A4 had increased sensitivity to cholinergic stimulation resulting in decreased

seizure threshold to cholinergic stimuli in hippocampal slices and increased sensitivity to the status epilepticus inducing cholinergic agent pilocarpine. They later went on to demonstrate that SLC10A4 is important for DA homeostasis and neuromodulation in vivo, and that mice lacking SLC10A4 are slightly hypoactive and display heightened locomotor sensitivity to the dopamine-releasing drug amphetamine and to tranylcypromine, a monoamine oxidase inhibitor that prevents DA degradation (Larhammar et al., 2015).

Here, we extended the scope of *Slc10a4* expression analyses, by quantifying *Slc10a4* mRNA in mouse brain regions identified by previous studies (Lein et al., 2007) and by assessing *Slc10a4* expression in two other major cell types of the brain, astrocytes and microglia. We have also generated a genetic mouse model of SLC10A4 loss by deletion of the *Slc10a4* gene. Using this mouse model, we further examined the consequences of *Slc10a4* loss on cognitive behaviors that are dependent on hippocampal or striatal dopaminergic function (Darvas and Palmiter, 2010, 2011; Melief et al., 2015), and which have been implicated in neurodegenerative diseases like Parkinson's (Goldman et al., 2013) and Alzheimer's disease (Albert et al., 2011). We further analyzed the effects of deleting *Slc10a4* on brain ACh levels.

EXPERIMENTAL PROCEDURES

Animals

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Washington. The murine *Slc10a4* gene has three exons, all of which contain protein coding regions (Fig. 1A). To generate *Slc10a4* null mice ($Slc10a4^{\Delta/\Delta}$), we first generated $Slc10a4^{lox/lox}$ mice which allow Cre-dependent deletion of the *Slc10a4* gene. $Slc10a4^{lox/lox}$ mice were generated by gene targeting. They have a triple hemagglutinin tag (3xHA) fused to the C-terminus protein coding region of exon 1 and loxP sites flanking exon 2 (Fig. 1B). We crossed $Slc10a4^{lox/lox}$ mice with the B6.129S4-$Meox2^{tm1(cre)Sor}$/J strain (Tallquist and Soriano, 2000) to delete one copy of exon 2 of the *Slc10a4* gene in early embryos ($Slc10a4^{\Delta/+}$, Fig. 1C). Thus generated $Slc10a4^{\Delta/+}$ mice were crossed with C57BL/6J mice to remove the Cre recombinase allele, followed by backcrossing to the C57BL/6J genetic background for 10 generations to generate breeding pairs used for the experiments presented in this manuscript. Only $Slc10a4^{\Delta/+}$ mice were used for breeding and produced litters of wild-type ($Slc10a4^{+/+}$) litter-mate control and $Slc10a4^{\Delta/\Delta}$ mice on a C57BL/6J background. Mice of both genders at the age of 3–5 month were used for all experiments. All mice were housed in groups of 3–5 animals under a 12-h, light–dark cycle (6 AM–6 PM) in a temperature-controlled environment with food and water available *ad libitum*.

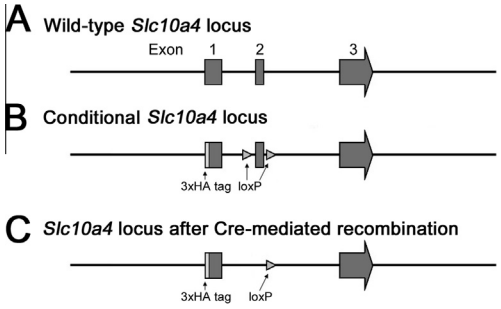

Fig. 1. Targeting strategy for generation of $Slc10a4^{\Delta/\Delta}$ and $Slc10a4^{\Delta/+}$ mice. (A) Wild-type *Slc10a4* locus. (B) Conditional *Slc10a4* with loxP sites flanking Exon 2. (C) $Slc10a4^{\Delta}$ locus lacking Exon 2 after Cre excision.

Reagents

RNA extraction kits (Ambion), TaqMan probes and primers (Applied Biosystems), and penicillin–streptomycin (Gibco) were purchased from Thermo Fisher Scientific (Waltham, MA). The iScript cDNA synthesis kit was purchased from Bio-Rad (Hercules, CA). DMEM/F12 medium and fetal bovine serum were purchased from HyClone (Logan, UT). Papain and DNase I were purchased from Worthington Biochemical (Lakewood, NJ). L-cysteine and poly-L-ornithine were purchased from Sigma–Aldrich (St. Louis, MO).

Slc10a4 expression

Quantification of Slc10a4 mRNA expression in $Slc10a4^{+/+}$ mouse brain. According to the Allen Mouse Brain Atlas (http://mouse.brain-map.org), *Slc10a4* mRNA is enriched in the striatum, midbrain and brainstem (Lein et al., 2007). To quantify expression of *Slc10a4* mRNA in these brain regions, we collected $Slc10a4^{+/+}$ tissue from striatum, the brain stem, and from the midbrain substantia nigra pars compacta (SNC) and ventral tegmental area (VTA). Tissue was immediately flash-frozen in liquid nitrogen and stored at −70 °C. Tissue was homogenized, followed by RNA extraction using Ambion PureLink®RNA kits, and then reverse-transcription with iScript cDNA synthesis kits. TaqMan probes and primers of *Slc10a4* were purchased from Applied Biosystems. Quantitative PCR (qPCR) was performed on an Applied Biosystems ViiA 7 Real-Time PCR System with the method of relative quantitation (Larionov et al., 2005) using normalization to glyceraldehyde phosphate dehydrogenase (GAPDH) expression (Li et al., 2015) and striatal *Slc10a4* mRNA expression as a calibrator.

Determination of brain cell-types expressing Slc10a4 mRNA. Primary mixed glial cultures were generated from 1- to 3-day-old $Slc10a4^{+/+}$ pups as described previously (Li et al., 2015). In brief, cerebral cortex was dissected from brains and remaining meninges were removed while in ice-cold phosphate-buffered saline (PBS). Cortical tissue was digested by a 30-min incubation in DMEM/F12 medium containing papain (15 U/ml), DNase I (200 mg/ml), EDTA (0.5 mM) and L-cysteine (0.2 mg/ml) at 37 °C, and then dissociated mechanically. The resulting cell suspension was plated on poly-L-ornithine-coated flasks and maintained at 37 °C and 5% CO_2 in DMEM/F12 supplemented with 10% fetal bovine serum, penicillin