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A Transgenic Mouse Model to Selectively Identify α_3 Na,K-ATPase Expressing Cells in the Nervous System

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Abstract

The α_3 Na⁺,K⁺-ATPase (α_3 NKA) is one of four known α isoforms of the mammalian transporter. A deficiency in α_3 NKA is linked to severe movement control disorders. Understanding the pathogenesis of these disorders is limited by an incomplete knowledge of α_3 NKA expression in the brain as well as the challenges associated with identifying living cells that express the isoform for subsequent electrophysiological studies. To address this problem, transgenic mice were generated on the C57BL/6 genetic background, which utilize the mouse α_3 subunit gene (*Atp1a3*) promoter to drive the expression of ZsGreen1 fluorescent protein. Consistent with published results on α_3 NKA distribution, a ZsGreen1 signal was detected in the brain, but not in the liver, with *Atp1a3*-ZsGreen1 transgenic mice. The intensity of ZsGreen1 fluorescence in neuronal cell bodies varied considerably in the brain, being highest in the brainstem, deep cerebellar and select thalamic nuclei, and relatively weak in cortical regions. Fluorescence was not detected in astrocytes or white matter areas. ZsGreen1-positive neurons were readily observed in fresh

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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Brain structures/centers mentioned in the text were annotated according to the Allen Mouse Brain Atlas (see <http://mouse.brain-map.org/static/atlas> and Table 1)

(unfixed) brain sections, which were amenable to patch-clamp recordings. Thus, the α_3 NKA-ZsGreen1 mouse model provides a powerful tool for studying the distribution and functional properties of α_3 NKA-expressing neurons in the brain.

Keywords

Atp1a3; sodium-potassium ATPase; transgenic mice; central nervous system; ZsGreen1

1.0 Introduction

The Na⁺,K⁺-ATPase (NKA) pump is a fundamental constituent of every cell in the body. It is responsible for active transport of Na⁺ and K⁺, and maintenance of the plasma membrane potential. The transporter is composed of α , β and γ subunits. The α subunit determines all major catalytic and transport properties of the pump including binding and hydrolysis of ATP, binding and trans-membrane translocation of Na⁺ and K⁺, and binding to selective pump inhibitors (e.g. cardiac glycosides). There are four known α subunit for NKA in mammals that are expressed in a spatiotemporal manner (Blanco and Mercer, 1998; Lingrel et al., 1990; Sweadner, 1989; Sweadner, 1991). The transporter containing the α_3 isoform (α_3 NKA) is essentially restricted to the nervous system (Dobretsov and Stimers, 2005; Sweadner, 1991). Within the nervous system, expression of α_3 NKA appears to be limited to neurons; glial cells contain the α_1 and α_2 isoforms, but not the α_3 isoform of the transporter (Cameron et al., 1994; Dobretsov and Stimers, 2005; Sweadner, 1991). Further complexity is provided by the assortment of NKAs present within cells. Some neurons express α_3 NKA either alone or in combination with other α isoforms of NKA, while others do not express any α_3 NKA, instead relying on other isoforms (primarily α_1 NKA alone) for function (Dobretsov and Stimers, 2005; Hieber et al., 1991; McGrail et al., 1991; Shyjan and Levenson, 1989; Watts et al., 1991).

Mice that are null for α_3 NKA, or harbor mutations in *Atp1a3* that perturb the expression and/or function of both alleles die shortly after birth (Clapcote et al., 2009; Ikeda et al., 2013; Lingrel et al., 2007; Moseley et al., 2007). In humans, *ATPIA3* mutations that result in haploinsufficiency are linked to grave neurological deficits including rapid-onset dystonia Parkinsonism (RDP) (Anselm et al., 2009; Brashear et al., 2007; de Carvalho et al., 2004; Lee et al., 2007; McKeon et al., 2007; Rodacker et al., 2006), alternate hemiplegia of childhood (AHC) (Heinzen et al., 2012; Hoei-Hansen et al., 2014; Panagiotakaki et al., 2010; Rosewich et al., 2012; Yang et al., 2014), cerebral ataxia, areflexia, pes cavus, optic atrophy and sensorineural hearing loss (CAPOS syndrome) (Demos et al., 2014; Heimer et al., 2015; Maas et al., 2016), or catastrophic early life epilepsy, episodic apnea, and postnatal microcephaly (Paciorkowski et al., 2015). Likewise, genetically engineered *Atp1a3*^{+/−} mice (Deandrade et al., 2011; Ikeda et al., 2017; Ikeda et al., 2013; Moseley et al., 2007), or mice that carry a missense mutation (D801Y, D801N or 1810N) that disrupts the enzymatic activity of one *Atp1a3* allele (Clapcote et al., 2009; Grafe et al., 1994; Hunanyan et al., 2015; Isaksen et al., 2017; Kirshenbaum et al., 2011), develop impaired movement, deficits in learning and memory, and in some cases, spontaneous or inducible hemiplegic episodes and tonic seizures (reviewed in Holm and Lykke-Hartmann, 2016; Isaksen et al., 2017).

Thus, a full complement of native (unmutated) α_3 NKA is necessary for normal nervous system function. These studies demonstrate that other NKA isoforms cannot compensate for impairment in the levels or activity of α_3 NKA. However, elucidating the specific roles for α_3 NKA in neurons has been hindered by a lack of means to identify cells containing this isoform that are readily compatible with approaches for functional analysis (i.e. electrophysiology) (Dobretsov and Stimers, 2010).

Current methods for identification of cells expressing α_3 NKA make use of *in situ* hybridization (ISH) and immunostaining techniques, or exploit the high sensitivity of this isoform to inhibition by cardiac glycosides (e.g. ouabain) in certain species, including rodents (Bottger et al., 2011; Dobretsov and Stimers, 2005). However, ISH and immunohistochemical detection must be performed subsequent to any electrophysiological investigation, requiring that the functional analysis to be performed blindly with respect to a neuron's α_3 NKA phenotype, and only after invasive recording with an intracellular or a patch-clamp electrode (Parekh et al., 2010). In addition, the signal generated by ISH or immunohistochemistry may be difficult to discern above background in neurons containing low levels of α_3 NKA (Bottger et al., 2011; Richards et al., 2007). Furthermore, due to the architectural complexity of the CNS, it is sometimes difficult to associate a particular signal with a given neuron (Dobretsov and Stimers, 2005; McGrail et al., 1991). In rodents α_3 NKA is highly sensitive to ouabain. Therefore the inhibitor has often been used to selectively identify α_3 NKA-positive cells in electrophysiological experiments (Richards et al., 2007; Ross and Soltesz, 2000). However, the number of cells capable of being studied in a given preparation is limited because of the risk of incomplete washout of the inhibitor. Moreover, ouabain-sensitive changes in membrane potential or current cannot be used to distinguish cells with NKAs that are affected equally by the inhibitor (i.e. α_1 and α_2 isoforms).

To facilitate functional studies of α_3 NKA in its native environment, we have generated a novel transgenic mouse model that utilizes the promoter of the mouse α_3 subunit gene (*Atp1a3*) to drive the expression of ZsGreen1 fluorescent protein (ZsGreen1). Presented here is an initial characterization of *Atp1a3*-ZsGreen1 mice demonstrating their use in mapping brain regions containing the α_3 NKA isoform, and in identifying α_3 NKA-positive neurons for electrophysiological studies.

2.0 Experimental procedures

2.1 *Atp1a3*-ZsGreen1 plasmid construction

A portion of the mouse *Atp1a3* gene (promoter and entire 5'-untranslated region) linked to the expression cassette from pZsGreen1-1 (Clontech, Palo Alto, CA, USA) was synthesized and cloned into the EcoRV site of pUC57 by GenScript (Piscataway, NJ, USA) to generate the *Atp1a3*-ZsGreen1 plasmid. The *Atp1a3* sequence spans positions -1578 to +177 (relative to the transcription start site), and is highly conserved between human, rat and mouse. It contains most (if not all) of the transcription regulatory elements mapped to date (Benfante et al., 2005; Henriksen et al., 2013; Li and Langhans, 2015; Murakami et al., 1997).

2.2 Production of Atp1a3-ZsGreen1 transgenic mice

Mice that harbor Atp1a3-ZsGreen1 transgene were generated onsite at the Transgenic Core Facility by microinjection of a 2,969 bp BglIII-AflIII fragment from the Atp1a3-ZsGreen1 plasmid into fertilized C57BL/6N (Charles River Laboratories) mouse eggs. All procedures involving the use of mice were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Research Council's Guide for the Care and Use of Laboratory Animals.

2.3 Genotyping

Genomic DNA was isolated from tail biopsies obtained from mice according to HotSHOT protocol (Truett et al., 2000). Mice were genotyped for the presence of the Atp1a3-ZsGreen1 transgene by PCR using the following primers: ZsGreen1-F, 5' -

CATGTACCACGAGTCCAAGTTCTAC-3'; ZsGreen1-R, 5' - TCAGCTTGTGCTGGATGAAGTG-3'.

PCR conditions were as follows: 94°C for 2 min followed by 35 cycles of amplification (94°C denaturation for 20 sec, 59°C annealing for 15 sec, 68°C elongation for 1 min).

Subsequently, PCR products were fractionated on a 1% agarose gel to determine those animals that harbor the transgene.

2.4 RT-PCR

Total RNA was isolated from whole brain of P2, P7, P14, P21 and P80 mice using Trizol (Invitrogen, Carlsbad, CA, USA). Any contamination with genomic DNA was eliminated through use of the DNA-free DNA Removal Kit (Invitrogen) according to the manufacturer's specifications. First-strand cDNA synthesis was generated with the iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA, USA) per supplier's instructions using 1.0 of total RNA in a final reaction volume of 20 µl. Conditions for reverse transcription (RT) were as follows: 5 min at 25°C, 30 min at 42°C, and then 5 min at 25°C. The cDNA reactions were aliquoted and stored at -70°C until further use. Semi-quantitative PCR was performed using KlenTaq LA DNA polymerase (DNA Polymerase Technology, St. Louis, MO, USA) according to the manufacturer's protocol. Each reaction (20 µl final volume) contained 1 µl of cDNA as template. The same forward primer was used to amplify both Atp1a₃ and ZsGreen1 Primers were as follows: Atp1a₃/ZsGreen1-F, 5' -GTCTGAACGCCGCTCTT-3'; Atp1a₃-R, 5' -CATTACCTGCATCTTTTCACC-3'; ZsGreen1-R, 5' -TCAGCTTGTGCTGGATGAAGTG-3'; 18S rRNA-F, 5' -CGCGTTCTATTTTGTGGT-3'; 18S rRNA-R, 5' -AGTCGGCATCGTTTATGGTC-3'. PCR conditions were similar to those described earlier for genotyping of transgenic mice, with minor modifications. The number of amplification cycles was 27 for Atp1a₃, 29 for ZsGreen1, and 18 for 18S rRNA, which were determined empirically to be within the linear range. Expression of the Atp1a3-ZsGreen1 transgene and endogenous *Atp1a3* gene is reported relative to that for 18S rRNA, as determined by Image J analysis.

2.5 Histology

Transgenic mice and wild type (non-transgenic) littermates were anesthetized with isoflurane and subsequently perfused with 0.05% heparin/0.9% NaCl, followed by 4%

paraformaldehyde-lysine- periodate fixative (PLP), pH 7.4. Brain, spinal cord and liver were removed and post-fixed in 4% PLP at 4°C overnight and then cryoprotected with 30% sucrose in phosphate buffered saline (PBS), pH 7.4, and stored at 4°C until use in confocal microscopy and immunofluorescence studies. For microscopy, coronal or sagittal slices (60 µm) were obtained with a cryostat and stored free floating in 0.1 M glycine/PBS with 0.02% Microcide at 4°C, prior to analysis. Antibodies for fluorescence and confocal microscopy were as follows: monoclonal antibody to calbindin-D-28K (1:2500 dilution; CalB; Cat. # C9848, clone CB955, Sigma Chemical Co., St. Louis, MO, USA) was used to selectively identify Purkinje cells in the cerebellum; monoclonal antibody to glutamate decarboxylase, GAD67 (1:500; Cat. # MAB5406, EMD Millipore Corp., Temecula, CA, USA) and glial fibrillary acidic protein (GFAP; 1:1000; Cat.# 173002, Synaptic Systems, Gottingen, Germany) antibody were used to identify GABAergic neurons and astroglia, respectively.

2.6 Image Analysis

Tissue sections were evaluated and images captured between 2.5–40× magnification using an upright BX51WI Olympus microscope (Olympus America, Melville, NY, USA) equipped with epi- fluorescent attachment and Chroma Technology, Corp. (Bellows Falls, VT, USA) 49002 EGFP/FITC/Cy2 filter cube (Em/Ex ratio 470/525) and a Coolsnap Cf digital camera (Photometrix, Tucson, AZ, USA) or a Zeiss Axioskop 40 microscope equipped with fluorescent light source and digital camera (Carl Zeiss Microimaging, Inc., NY, USA). Confocal images were collected from a Zeiss Pascal confocal microscope with a 20× objective.

The confocal acquired image stacks (z-stacks) were reconstructed into 3D image projections and analyzed using the Imaris software (Bitplane AG, Zurich, Switzerland). Analysis of all other images was conducted using the (open source) Fiji Image J software package (see (Schindelin et al., 2012) and www.imagej.net). Whenever required, images were stitched together using a pairwise stitching Fiji Image J plugin (Preibisch et al., 2009). Identification of brain regions labeled with ZsGreen1 was based on the Allen Mouse Brain Atlas (AMB atlas) available from: <http://mouse.brain-map.org> (© 2015 Allen Institute for Brain Science, (Lein et al., 2007)). All images used for semi-quantitative analysis and subject-to-subject comparison of the labeling intensity in various brain regions were 1300 × 1030 pixel images captured with a 2.5× objective (5.20 × 4.12 mm or 16 µm² of the section area per pixel). Images captured at higher resolutions were used only for within-image evaluation of labeling.

For semi-quantitative analysis of transgene expression in different brain regions having a convex shape (e.g. most subcortical brain structures and nuclei), regions of interest (ROI) were outlined with the Image J polygon selection tool, and ROI manager mean pixel intensity of fluorescence was determined taking into account the integral measure of size, density, and intensity of fluorescence of ZsGreen1- positive cells within a selected region (see Fig. 1). A segmented line tool was also used for analysis of laminated structures such as cerebral or cerebellar cortex, olfactory bulb, and hippocampal formations. This Image J tool allows setting the width of the selection line (in pixels) and returns the mean intensity per pixel of selected area (“Analyze: Measure” menu command) or line selection pixel intensity

profile where each step of the line represented by mean line width pixel intensity (“Analyze: Plot Profile” menu command). No adjustments were made to image contrast or brightness, however the rolling ball (10 pixels) background subtraction protocol was applied before any measurement to remove background signal. Pixel intensity is expressed in units of 256 shades of gray scale.

2.7 Electrophysiological recording

For electrophysiological experiments, animals were anesthetized with isoflurane prior to decapitation and the brain was quickly removed. Sagittal sections (350 μ m) of hippocampus were cut using a vibratome stage (Leica VT1200S) at 4°C in oxygenated slicing solution containing the following (in mM): 120 sucrose, 65.5 NaCl, 2 KCl, 1.1 KH₂PO₄, 25 NaHCO₃, 10 D-glucose, 1 CaCl₂, and 5 MgSO₄. The slices were then stored for at least 60 min at room temperature in oxygenated artificial cerebrospinal fluid (ACSF; osmolality 290–300 mOsm) in an incubation chamber containing the following (in mM): 119 NaCl, 2.5 KCl, 1 KFI₂PO₄, 25 NaHCO₃, 10 D-glucose, 2.5 CaCl₂, and 1.3 MgSO₄. Subsequently, slices were transferred to the recording chamber and mounted on the stage of a microscope. Current-clamp (whole-cell) recordings were performed using infrared differential interference contrast (IR-DIC) microscopy and a 40X water-immersion objective. Slices were continuously perfused with ACSF solution saturated with 95%O₂-5%CO₂ at 30°C. Patch electrodes (final resistance 5–7 M Ω) were pulled from borosilicate glass (Sutter Instruments, Novato, CA, USA) on a horizontal Flaming-Brown microelectrode puller (model P-97, Sutter Instruments), using a 3-stage pull protocol. Voltage and current signals were recorded using a Multiclamp-700B amplifier (Molecular Devices, Sunnyvale, CA, USA). During a typical experiment (20 to 60 min), the access resistance was monitored with a 10 ms and –10 mV test pulse delivered at 2 min intervals. The experiment was terminated if the series resistance increased by 25% from its original value. Currents were low-pass filtered at 2 kHz, digitized at 5 kHz (Digidata 1440-A), acquired using Clampex 10.2 software, and analyzed off-line with Clampfit 10.2 software (Molecular Devices).

2.8 Statistical analysis

Data are presented as mean $\pm$ SE. One-way repeated-measures analysis of variance (RM-ANOVA) with Tukey’s multiple comparison post-test or two-way RM-ANOVA with a Bonferroni’s post hoc tests and/or Student’s t-test were performed using GraphPad Prism 5.0 (La Jolla, CA, USA), accordingly. Statistical graphs were prepared using OriginPro 9.0 software (OriginLab, Northampton, MA, USA).

3.0 Results

3.1 Atp1a3-ZsGreen1 transgenic mouse lines

Two lines of transgenic mice were established that express the Atp1a3-ZsGreen1 transgene. The lines are designated 838 and 840 based on numbering from the Transgenic Core Facility. Both lines of mice exhibited normal growth and breeding capacity, and did not display any outward phenotype, suggesting that neither the transgene nor its product (ZsGreen1) cause little (if any) interference with expression and/or function of the endogenous α_3 subunit of NKA. As expected, mating of hemizygous transgenic mice with

wild-type animals resulted in roughly 50% of the progeny being transgenic. All of the studies presented here were performed with mice from line 840, although similar results (data not shown) were obtained with line 838.

3.2 Expression of the *Atp1a3*-ZsGreen1 transgene is consistent to that of the endogenous *Atp1a3* gene

Developmental expression of the *Atp1a3*-ZsGreen1 transgene was evaluated by RT-PCR analysis using RNA isolated from brain or liver, and compared to that from the endogenous *Atp1a3* gene. As shown in Fig. 2, younger mice (P0-P14) expressed the transgene and endogenous *Atp1a3* gene to higher levels in brain than did older mice (P21-P80) ($p < 0.01$ by two-sample t-test; Fig 2B). Neither the transgene nor the endogenous gene was expressed in liver, throughout development, as expected (Blanco, 2005).

3.3 Mapping of fluorescent signal in *Atp1a3*-ZsGreen1 mouse brain

To discern which cells express the *Atp1a3*-ZsGreen1 transgene, frozen tissue sections were obtained from male mice (2–3 months old) and analyzed for fluorescence. Representative low-magnification images are shown in Fig. 3. Only a diffuse background fluorescent signal was observed in liver (Fig. 3A,B), while cerebellar brain sections demonstrated bright punctate labeling over the Purkinje cell layer (PJ) and deep cerebellar nuclei (DCN) region (Fig. 3C,D). Punctate labeling was also observed in PJ and DCN cerebellar and brainstem regions of an image taken at lower magnification (2.5×) from a different *Atp1a3*-ZsGreen1 mouse (Fig. 3E,F). The fluorescent signal is confined to discrete populations of neuronal cells. No ZsGreen1-specific labeling was observed in regions enriched for glial cells such as cerebellar white matter tracts (Fig. 3E,F), although this as well as in other myelin-enriched brain regions such as corpus callosum showed intense background fluorescence that is likely due to broad-spectrum myelin auto-fluorescence routinely observed in formaldehyde- or glutaraldehyde-fixed tissues (Baschong et al., 2001; Christensen et al., 2014; Oliveira et al., 2010), which was effectively removed by the rolling ball background subtraction method. As expected, there was no specific signal (i.e., punctate fluorescent labeling) observed in the brain of a non-transgenic (WT) littermate (Fig. 3 G,H). As shown in Fig. 4, there was no overlap between the ZsGreen1 signal in brain and GFAP staining, indicating that the transgene is not expressed in astroglia.

3.4 Subcellular localization of ZsGreen1 fluorescence in brain

At the subcellular level, the ZsGreen1 signal in neurons appeared punctate and primarily restricted to the soma (Fig. 5). However, fluorescence was observed in some neuronal processes, typically limited to the proximal portion (Fig. 5A and B). Whether the punctate signal is due to specific trafficking of ZsGreen1 fluorescent protein to a subcellular compartment or simply the result of its hydrophobic nature is unknown, although punctate cytoplasmic has been observed in other (unrelated) ZsGreen1 transgenic mouse models (Wouters et al., 2005). Because most of the fluorescent signal is localized to the cell body, it makes it easier to identify the centers within the CNS that express the transgene.

3.5 ZsGreen1 fluorescence in brain regions of Atp1a3-ZsGreen1 mice

3.5.1 Cerebral nuclei and brainstem—There was a statistically significant gradient in the distribution of ZsGreen1 fluorescence in the rostro- caudal direction of subcortical regions (Fig. 6B; two-way RM ANOVA, $F_{(8,32)} = 26.6$, $p < 0.01$) and by brain structure/territory (Fig. 6C; two-way RM ANOVA effect of structure, $F_{(11,44)} = 26.6$, $p < 0.01$). In both male and female transgenic mice, the frontal brain and cortical regions displayed the lowest amount of fluorescence, while midbrain and hypothalamus were the most intensively labeled brain structures (see Tables 2 and 3 for statistical comparison). No sex-related differences were detected. Therefore the sexes were combined for analysis of ZsGreen1 fluorescence intensity in the cerebellar nuclei and brain stem sub-regions (Fig. 7).

The midbrain parabigeminal (PBG) nucleus demonstrated the highest level of fluorescent intensity (20 gray scale units) of all the brain sub-structures examined in Atp1a3-ZsGreen1 mice, as shown in Fig. 7. This nucleus, along with 11 other nuclei (PF, AV and AD in thalamus; DN in cerebellum; III, RN, ND, DR in midbrain; MARN, XII and RM/RO in medulla), constituted the top third (>13.3 gray scale units) for signal intensity.

3.5.2 Cerebral Cortex—As indicated earlier, the cerebral cortex regions generally exhibited very low ZsGreen1 -specific fluorescence at low magnification, with the notable exception of the dorsal and ventral borders of the accessory and main olfactory bulbs (rostro-ventral border in some sections). These regions exhibited a very bright fluorescent signal adjacent to, and sometimes overlapping, the glomerular layers of the respective structures (Fig. 8A). However, at higher magnification, the signal appeared to emanate from optic nerve fibers rather than glomerular layer cells (Fig. 8a1 and a2). While other cortical regions showed only scattered fluorescent labeling, if any, at low magnification (Figs. 1, 8A,B, and 9A) some ZsGreen1-expressing cells were detected in cortical structures when inspected at 10× or higher magnification (Fig. 8 and 9). Taken together, these results demonstrate that density (and perhaps size) of ZsGreen1-positive cells is higher in subcortical regions of brain than in the cortex.

Like most cortical structures, the hippocampus showed generally weak fluorescence at low-magnification except perhaps in the polymorphic cell layer of the dentate gyrus (Fig. 1,4, 8B, 9A). Examination at higher magnification confirmed the presence of ZsGreen1-positive neurons in the polymorphic cell layer of the dentate gyrus (Fig. 9B). In addition, many ZsGreen1 -positive neurons could be seen in the subiculum and postsubiculum, with a sprinkling of fluorescent cells in the stratum oriens, pyramidale and radiatum of the hippocampus (Fig. 9B). Notably, the soma of many of the ZsGreen1- labeled cells in the pyramidal layer was significantly greater than the majority of non-labeled pyramidal neurons, suggesting that these large cells are either interneurons or a subtype of pyramidal cells (see arrows in Fig. 9C).

3.5.3 Cerebellum—In the adult cerebellar cortex, ZsGreen1 fluorescent labeling was essentially limited to the Purkinje (PJ) cell layer. The molecular and granular layers showed little, if any, labeling above background (Fig. 3), even when examined at higher magnification (Fig. 5D). The fluorescent signal in the PJ cell layer was compared between

the different cerebellar lobules (Fig. 10). The Image J segmented line tool (25 μm -thick) was used to trace the PJ cell layer in each lobule as illustrated in Fig. 10B (semitransparent line shown on the right side). The mean pixel labeling intensity within the area, as well the plot profile (mean pixel intensity along a line), was measured following rolling ball background subtraction. The number of PJ neurons with a strong ZsGreen1 signal was determined and expressed in density units (number of neurons per mm of PJ layer length) by counting the peaks equal to or greater than 10 pixel intensity units (horizontal dashed line in Fig. 10E). Importantly, most but not all PJ neurons expressed ZsGreen1. This is illustrated by the confocal image provided in Fig. 5D and in the overlays of images captured with GFP and the rhodamine filter block in Tg mice brain sections that were stained for calbindin - a marker of PJ neurons; arrows point three of such neurons in Fig. 10C and D. To simplify the counting of ZsGreen1-negative PJ neurons, the original images were preprocessed using the “Sharpen and Find Edges” Image J protocols before the overlay (Fig. 10D). The number of ZsGreen1 negative neurons was expressed as a percentage of the total calbindin-labeled neurons in the PJ cell layer of a given cerebellar lobule. The mean fluorescent intensity (Fig. 10F) and density of strongly- labeled neurons (Fig. 10G) in the PJ cell layer were lower in lobule XII than other cerebellar lobules, but these differences were not statistically significant (One-way RM ANOVA: $F_{(7,23)} = 1.229$; $p=0.322$ for intensity and $F_{(7,23)} = 2.431$; $p=0.124$ for density of strongly labeled cells). However, the percentage of ZsGreen1-negative cells was significantly higher in lobule VII ($47.3\% \pm 3.5$; RM ANOVA $F_{(7,23)} = 6.277$, $p<0.001$, post-hoc Tukey test; $p<0.05$; Fig. 10H) compared with the other lobules, which varied between $22.4\% \pm 2.8$ and $31.1\% \pm 1.8$ (lobules II and VIII, respectively) as shown in Fig. 10H.

3.4 Electrophysiological studies

The results presented thus far were obtained using tissue fixed in formaldehyde. To confirm that ZsGreen1 labeling permits the identification of neurons of interest in functionally competent cells, we conducted a limited set of patch-clamp experiments. Two adult *Atp1a3*-ZsGreen1 mice were used for these studies. The hippocampus was dissected from brain, sectioned sagittally into 300 μm slices, and the activity of CA3 striatum radiatum interneurons was recorded in the current-clamp mode. Fig. 11 shows representative recordings of activity in neurons either possessing or lacking ZsGreen1 fluorescence. This result, supported by two more similar recordings from ZsGreen1-positive cells, indicates the viability of ZsGreen1-positive neurons, which did not appear to be compromised by the expression of the florescent protein. Whether these neurons exhibit unique physiological and pharmacological properties as compared to ZsGreen1-negative neurons of the same type will be examined in future electrophysiological studies, which are beyond the scope of this initial characterization of the transgenic model.

4.0 Discussion

Traditional approaches for mapping $\alpha_3\text{NKA}$ expression in the CNS are labor intensive, and typically incompatible with functional analysis of the transporter in its native environment. These approaches include *in situ* hybridization, immunohistochemistry, and differential sensitivity of the α isoforms of NKA to inhibitors such as ouabain. To address this problem we have generated a transgenic mouse model that utilizes the α_3 subunit (*Atp1a3*) promoter

to drive expression of ZsGreen1 fluorescent protein (ZsGreen1) to identify α_3 NKA neurons. This report provides a detailed characterization of the distribution of the ZsGreen1 signal in brain, as well as the utility of the transgenic model for electrophysiological study. The Atp1a3-ZsGreen1 mice provide an important tool to elucidate the functional significance of this isoform in neurons. In addition, these mice could provide insight into a variety of genetic diseases associated with mutations in α_3 NKA. While comparison of the distribution of α_3 NKA neurons in Atp1a3-ZsGreen1 mice with data from the current literature based on ISH or immunohistochemistry are challenging due to technical differences, there are some commonalities as discussed below for the various brain regions.

4.1 Cerebral and brainstem nuclei

Two earlier studies used ISH to determine the distribution of α_3 NKA mRNA in subcortical brain regions in rat. In the first report (Watts et al., 1991), labeling of cerebral nuclei and brain stem regions with an α_3 NKA probe was described as “heavy and discrete”, but the nuclei/regions were not specified. In the other study (Hieber et al., 1991), the α_3 NKA probe was reported to label cerebellar nuclei and the thalamus with similar intensity. However, this study noted that in the brainstem “some structures, such as the substantia nigra pars compacta, the red nucleus, and the motor nucleus of III, were particularly intensely labeled.” Bottger et al. performed a comprehensive immunohistochemical analysis of α_3 NKA distribution in mouse brain, which included many subcortical structures (Bottger et al., 2011). Our findings are in general agreement with these studies in that a ZsGreen1 signal (see Figs. 6 and 7) was detected in many of the subcortical brain regions/nuclei where hybridization- or immuno-labeling for α_3 NKA was described as strong and cell body specific (as opposed to punctate, neuropile or fiber labeling).

4.2 Olfactory bulb and cerebral cortex

Immunolabeling for α_3 NKA in the main and accessory olfactory bulb in mouse was described as punctate neuropile and cell body staining (glomerular layer) or punctate staining (external plexiform layers of accessory and main olfactory bulbs and anterior olfactory nucleus) (Bottger et al., 2011). With our study, a ZsGreen1 signal in these areas could only be detected under high magnification. In a sharp contrast to the study by Bottger et al. (2011), a very strong ZsGreen1 signal was observed in the white matter regions of the main and accessory olfactory bulb (Fig. 8). This was the only occasion where it was difficult to interpret whether the ZsGreen1 signal was emanating from the neuronal cell body or processes.

Previous *in situ* hybridization studies with rat brain cortex reported heaviest labeling for α_3 NKA in cortical layers II and V (Watts et al., 1991) or layer V only (Hieber et al., 1991), while in another study (McGrail et al., 1991), which utilized immunohistochemistry, demonstrated the most prominent immunostaining for α_3 NKA in cortical layers II-V from rat, where mostly large pyramidal neurons appeared to be labeled. With an immunohistochemistry study (Bottger et al., 2011) in mouse brain, neocortex staining was described as “dense punctate staining that sometimes appeared as possible neuronal plasma membrane staining” in the cortical layers III and V. The authors reported that the staining increased, caudally. Our data can neither confirm nor dispute these observations. Compared

with many of the other brain regions, the ZsGreen1 signal in cortex was primarily too weak to be detected at low magnification, although the signal is present in some of neurons of the cerebral cortex (Fig. 8, 9). However, the sub-regional distribution of these cells awaits future study.

4.3 Hippocampal formation

One of the major controversies in the relevant literature relates to the expression of α_3 NKA within the hippocampal formation. *In situ* hybridization studies (Chauhan and Siegel, 1996; Hieber et al., 1991; Watts et al., 1991) have detected high levels of α_3 NKA transcripts in pyramidal and dentate gyrus granular cell layers (see also Allen Brain Atlas). In addition, medium and large clusters of “grain label” were observed in the stratum oriens and at the border of the stratum radiatum and stratum lacunosum-moleculare of the CA1-CA3 regions in hippocampus (Chauhan and Siegel, 1996). The medium clusters were identified as interneurons, while the large clusters corresponded to basket cells. Immunostaining studies also demonstrated intense staining for α_3 NKA outlining the bodies of rat hippocampus pyramidal cells (Cameron et al., 1994; McGrail et al., 1991; Pietrini et al., 1992) and dentate gyrus granule cells (Cameron et al., 1994). However with a more recent immunohistochemistry study (Bottger et al., 2011), only scattered α_3 NKA-positive cell bodies were observed in all but the molecular layers of hippocampus. Immuno-positive cell bodies were also noted in the subiculum and pre- and para-subiculum. Based on sensitivity of the resting membrane potential and AP discharge characteristics to low concentrations (30 μ M) of ouabain or strophanthidin, only the interneurons located at the border of the molecular and granular layers of dentate gyrus, but not granule cells, had functional α_3 NKA activity (Ross and Soltesz, 2000). A similar conclusion was reached with respect to interneurons and pyramidal cells of the subiculum based on differences in the electrophysiological response of these cells to low concentrations of ouabain (patch-clamp pump current recordings), combined with *in situ* hybridization and immunohistochemistry results for α_3 NKA (Richards et al., 2007). Our data regarding the distribution of ZsGreen1 in hippocampus are in concordance with these latter studies, which indicate that α_3 NKA is expressed in some interneurons of the hippocampus and dentate gyrus, but rarely (if at all) in pyramidal and granular cells (Fig. 9).

4.4 Cerebellum

Previous studies report divergent findings with respect to expression of α_3 NKA in cerebellum. In one *in situ* hybridization study (Watts et al., 1991), an α_3 NKA probe resulted in moderate labeling of the granule cell layer and strong labeling of PJ cells, but labeling of other cell types was not mentioned. In a similar study (Hieber et al., 1991) which also investigated the cerebellum from rat, the antisense probe for α_3 NKA transcripts strongly labeled Purkinje cells as well, but it also labeled basket and stellate cells, and modest labeling was seen in the granule cell layer. Yet another *in situ* hybridization study mapping α_3 -NKA transcripts in the rat cerebellum (Chauhan and Siegel, 1997), showed large clusters of label on stellate, basket, Golgi, and Purkinje cells, with Purkinje cells being the most prominently labeled.

In immunostaining studies (Genet and Kado, 1997; McGrail et al., 1991), an α_3 NKA-specific antibody labeled cerebellar Purkinje cell bodies in rat. Prominent staining was also observed in basket cell processes, with occasional ring-shaped staining of the basket cell soma. No labeling of granule cells was observed in these studies. However, another immunostaining study (Cameron et al., 1994) reported labeling of the plasma membrane in all cells types of the cerebellar cortex from rat. Conversely, immunohistochemistry performed in mouse cerebellum, demonstrated strong α_3 NKA-specific immunoreactivity in the PJ cell layer, with only punctate labeling of the molecular and granular layers (Bottger et al., 2011).

Our study with *Atp1a3-ZsGreen1* mice suggest that in the cerebellar cortex, α_3 NKA is expressed exclusively in PJ cells. Notably, our study reveals a previously unknown feature. We observed two distinct types of PJ cells; those that express the *ZsGreen1* reporter and other lacking the fluorescent signal. While *ZsGreen1*-negative cells accounted for roughly 30% of all PJ neurons, their relative abundance varied depending upon the cerebellar lobule; lowest (22%) in lobule II and highest in the cerebellar vermis, lobule VII (47%; Fig. 10). It is known that cerebellar PJ neurons express lobule-specific differences in excitability and firing patterns (Kim et al., 2012), and these differences may, in part, be due to NKA activity (Forrest et al., 2012; Genet and Kado, 1997). Having said so however, such alternatives as chromosome silencing or role of epigenetic effects in aforementioned apparent lobule-specific differences of *ZsGreen1* labeling of PJ neurons may not be excluded at this point.

4.5 Relevance to human diseases

There are a wide variety of symptoms in human diseases associated with mutations in the *ATP1A3* gene (Rosewich et al., 2012). AHC and RDP diseases are caused by mutations that result in a loss of α_3 NKA expression or its activity and the most common symptoms in these diseases are dystonia with an obvious rostrocaudal gradient, dysarthria and drooling that usually have early (childhood) and abrupt onset associated with stress, exercise, and hypo- or hyper-thermia. Other common symptoms for RDP (which also occur in many cases of AHC) are hypomimia, mutism, atactic gait and bradykinesia. Nystagmus is also characteristic of AHC and prevalent in RDP patients. Interestingly, many of the subcortical centers identified in the current study having a substantial *ZsGreen1* signal (indicative of α_3 NKA expression), may be directly linked to some of the symptoms above (for example abnormal function of PBG, ND or PRP to nystagmus; oculomotor nerve nucleus to hypomimia, hypoglossal nerve nucleus to dysarthria, drooling and mutism; red and dentate nuclei to dystonia and gait problems). Other associations are less straightforward, but dysfunction of the raphe nuclei (including the hypothalamus as the most *ZsGreen1* labeled subcortical region) may potentially add to the stress-associated onset of AHC and RDP. Normal function of the anterodorsal and anteroventral thalamic nuclei, which are negatively affected in some cases of AHC, are reported to be important for learning and memory (Van and Wyss, 1995). The thalamic parafascicular nucleus, which emanated a strong *ZsGreen1* signal in *Atp1a3-ZsGreen1* mice, has been implicated in temporal lobe epilepsy (Langlois et al., 2010), and may likewise be relevant in some cases of AHC and RDP complicated by seizures. Crossing *Atp1a3-ZsGreen1* transgene into mice carrying the *Myshkin* (181 ON), *Mashloul* (D801N) or D801Y mutations in *Atp1a3* (mouse models of α_3 NKA insufficiency-

related disorders; see (Isaksen et al., 2017)) and examining the functional activity in ZsGreen1-positive neurons may provide insight to the pathophysiology underlying AHC and RDP.

Finally, our study provided no evidence for a statistically significant difference in the ZsGreen1 signal between males and females. So far, no gender bias in the prevalence or severity of symptoms of AHC and RDP has been reported. However, in mice lacking one functional copy of *Atp1a3* gene, stress produced signs of sensory-motor impairment only in female animals (Deandrade et al., 2011).

4.6 Conclusions

Here we describe the generation and initial characterization of a unique transgenic mouse model that affords easy identification of α_3 NKA-expressing neurons in brain through expression of ZsGreen1 fluorescent protein. The mouse α_3 subunit gene (*Atp1a3*) promoter (including nearly 1.6 kb of proximal 5'-flanking DNA and all of the 5'-untranslated sequence) was used to drive expression of ZsGreen1 as this sequence is highly conserved between species, and contains all of the known transcription factor binding sites (as well as most of the methylation sites) responsible for cell type-specific control (Henriksen et al., 2013; Li and Langhans, 2015). The pattern of ZsGreen1 expression in the CNS was largely consistent with that for α_3 NKA as reported in other studies (see discussion above), although there were a few difference including the presence of a strong ZsGreen1 signal in the olfactory bulb territory lacking neurons, but enriched in olfactory afferent fibers and glial cells.

Expression of ZsGreen1 did not cause any gross untoward phenotype in the *Atp1a3*-ZsGreen1 mice as expected, and did not comprise the functional activity in neurons as assessed by our initial electrophysiological analysis in brain slices (Fig. 11). Additional studies will be essential for understanding the functional significance of α_3 NKA expression in specific sub-populations of neurons, as well as to investigate the pathophysiology underlying neurological disorders with mutations that cause a deficiency (RDP, AHC) or gain of function (CAPOS) of the α_3 NKA transport mechanism.

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Maxim Dobretsov devised the idea of experiments, analyzed the data and drafted the manuscript. Abdallah Hayar and Maxim Kozhemyakin conducted the electrophysiological experiments, analyzed the data and helped in editing the manuscript. Kim E. Light and Dwight R. Pierce performed the immunostaining and confocal image analysis, and helped to edit the manuscript. Neriman T. Kockara and Pankaj Patyal were responsible for maintaining the mouse colony, genotyping the animals and drafting of portions of the manuscript. Neriman T. Kockara also performed the RT-PCR analysis. Patricia A. Wight designed the *Atp1a3*-ZsGreen1 transgene and played a leading role in all aspects of molecular biology associated with the project. She contributed significantly to writing the manuscript.

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Abbreviations

α_3NKA	α_3 isoform of the sodium-potassium ATPase
AHC	alternative hemiplegia of childhood
AMB	Allen mouse brain atlas
CAPOS	cerebral ataxia, areflexia, pes cavus, optic atrophy and sensorineural hearing loss syndrome
DCN	deep cerebellar nuclei
ISH	<i>in situ</i> hybridization
PJ	cerebellar Purkinje cells or layers
RDP	rapid onset Dystonia-Parkinsonism
Tg	transgenic
WT	wild type

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- Transgenic mice have been generated that use the mouse *Atp1a3* promoter to drive expression of ZsGreen1 fluorescent protein.
- ZsGreen1 fluorescent neurons are readily identifiable in both fixed and unfixed tissue from brain.
- The model provides a novel tool to elucidate the distribution and functional properties of α_3 NKA- expressing neurons.

Figure 1. Quantification of ZsGreen1 fluorescence in brain regions from an Atp1a3-ZsGreen1 transgenic mouse at 3 months of age.
(A) Representative florescent image of a frozen brain section (60 μ m) obtained from a Atp1a3-ZsGreen1 male mouse (approximate Allen's Mouse Brain Reference Atlas (AMB) level 75). Brain regions of interests (ROI) have been outlined using the Fiji Image J ROI Manager tool and are based on the AMB. (B) Fluorescent signal remaining after subtraction of the background intensity as calculated by the rolling ball method (see Experimental Procedures for more details). (C) Intensity of labeling of ROIs shown in panels A and B (sorted by brain region and then by intensity). Abbreviations (order as in Panel C): White

matter tracts: fi - fimbria, st - stria terminalis, cpd - cerebellar peduncle, fr - fasciculus retroflexus; Isocortex: AUD- auditory cortex, TEa-ECT-PERl - temporal association area and perirhinal and entorhinal cortical areas, SSp - primary somato-sensory cortex; Olfactory areas: PIR - piriform area, PAA - piriform amygdalar area, COA - cortical amygdalar area, posterior part; Retrohippocampal region and hippocampus: ENTI - entorhinal cortical area, lateral part, CA - Ammon's horn; DG - dentate gyrus; Cortical subplate - CTXsp; Cerebral nuclei: CP - caudate putamen, MEApv - medial amygdalar nucleus, posteroventral part, MEApd - medial amygdalar nucleus, posterodorsal part; Thalamus and Epithalamus: PO - posterior complex of the thalamus, LGd - lateral geniculate complex, dorsal part, VP - ventral posterior complex of the thalamus, LP - lateral posterior nucleus, RT - reticular nucleus; LGv - lateral geniculate complex, ventral part, MT - medial thalamic nuclei, LH - lateral habenula; VM- ventral medial nucleus, PF - parafascicular nucleus; Hypothalamus: STN - subthalamic nucleus; HY- hypothalamus major proper; Zl- zona inserta

Figure 2. Relative levels of Atp1a3-ZsGreen1 and Atp1a3 mRNA expression in transgenic (Tg) and wild type (WT) littermates

(A) Gel picture of RT-PCR results from Tg and WT mice at the indicated postnatal days (P) of age. Each lane represents a unique animal except for the no template (H₂O) control. The 18S rRNA product was used as a reference for normalization. Both the Atp1a3-ZsGreen1 transgene and endogenous Atp1a3 gene were expressed well in brain, but not in liver. (B) Plot of the mean level of expression for Atp1 a3- ZsGreen1 and Atp1a3 relative to 18S rRNA in brain during development as determined by measuring the band intensities in Panel A. Numbers next to Atp1a3 data points indicate sample size (i.e. number of animals analyzed). Both the transgene and endogenous Atp1a3 gene demonstrate a similar pattern of temporal expression in brain. (C) Plot of the band intensities between Atp1a3-ZsGreen1 and Atp1a3 signals for each animal in Panel A. Shown is a linear regression (solid line) of the

data obtained from transgenic mice (filled circles) flanked by the 95% confidence limits (dotted lines). Open circles indicate data obtained with non-transgenic (WT) mice.

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Figure 3. ZsGreen1 fluorescence in tissues from Atp1a3-ZsGreen1 transgenic mice
 (A, B) Image captured at 5 $\times$ magnification of frozen sections (30 μ m) from liver of an Atp1a3-ZsGreen1 Tg mouse. Panel A represents the original image and Panel B is the same image after performing rolling ball background subtraction. (C, D) Image of the cerebellum from the same Tg mouse as in Panels A and B. Panel C represents the original image, while the image in Panel D is after rolling ball background subtraction. (E-H) Images of cerebellum captured at 2.5 $\times$ magnification of a brain slice (60 μ m) obtained from an Atp1a3-ZsGreen1 Tg mouse (E, F) and a non-transgenic littermate (G, H) cut at approximately the

same sagittal level. Panels E and G are original images and Panels F and H represent the corresponding images after rolling ball background subtraction. Labels: DCN - deep cerebellar nucleus, PJ - Purkinje cell layer, wt - white matter tracts. The scale bar in Panel A represents 2 mm and is applicable for Panels A-D. The scale bar in Panel E represents 1 mm and is applicable for Panels E-H.

Figure 4. Atp1a3-ZsGreen1 mouse brain section (60 μ m) stained with GFAP

The image was taken at 20 $\times$ magnification and does not show any co-localization of ZsGreen1 fluorescence (green) with immunostaining for GFAP (red) in the dentate gyrus (A), deep cerebellar nucleus (B), or parabigeminal nucleus (C). The scale bar (100 μ m) is indicated in Panel C, which is the same for all images. DAPI staining (blue) in Panel A specifies cell nuclei.

Figure 5. High magnification of ZsGreen1 fluorescence in Atp1a3-ZsGreen1 mouse brain
 (A) Central amygdalar nucleus; arrow indicates a neuronal process. (B) Zona Inserta. (C) Cerebellar Purkinje cell layer; arrows indicates cell nucleus. Images depicted in Panels A-C were taken with a 40× objective and share the same scale as shown in Panel C. (D) Three-dimensional reconstruction of PJ cell layer based on the Z-stack of confocal images taken from a cerebellar section (60 μm ; Scale bar = 15 μm). The section was stained with a calbindin D28k antibody to identify PJ cell bodies and axonal trees (red). Yellow is overlay of ZsGreen1 signal and red staining for calbindin. Arrows point to cells having no detectable ZsGreen1 signal.

Figure 6. Fluorescent intensity in brain regions of *Atp1a3-ZsGreen1* mice

(A) Schematic illustration of coronal frozen sections (60 μ m) isolated from the brains of *Atp1a3-ZsGreen1* mice at 3 to 3.5 months of age ($n = 3$ per sex). Arrows indicate the relative location of the brain slices. At least three sections per mouse were analyzed for fluorescence in subcortical structures and major brain regions. (B) *ZsGreen1* fluorescence in subcortical areas (gray shaded areas in the illustration on the left side). Mean pixel intensity indicates the average amount after rolling ball background subtraction of the combined area(s) as noted. Brain regions according to the AMB atlas: cerebral nuclei (CN),

hypothalamus (HY), medulla (MY), midbrain (MB), pons (P), thalamus (TH). (C) ZsGreen1 fluorescence in major brain sub-regions (gray shaded areas depicted in the illustration to the left) according to the procedure used in Panel B, except mean pixel intensity is plotted against a particular brain sub-region. The level of the AMB atlas selected was based on being most representative for each brain region (e.g. AMB atlas coronal layers 85–93 for MB and 61–69 for TH). Additional abbreviations: cerebellum (CB), cortical subplate (CTXsp), hippocampal formation (HIF), isocortex (ISO), midbrain (MB), olfactory-related cortical area (OLF), pallidum (PAL), striatum (STR).

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Figure 7. Intensity of ZsGreen1 fluorescence within brain sub-regions of Atp1a3-ZsGreen1 mice (A) pallidum/striatum, (B) thalamus and epithalamus, (C) hypothalamus and cerebellum, (D) midbrain, (E) pons, (F) medulla. Each column represents the mean level of fluorescence per specified sub-region studied in sections from 4 to 6 animals. Sub-regions were based on the presence of at least one section per brain in which the borders of the nuclei could be reliably determined. Nuclei whose borders could not be established due to its relative small size were not studied or were studied as a part of a larger CNS subdivision. For example, most hypothalamic nuclei except for zona inserta (ZI), supramammillary (SUM), medial mammillary (MM) and subthalamic (STN) nuclei were studied by grouping them into a single hypothalamic (HY) region of interest. Results are presented as mean pixel intensity ($\pm$ SD) from coronal sections (60 μ m) according to the procedure described in Fig. 1. Horizontal dashed lines represent mean + 3SD pixel intensity value (4.027 shades of gray) measured in 18 randomly selected white matter regions (such as corpus callosum, fimbria or cerebellar peduncle, see Fig. 1; 3 ROI per brain) having only background levels of fluorescence. Thus, brain nuclei with a mean intensity exceeding this value are considered to

have specific fluorescence due to expression of ZsGreen1 protein with more than 99% certainty. See Table 1 for a complete list of abbreviations for brain sub-regions.

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Figure 8. Global fluorescence in cortical areas of *Atp1a3-ZsGreen1* mouse brain

(A) Image of a sagittal section (60 μ m) of the olfactory bulb and adjacent brain regions (sagittal AMB atlas, layer 15) captured at low magnification (2.5 $\times$; scale bar = 0.5 mm). Boxes indicate areas captured at higher magnification (1 $\times$) with the images on the right side (scale bar in Panel a1 represents 100 μ m). Abbreviations: glomerular (gl), mitral (mi), and granular (gr) cell layers of the main (MOB) and accessory (AOB) olfactory bulb; anterior olfactory nucleus (AON); fiber tracts (ft). (B) Image of a coronal section (200 μ m) of cortical structures and adjacent brain regions (coronal AMB atlas, layer 60) captured at low

magnification (2.5x; scale bar scale bar = 0.5 mm). Boxes indicate areas captured at higher magnification (10x) with the images on the right side (scale bar in Panel b1 represents 100 μ m). Abbreviations: centrolateral thalamic nucleus (CL), caudate putamen (CP), globus pallidus, external portion (GPe), hippocampal formation (HIP), laterodorsal thalamic nucleus (LD), lateral habenula (LH), medial habenula (MH), motor cortex (MO), paracentral thalamic nucleus (PCN), retrosplenial cortex (RSP), reticular nucleus of thalamus (RT), somatosensory cortex (SS), ventral group of the dorsal thalamus (VENT).

Figure 9. ZsGreen1-labeled cells in hippocampus

(A) Low power image of a frozen sagittal section (60 μ m) at AMB atlas sagittal level 10. The rectangle specifies the hippocampal region, which is shown at higher (5 $\times$ magnification in Panel B. Designated structures: interposed nucleus (IP), parabigeminal nucleus (PBG), substantia nigra (SNR), zona incerta (ZI), globus pallidus external part (GPe) and substantia innominata (SI). (B) Enlarged boxed area from Panel A containing the hippocampal region. Designated structures: molecular (mo) and polymorphic (po) layers of the dentate gyrus; hippocampal lacunosum-moleculare (slm), radiatum (sr) and oriens (so) layers; subiculum

(SUB) and postsubiculum (POST). Arrows point to three ZsGreen1-positive cells in the pyramidal layer region which are shown at higher (10x) magnification in Panel C. (C) The majority of the neurons within the hippocampal pyramidal layer are ZsGreen1 -negative, with a few exceptions (arrows indicate ZsGreen1 fluorescent cells).

Figure 10. ZsGreen1 labeling in cerebellum

(A) Low power image of a frozen section (60 μ m; scale bar 0.5 mm) of cerebellum (AMB atlas sagittal layer 20–21) stained with antibodies against calbindin to identify Purkinje (PJ) neurons. Roman numerals indicate the different cerebellar lobules. (B) Image of lobule II at higher (20 $\times$) magnification (scale bar for images B–D is 100 μ m, as depicted in Panel D). The granular (gr) and molecular (mo) layers of cerebellum are indicated. A semi-transparent selection line has been drawn over a portion of the PJ cell layer towards the right side of Panel B to illustrate how the area was traced in order to determine the average pixel intensity

and pixel intensity profile within a given lobule. (C) Overlay of images for lobule II. Green indicates ZsGreen1-positive cells, while red indicates cells that express calbindin. Cells expressing both ZsGreen1 and calbindin are yellow. (D) Image from Panel C after Sharpen and Find Edges pre-processing and rolling ball background subtraction protocols in Image J. Arrows in Panels C and D indicate ZsGreen1 -negative PJ neurons. (E) Linear ZsGreen1 labeling intensity profile in the PJ cell layer for all of lobule II. Horizontal dashed line indicates arbitrary cut-off value (10 pixel intensity units) used to denote strongly labeled PJ neurons. (F) Mean pixel intensity for the PJ cell layer within different cerebellar lobules (n = 3 animals). (G) Counts (number per PJ cell layer length in mm) of strongly labeled PJ neurons in the different cerebellar lobules. (H) Percentage of ZsGreen1-negative neurons within the PJ cell layer of a given lobule. Asterisk indicates a significant difference ($p < 0.05$) between the amount of ZsGreen1 -negative cells in lobule VII compared with other lobules.

Figure 11. Identification and patch clamp recording (current clamp mode) from ZsGreen1 - positive and negative interneurons in the striatum radiatum of the hippocampus
 (A) ZsGreen1-positive neuron and its response to a depolarizing current ramp. (B) ZsGreen1-negative interneuron from the same animal, and its response to a depolarizing current ramp. Scale bars for all images are 25 μm. Scale bars for current command and voltage response (vertical bars) traces are 30 pA and 20 mV, respectively. Time scale (horizontal bars) is 200 ms.

Table 1:List of annotations of mouse brain structures and centers mentioned in the text (see also mouse.brain-map.org)

ACB	Nucleus accumbens	ICe	Inferior colliculus, external nucleus
AD	Anterodorsal nucleus of thalamus	III	Oculomotor nucleus
AI	Agranular insular cortex	IO	Inferior olivary complex
AMd	Anteromedial nucleus, dorsal part	IP	Interposed nucleus
AOB	Accessory olfactory bulb	IRN	Intermediate reticular nucleus
AP	Area postrema	ISO	Isocortex
AUD	Auditory cortex	LAV	Lateral vestibular nucleus
AV	Anteroventral nucleus of thalamus	LD	Lateral dorsal nucleus of thalamus
CB	Cerebellum	LGd	Dorsal part of the lateral geniculate
CEA	Central amygdalar nucleus	LGv	Ventral part of the lateral geniculate
CL	Central lateral nucleus of the thalamus	LH	Lateral habenula
CM	Central medial nucleus of the thalamus	LHA	Lateral hypothalamic area
CN	Cerebral nuclei	LPLR	Lateral posterior nucleus of the thalam laterorostral
CP	Caudate putamen	LPMR	Lateral posterior nucleus of the thalam mediorostral
CS	Superior central nucleus raphe	LS	Lateral septal nucleus
CTXpl	Cortical subplate	MARN	Magnocellular reticular nucleus
DCO	Dorsal cochlear nucleus	MB	Midbrain
DG	Dentate gyrus	MDRNd	Medullary reticular nucleus, dorsal p
DN	Dentate nucleus	MDRNv	Medullary reticular nucleus, ventral p
DR	Dorsal nucleus raphe	MEA	Medial amygdalar nucleus
ENT	Entorhinal area	MEApd	Medial amygdalar nucleus, posterodo
FN	Fastigial nucleus	MY	Medulla
GENd	Geniculate group, dorsal thalamus	MG	Medial geniculate complex
GPe	Globus pallidus, external segment	MH	Medial habenula
GPi	Globus pallidus, internal segment	MM	Medial mammillary nucleus
GRN	Gigantocellular reticular nucleus	MO	Motor cortex
GU	Gustatory cortex	MOB	Main olfactory bulb
HIF	Hippocampal formation	MRN	Midbrain reticular nucleus
HIP	Hippocampus	MS	Medial septa l nucleus
HY	Hypothalamus	MV	Medial vestibular nucleus
ICc	Inferior colliculus, central nucleus	NB	Nu. of the brachium of the inferior colli
ICd	Inferior colliculus, dorsal nucleus	ND	Nucleus of Darkschewitsch
NDB	Diagonal band nucleus	RN	Red nucleus
NLL	Nucleus of the lateral lemniscus	RR	Midbrain reticular nucleus, retrorubral
NTS	Nucleus of the solitary tract	RSPd	Dorsal retrosplenial cortex
OLF	Olfactory related cortical area	RT	Reticular nucleus of thalamus
P	Pons	SCm	Superior colliculus, motor related
P5V	Principal sensory nucleus of the trigeminal	SCs	Superior colliculus, sensory related
PAG	Periaqueductal gray	SI	Substantia innominata
PAL	Pallidum	SNC	Substantia nigra, compact part

PARN	Parvicellular reticular nucleus	SNr	Substantia nigra, reticular part
PB	Parabrachial nucleus	SOC	Superior olivary complex
PBG	Parabigeminal nucleus	SPA	Subparafascicular area
PCG	Pontine central gray	SPFm	Subparafascicular nucleus, magnocell
PCN	Paracentral nucleus	SPVC	Spinal nucleus of the trigeminal, caud
PF	Parafascicular nucleus	SPVI	Spinal nucleus of the trigeminal, interp
PG	Pontine gray	SPVO	Spinal nucleus of the trigeminal, oral p
PGRNd	Paragigantocellular reticular nucleus, dorsal part	SS	Somatosensory cortex
PGRNI	Paragigantocellular reticular nucleus, lateral part	STN	Subthalamic nucleus
PH	Posterior hypothalamic nucleus	STR	Striatum
PMd	Dorsal premammillary nucleus	SUB	Subiculum
PMv	Ventral premammillary nucleus	SUM	Supramammillary nucleus
PO	Posterior complex of the thalamus	SUV	Superior vestibular nucleus
POST	Postsubiculum	TH	Thalamus
PPN	Pedunculopontine nucleus	TMd	Tuberomammillary nucleus, dorsal
PRE	Presubiculum	TRN	Tegmental reticular nucleus
PRNc	Pontine reticular nucleus, caudal part	VCO	Ventral cochlear nucleus
PRNr	Pontine reticular nucleus, rostral part	VII	Facial motor nucleus
PRP	Nucleus prepositus	VIS	Visual cortex
PRT	Pretecal region	VM	Ventral medial nucleus of the thalamu
PTLp	Posterior parietal association cortex	VP	Ventral posterior complex of the thala
PVp	Periventricular hypothalamic nu., posterior part	VTa	Ventral tegmental area
RE	Nucleus of reunions	XII	Hypoglossal nucleus
RM-RO	Nucleus raphe magnus-obscurus	ZI	Zona inserta

Table 2:

Effect of rostro-caudal subcortical region position on the intensity of ZsGreen1 labeling *

AMB atlas **	44–53	54–63	64–73	74–83	84–93	94–103	104–113	114–123	124–132
	0.5	–0.6	–1.6	–2.6	–3.6	–4.6	–5.6	–6.6	–7.6
44–53		no	yes	yes	yes	yes	yes	yes	yes
54–63			no	yes	yes	yes	yes	yes	yes
64–73				yes	yes	yes	no	yes	yes
74–83					yes	no	no	no	no
84–93						no	yes	yes	yes
94–103							no	no	no
104–113								no	no
114–123									no
124–132									

* yes indicates statistically significant difference ($p < 0.05$; One-way RM ANOVA, Tukey's multiple comparison posttest)

** numbers in the second header line indicate approximate distance of the center of the block from bregma in C57Bl/6J male mouse at 2 months of age, as that used in the Allen Mouse Brain (AMB) atlas.

Table 3:Effect of brain region on the intensity of ZsGreen1 labeling^{*}

Brain region	CTXsp	OLF	HIF	STR	CB	TH	PAL	MY	PN	MB	HY
ISO	no	no	no	no	no	yes	yes	yes	yes	yes	yes
CTXpl		no	no	no	no	no	yes	yes	yes	yes	yes
OLF			no	no	no	no	yes	yes	yes	yes	yes
HIF				no	no	yes	yes	yes	yes	yes	yes
STR					no	no	yes	yes	yes	yes	yes
CB						no	no	yes	yes	yes	yes
TH							no	no	no	yes	yes
PAL								no	no	yes	yes
MY									no	no	yes
PN										no	yes
MB											no

^{*} yes indicates statistically significant difference ($p < 0.05$; One-way RM ANOVA, Tukey's multiple compa posttest)

For abbreviations see Table 1 and legend to Fig. 5C.