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STAM2, a member of the endosome-associated complex ESCRT-0 is highly expressed in neurons

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ABSTRACT

STAM2 (signal transducing adaptor molecule 2), a subunit of the ESCRT-0 complex, is an endosomal protein acting as a regulator of receptor signaling and trafficking. To analyze STAM2 in the nervous system, its gene expression and protein localization in the mouse brain were identified using three methods: mRNA *in situ* hybridization, immunohistochemistry, and *via lacZ* reporter in frame with *Stam2* gene using the gene trap mouse line $Stam2^{Gt1Gaj}$. STAM2 intracellular localization was analyzed by subcellular fractionation and co-immunofluorescence using confocal microscopy. *Stam2* was strongly expressed in the cerebral and cerebellar cortex, hippocampal formation, olfactory bulb, and medial habenula. The majority of STAM2-positive cells co-stained with the neuronal markers. In neurons STAM2 was found in the early endosomes and also in the nucleus. The other members of the ESCRT-0 complex co-localized with STAM2 in the cytoplasm, but they were not present in the nucleus. The newly identified neuron-specific nuclear localization of STAM2, together with its high expression in the brain indicated that STAM2 might have a specific function in the mouse nervous system.

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1. Introduction

STAM2 (signal transducing adaptor molecule 2) is a 523 amino acid-long protein encoded by *Stam2* gene that belongs to the STAM protein family. STAM2 has a characteristic domain organization consisting of a VHS domain (Vps-27/Hrs/Stam) at its N-terminus, as well as a UIM (ubiquitin-interacting motif), SH3 (Src homology 3), CC ("coiled coil"), SSM (STAM-specific motif) and C-terminal ITAM (immunoreceptor tyrosine-based activation motif) domain (Endo et al., 2000; Kato et al., 2000; Lohi and Lehto, 2001; Mizuno et al., 2003, 2004).

STAM2 is suggested to play a regulatory role in the endosomal sorting of ubiquitinated membrane proteins. Together with STAM1 and HRS (hepatocyte growth factor-regulated tyrosine kinase substrate) it forms the endosome-associated complex ESCRT-0 (endosomal sorting process required for transport-0) implicated in the sorting of mono-ubiquitinated endosomal cargo. Cargo destined toward the lysosomes for degradation is subjected to the ESCRT machinery, which consists of four protein complexes. A ubiquitinated tag is initially recognized by the ESCRT-0 complex, followed by ESCRT-I, ESCRT-II and ESCRT-III (Hurley, 2010; Roxrud et al., 2010; Williams and Urbé, 2007). A ubiquitin tag can be removed by specific deubiquitinating enzymes: AMSH (associated molecule with the SH3 domain of STAM) and UBPY (ubiquitin-specific protease Y). Both of them bind the SH3 domain of STAM (Kato et al., 2000) and this interaction is involved in endosomal cargo sorting.

ESCRT dysfunction, aberrant endosomal trafficking, and non-proper ubiquitination and deubiquitination are related to many neurodegenerative diseases (Lee and Gao, 2012; Rusten and Simonsen, 2008; Saksena and Emr, 2009; Stuffers et al., 2009). There is increasing evidence that endosomal dysfunction of the most upstream component of the ESCRT system is involved in neuronal cell degeneration (Lee and Gao, 2012). STAM1 and HRS conditional knock-out mice were shown to display a loss of neurons in the CA3 region of the hippocampus (Tamai et al., 2008; Yamada et al., 2001). Knockdown of AMSH or UBPY also impaired deubiquitination in neurons; deletion of AMSH led to neuronal loss in the CA1 subfield of the hippocampus, while the loss of UBPY resulted in embryonic lethality (Ishii et al., 2001; Niendorf et al., 2007). STAM2 knockout mice show no abnormalities at birth, but double knockout of

Abbreviations: STAM, signal transducing adaptor molecule; VHS domain, Vps-27/Hrs/Stam domain; UIM, ubiquitin-interacting motif; SH3, Src homology 3; CC, coiled coil; SSM, STAM-specific motif; ITAM, immunoreceptor tyrosine-based activation motif; HRS, hepatocyte growth factor-regulated tyrosine kinase substrate; ESCRT, endosomal sorting process required for transport; AMSH, associated molecule with the SH3 domain of STAM; UBPY, ubiquitin-specific protease Y; EEA1, early endosome antigen 1.

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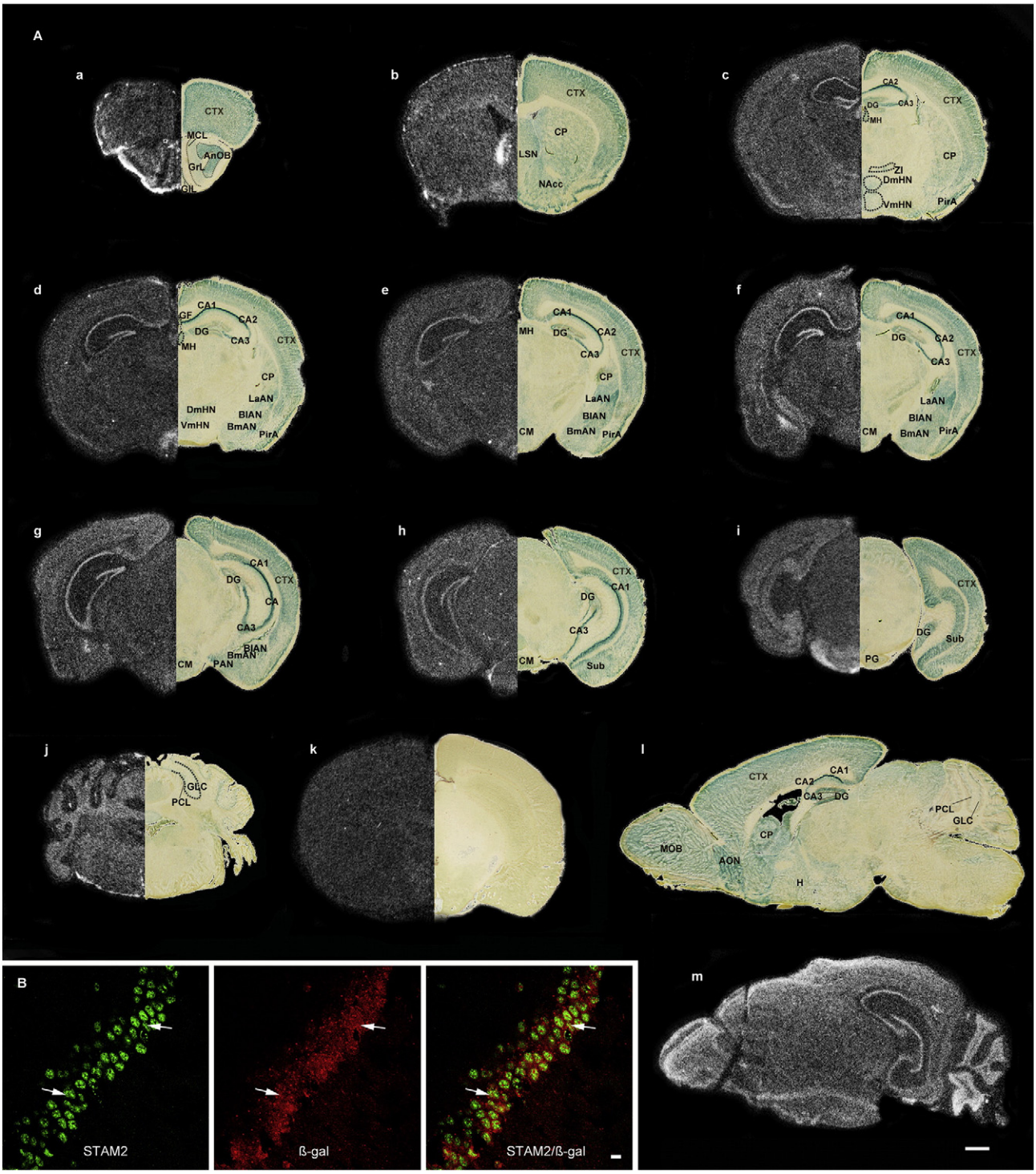


Fig. 1. *Stam2* expression in the adult mouse brain detected by mRNA *in situ* hybridization and β-galactosidase activity. (A a–k) Serial coronal brain cryosections analyzed by mRNA *in situ* hybridization and *lacZ* staining detected by X-gal. The hemisphere on the left represents *in situ* hybridization autoradiographs of 20 μm-thin coronal sections of wild type ($Stam2^{+/+}$) mouse brain, whereas the hemisphere on the right represents β-galactosidase activity staining performed on 50 μm-thin coronal cryosections of $Stam2^{Gt1Gaj/Gt1Gaj}$ mouse brain. (k) Specificity of X-gal staining was confirmed by the absence of the signal in the wild type ($Stam2^{+/+}$) brain sections incubated with X-gal, while specificity of mRNA *in situ* hybridization detection was confirmed by the absence of the hybridization signal in the tissue sections incubated with the *Stam2 sense* probe. (l, m) Representative sagittal section provides an overall view of the distribution of *Stam2* mRNA in wild type (m) and $Stam2^{Gt1Gaj}$ (l) mouse brains detected by *in situ* hybridization (m) and X-gal staining (l). Bar = 1 mm. (B) Confocal photomicrographs showing the co-localization of STAM2 and β-galactosidase in the pyramidal cells of the CA3 region of the hippocampus. Overlap in the distribution of STAM2 and β-galactosidase proteins (arrows) confirmed that *lacZ* insertion in the *Stam2* gene reflected *Stam2* expression. Bar = 10 μm. AnOB — anterior olfactory bulb, BlAN — basolateral nucleus of amygdala, BmAN — basomedial nucleus of amygdala, CA1 — CA1 pyramidal layer of hippocampus, CA2 — CA2 pyramidal layer of hippocampus, CA3 — CA3 pyramidal layer of hippocampus, CM — corpus mamillare, CP — caudoputamen, CTX — neocortex, DG — dentate gyrus, Sub — subiculum, DmHN — dorsomedial hypothalamic nucleus, GF — gyrus fasciolaris, GLC — granular layer of cerebellum, GlL — glomerular layer of the main olfactory bulb, GrL — granular layer of the main olfactory bulb, LaAN — lateral nucleus of amygdala, LSN — lateral septal nucleus, MCL — mitral cells layer of the main olfactory bulb, MH — medial habenula, NAcc — nucleus accumbens, PAN — posterior nucleus of amygdala, PCL — Purkinje cell layer of cerebellum, PGM — pontine gray matter, and VmHN — ventromedial hypothalamic nucleus.

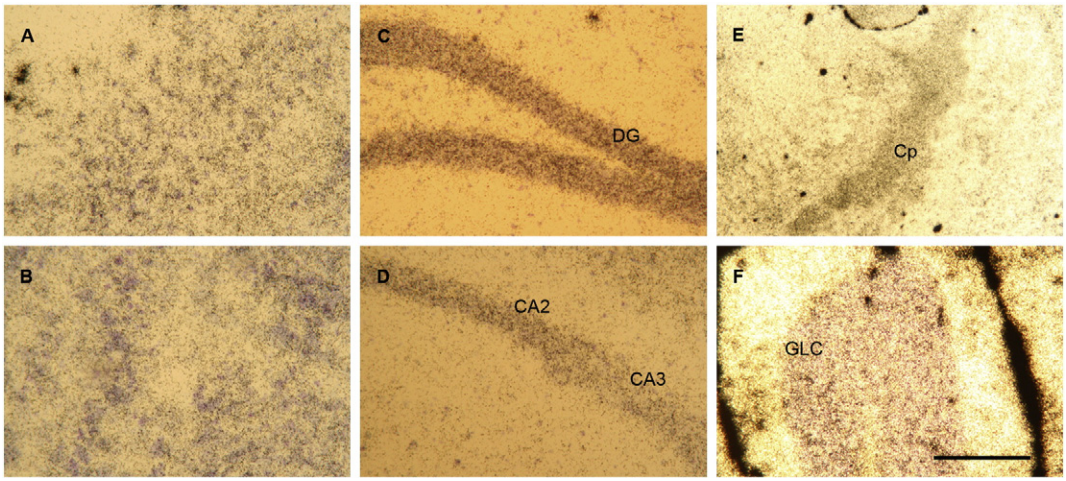


Fig. 2. Representative higher magnification images of coronal brain sections showing *Stam2* mRNA expression in selected brain regions. Sections are counterstained with cresyl violet. (A) Glomerular layer of the olfactory bulb, (B) granular layer of the olfactory bulb, (C) dentate gyrus (DG), (D) CA2–CA3 transition region of hippocampus, (E) caudoputamen (Cp), and (F) cerebellum; GLC — granular cell layer. Bar = 1 mm.

STAM1 and STAM2 is embryonically lethal due to a defect in ventral folding morphogenesis, implying that STAMs are indispensable for embryo development and survival (Yamada et al., 2002).

Our recent study demonstrated strong *Stam2* expression in the developing nervous system, particularly in differentiating layers of the telencephalic cortex and hippocampal formation (Ćurlin et al., 2012). These findings, together with the strong *Stam2* expression observed in the enteric nervous system and the brain regions related to olfaction (Čunko et al., 2008; Kapuralin et al., 2012), indicated a need to clarify *Stam2* expression in the central nervous system.

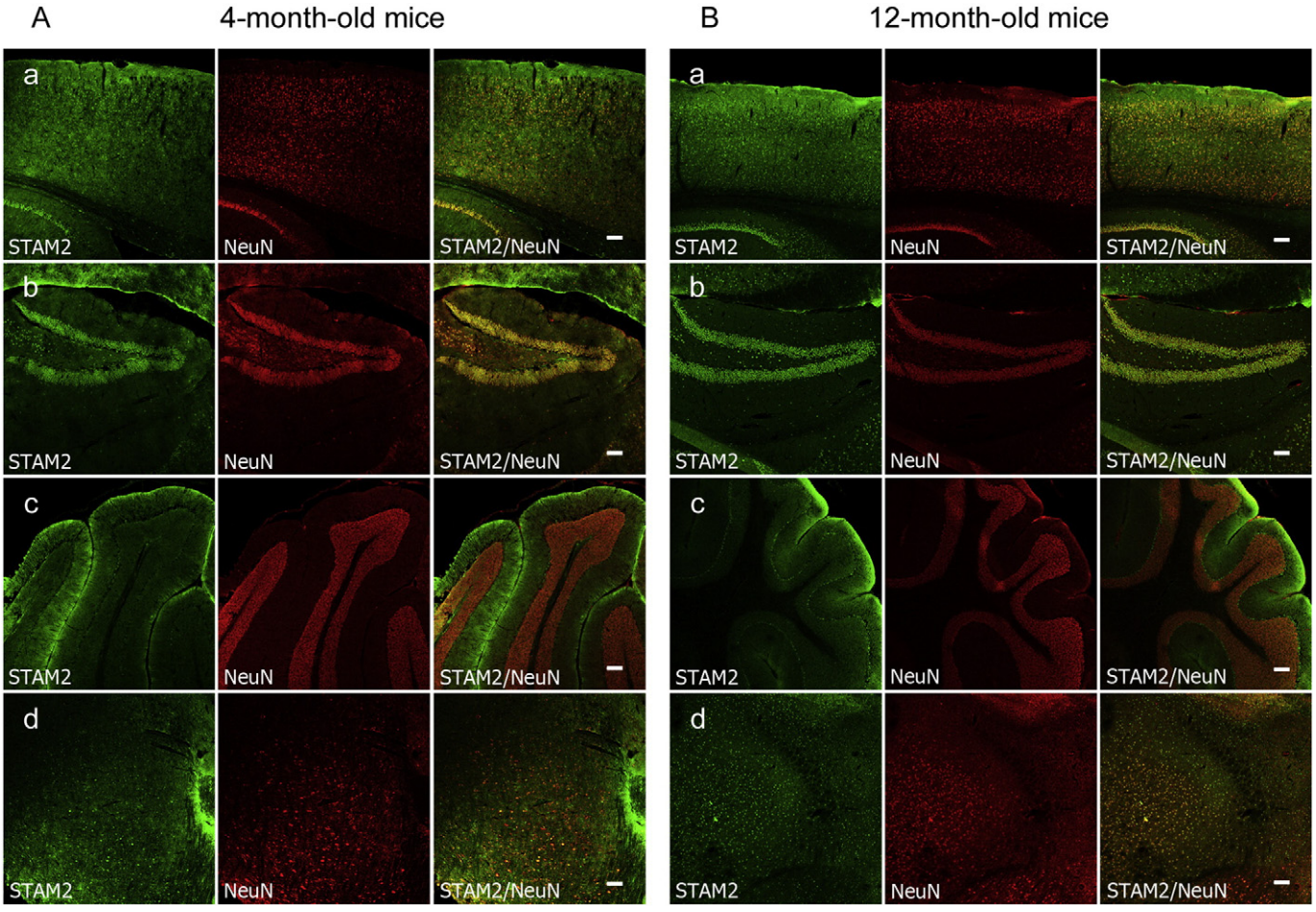


Fig. 3. STAM2 and NeuN immunostaining in the different brain regions of 4-month-old (A) and 12-month-old (B) mice; (a) cortex, (b) dentate gyrus, (c) cerebellum, (d) striatum. All bars are 100 μm.

In the present study we described the precise anatomical distribution and subcellular localization of STAM2 in the adult mouse brain using radioactive mRNA *in situ* hybridization and immunohistochemistry, and indirectly *via lacZ* reporter gene activity in the gene trap mouse line *Stam2*^{Gt1Gaj}. Together with endosomal STAM2 localization, we revealed a nuclear localization of STAM2 in neurons.

2. Material and methods

2.1. Animals

Experiments were performed on 4 to 6 month (considered as adult) and 12 month old (considered as old) male mice of wild type inbred strain C57Bl/6NCrl and the gene trap line *Stam2*^{Gt1Gaj}, kept on the same background. Genotyping of transgenic animals was performed with PCR using tail genomic DNA. The experiments were approved by the institutional Ethical Committee, and they were in line with the EU directive 2010/63/EU. Every effort was taken to minimize the number and suffering of animals.

The transgenic mouse line *Stam2*^{Gt1Gaj} was generated by a gene trap method (Skarnes et al., 1992; Thomas et al., 2000). A targeting vector pKC199βgeo, containing a mouse Hoxc9 splice acceptor sequence and fused promoterless genes *lacZ* and *neoR*, was integrated randomly into embryonic stem cells. The resulting insertion in *Stam2* gene was in the intron between exons 2 and 3 (Ćurlin et al., 2006, 2012).

Table 1
Relative levels of expression of *Stam2* in the adult mouse brain observed using *in situ* hybridization, *lacZ* staining and immunohistochemistry.
"–" no signal, "+" low signal, "++" moderate signal, "+++" high signal of *Stam2*. ISH: *in situ* hybridization with *Stam2* probe, *lacZ*: *lacZ* reporter staining using X-gal, IHC: immunohistochemistry with STAM2 antibody.

Brain region	ISH	*lacZ*	IHC
Telencephalon			
Neocortex			
Layer I	–	–	–
Layer II	+++	+++	+++
Layer III	+++	+++	+++
Layer IV	+	+	+
Layer V	++	++	++
Layer VI	++	++	++
Hippocampal formation			
Pyramidal layer CA1	+++	+++	+++
Pyramidal layer CA2	+++	+++	+++
Pyramidal layer CA3	++	++	++
Dentate gyrus	+++	++	+++
Subiculum	++	++	++
Gyrus fasciolaris	+++	+++	+++
Amygdala			
Lateral nucleus of amygdala	++	++	++
Basolateral nucleus of amygdala	++	++	++
Basomedial nucleus of amygdala	++	++	++
Posterior nucleus of amygdala	++	++	++
Olfactory areas			
Main olfactory bulb, granular layer	++	++	++
Main olfactory bulb, glomerular layer	+++	+++	+++
Main olfactory bulb, *stratum plexiformes internum*	+	+	+
Main olfactory bulb, *stratum plexiformes externum*	–	–	–
Main olfactory bulb, mitral cells layer	+	+	+
Anterior olfactory bulb	+++	+++	+++
Auxiliary olfactory bulb	+	+	+
Piriform area	+++	+++	+++
Striatum			
Caudoputamen	++	++	++
Lateral septal nucleus	+	+	+
Nucleus accumbens	+	+	+
Diencephalon			
Epithalamus			
Medial habenula	+++	+++	+++
Hypothalamus			
Ventromedial hypothalamic nucleus	+++	++	+++
Dorsomedial hypothalamic nucleus	+++	++	+++
Zona incerta	+	+	+
Corpus mamillare	++	+	++
Metencephalon			
Cerebellum			
Molecular layer	–	–	–
Purkinje cell layer	+	+	+
Granular layer	+++	+++	+++
Pons			
Pontine gray matter	+	+	+
Pontine nuclei	+	+	+

2.2. Primary neuronal cell culture

The primary neuronal cell culture was obtained from the wild-type C57Bl/6NCrl newborn mice (P0), sacrificed by decapitation. The cortex was dissected and minced with scissors in Hank's balanced salt solution (HBSS; Gibco). Dissociation of neuronal tissue and single-cell suspension was obtained using accutase (Gibco) for 15 min at 37 °C. The suspension was centrifuged, supernatant discarded and cells resuspended in DMEM/F-12 + GlutaMAX medium supplemented with N2, B27 and penicillin/streptomycin (all from Gibco). Cells were plated on poly-D-lysine (0.1 mg/ml, 18 h at 37 °C; Sigma) and laminin (10 μg/ml, 4 h at 37 °C; Sigma) coated 6-well plates (for subcellular fractionation) or on coated coverslips in 24-well plates (for immunocytochemistry) and maintained in the medium at 37 °C in humidified atmosphere of 5% CO_2 for 6 and 14 days. The plating density was 0.4×10^6 cells/ml.

2.3. Detection of β-galactosidase activity

Transcardial perfusion with PBS (phosphate-buffered saline) and fixative containing 2% formalin and 0.2% glutaraldehyde (Sigma-Aldrich) in 0.01 M PBS (pH 7.4) was performed on adult transgenic animals anesthetized intraperitoneally with Avertin (500 mg/kg). Brains were dissected and postfixed by immersion in the same fixative for 1 h on ice. After rinsing in PBS, brains were frozen to −80 °C in prefreezed isopentane (Sigma-Aldrich) and cut with a cryostat (Leica CM1850) into 50–60 μm thick sections mounted subsequently on SuperFrost microscope slides (Menzel-Glaser) and air-dried for 1–3 h. After rinsing in PBS, sections were incubated in the X-gal staining solution (0.5 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 10 mM potassium ferricyanide, 10 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.01% sodium deoxycholate, and 0.01% Igepal; all from Sigma-Aldrich in 0.01 M PBS (pH 7.4)) under light protection at 37 °C overnight. The stained sections were rinsed in PBS, dehydrated in ascending concentrations of ethanol, cleared in Histo-Clear (National Diagnostics) and coverslipped with Histomount (National Diagnostics). β-Galactosidase activity was visualized under the microscope (Olympus AX70) and documented by digital photography (Canon EOS 400D) or using a computer scanner (Epson Effection 4870 Photo).

2.4. mRNA in situ hybridization

mRNA *in situ* hybridization was performed according to a modified method described previously (Wittmann et al., 2009). In brief, synthetic DNA oligonucleotides complementary to the mRNA sequence of mouse *Stam2* (5′-CCT CTG TGA TTT TCT CCT TGC CAC CAG TTG GCG TCA CTG TCA TCC-3′; Microsynth) were labeled with [^{35}S]-dATP (PerkinElmer) using terminal deoxynucleotidyltransferase (Roche).

C57Bl/6NCrl wild type mice were sacrificed by cervical dislocation and the brains were quickly dissected and immediately frozen in isopentane (Sigma-Aldrich). 20 μm-thick coronal and sagittal sections were cut and thaw-mounted on poly-L-lysine coated slides and stored at −20 °C until use. Frozen sections were rapidly immersed into ice-cold 2% paraformaldehyde for 10 min, shortly rinsed in PBS, transferred to 0.25% acetic anhydride in 0.1 M triethylamine hydrochloride for

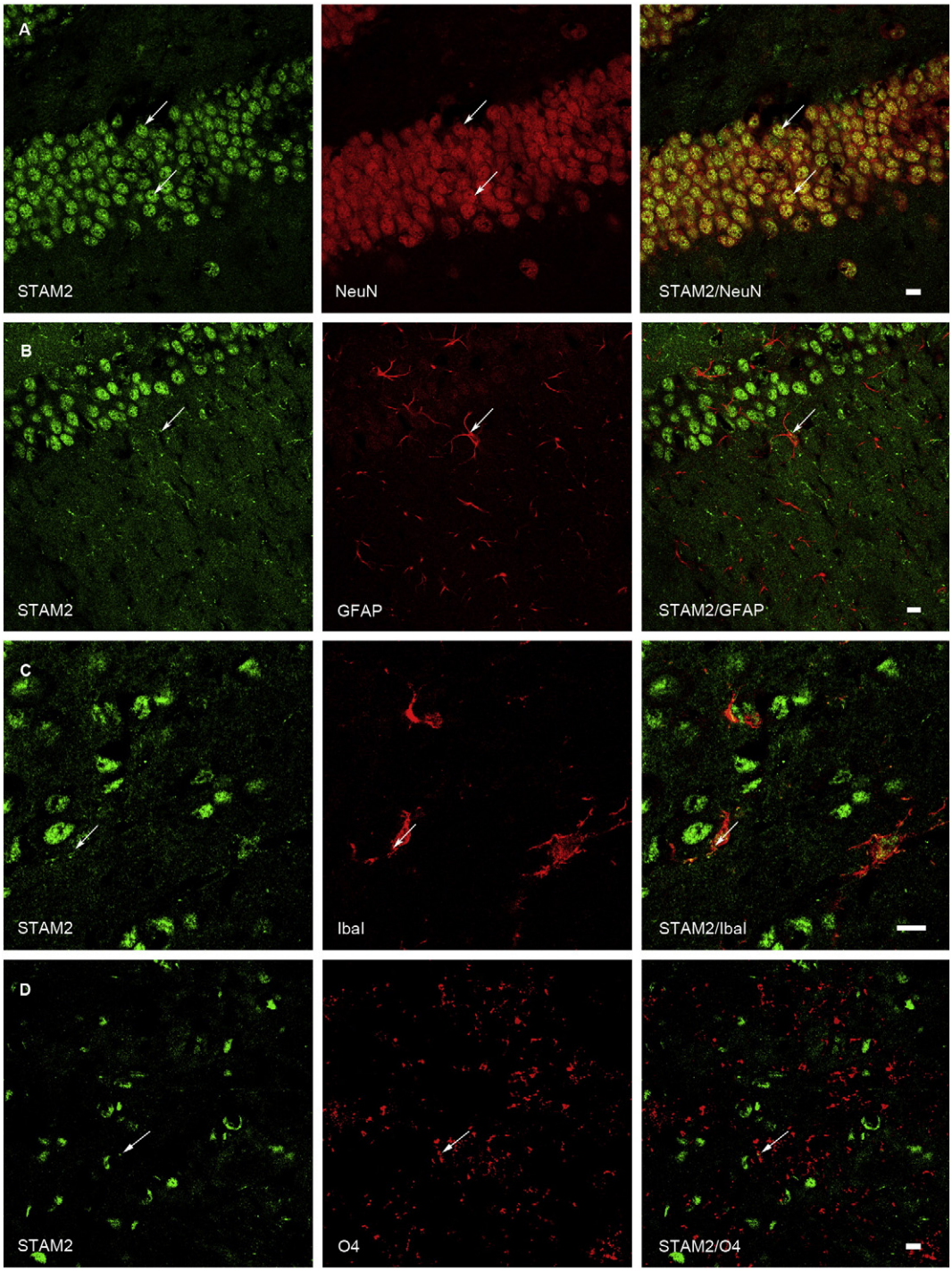
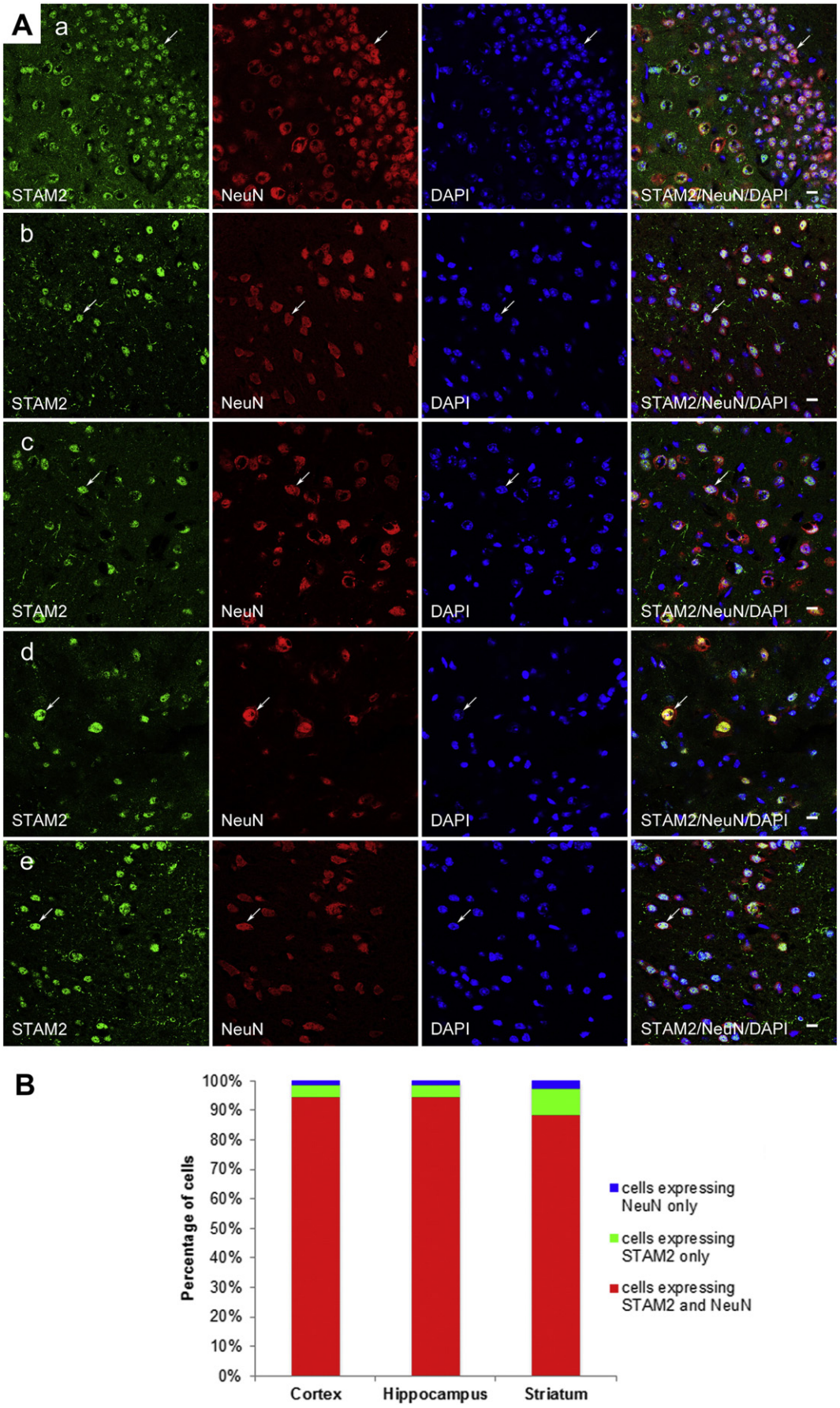


Fig. 4. STAM2 localization in neurons and glial cells of the central nervous system. (A) STAM2 immunoreactivity was highly localized in neurons (arrows), which was demonstrated with the neuronal marker NeuN. STAM2 was found in the cytoplasm and the nucleoplasm. (B, C) STAM2 localization in the cytoplasm of astrocytes (B) and microglia (C) (arrows) visualized by GFAP and IBA1 antibody. (D) A very faint STAM2 signal (arrows) was found in O4-positive cells, indicating the absence of high *Stam2* expression in the oligodendrocytes. All bars are 10 μm.

10 min at room temperature, dehydrated by ethanol series, and delipidated with chloroform. Hybridization was performed for 18 h at 53 °C with a labeled oligonucleotide probe ($0.8–10^6$ cpm) in 50 μl hybridization buffer (1× SSC (saline sodium citrate buffer), 0.05 M sodium-phosphate buffer, 1× Denhard solution, 0.25 mg/ml tRNA, 0.5 mg/ml salmon sperm DNA, 1 mM EDTA, and 0.01% SDS; all from Sigma-Aldrich). The next day the sections were rinsed in 0.5× SSC buffer with 0.5 mM DTT (dithiothreitol) at 60 °C. They were further dipped in 70% ethanol, dried, and exposed to Kodak BioMax MR Film for 2–3 weeks at room temperature. Slides with the sections were

Fig. 5. STAM2 and NeuN double-labeling reveals co-localization of STAM2 and NeuN in different brain regions (arrows). (A) (a) (hippocampus), (b) cortex, (c) olfactory bulb, (d) striatum, (e) cerebellum. All bars are 10 μm. (B) Quantitative analysis of STAM2 and NeuN immunoreactivity in the region of the cortex, hippocampus and striatum confirmed that the majority of STAM2-positive cells were neurons.


A
a
b
c
d
e
STAM2
NeuN
DAPI
STAM2/NeuN/DAPI
B
Percentage of cells
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Cortex
Hippocampus
Striatum
cells expressing NeuN only
cells expressing STAM2 only
cells expressing STAM2 and NeuN

subsequently autoradiographed using autoradiography emulsion, type NTB (Kodak) for 3–6 weeks at 4 °C. The X-ray films and dipped sections were developed with D-19 developer (Kodak). Films were further analyzed by computer scanning (Epson Effection 4870 Photo). Slides with the sections were counterstained lightly with cresyl violet (Sigma-Aldrich), dehydrated, cleared in Histo-Clear, and coverslipped with Histomount. Autoradiographs were visualized under the microscope (Olympus AX70) and digitalized with Canon EOS 400D.

Hybridization with the respective *sense Stam2* oligonucleotides (5′-GGA TGA CAG TGA CGC CAA CTG GTG GCA AGG AGA AAA TCA CAG AGG-3′; Mycrosinth) was included as a control.

2.5. Immunohistochemistry and immunocytochemistry

Wild type mice were anesthetized using Avertin (500 mg/kg) and then perfused transcardially with PBS and 4% paraformaldehyde (pH 7.4). Brains were isolated and further fixed by immersion in the same fixative at 4 °C overnight. They were washed in PBS and transferred to 10% sucrose followed by 30% sucrose in PBS at 4 °C. Coronal and sagittal 20 μm-thick sections were cut with a cryostat and thaw-mounted on Superfrost Plus (Menzel Glaser) coated slides.

For immunocytochemical analysis, neuronal cells isolated from the cerebral cortex were fixed in 4% paraformaldehyde (pH 7.4) for 15 min at 4 °C. After rinsing in PBS, cells were ready for immunolabeling.

Tissue sections and cells were immunolabeled with the primary antibodies against STAM2 (rabbit polyclonal, diluted 1:200, Abcam, ab63372; rabbit polyclonal, diluted 1:200, Bioworld Technology, BS1807; and rabbit polyclonal, diluted 1:200, Santa Cruz Biotechnology, sc-98681), AMSH (mouse monoclonal, diluted 1:50, Santa Cruz Biotechnology, sc-271641), EEA1 (mouse monoclonal, diluted 1:500, BD Transduction Laboratories, 610457), GFAP (chicken polyclonal, diluted 1:300, Abcam, ab4674), HRS (mouse monoclonal, diluted 1:200, Santa Cruz Biotechnology, sc166843), IBA1 (goat polyclonal, diluted 1:500, Abcam, ab5076), MAP2 (chicken polyclonal, diluted 1:1200, Abcam, ab5392), NeuN (mouse monoclonal, diluted 1:100, Millipore, MAB377), O4 (mouse monoclonal, diluted 1:200, Millipore, MAB345), STAM1 (goat polyclonal, diluted 1:100, Santa Cruz Biotechnology, sc-6919), UBPY (mouse monoclonal, diluted 1:50, Santa Cruz Biotechnology, sc-365481) and β-galactosidase (chicken polyclonal, diluted 1:250, Abcam, ab9361). Primary antibodies were diluted in PBS containing 0.2% Triton-X-100 (Sigma-Aldrich) and 2% serum from the species in which the secondary antibody was raised at 4 °C overnight. After rinsing in PBS, sections were further immunolabeled with the fluorescent secondary antibodies: Alexa Fluor 488 goat anti-rabbit (Invitrogen, A11008), Alexa Fluor 488 donkey anti-rabbit (Invitrogen, A21206), Alexa Fluor 488 rabbit anti-mouse (Invitrogen, A11059), Alexa Fluor 546 rabbit anti-chicken (Invitrogen, A11040), Alexa Fluor 546 rabbit anti-mouse (Invitrogen, A11003), Alexa Fluor 546 donkey anti-goat (Invitrogen, A11056) and Alexa Fluor 546 rabbit anti-goat (Invitrogen, A21085). All secondary antibodies were diluted in PBS at a concentration of 1:500 (for the brain tissue) or 1:1000 (for the cell culture). Incubation with the secondary antibody was performed for 2.5 h at room temperature. DAPI (Roche) was used as a nuclear counterstain. Finally, the sections and the cells were rinsed in PBS and coverslipped using Fluoromount (Sigma-Aldrich). Fluorescent mono and double-immunolabeling was analyzed by confocal microscope (Zeiss LSM 510 Meta).

2.6. Subcellular fractionation

Subcellular fractions were prepared from a primary neuronal cell culture obtained from the cortex of wild type C57Bl/6NCrl newborn mice with an NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific) as per the manufacturer's instructions. All lysis buffers were supplemented with Halt™ Protease Inhibitor Cocktail (Thermo Scientific).

Briefly, accutase (Gibco) was added to the wells of the 6-well plate where the neurons were cultured. After 10 min, cells were washed with PBS and transferred to a 1.5 ml microcentrifuge tube and pelleted by centrifugation at 500 *g* for 3 min. Ice cold cytoplasmic extraction reagent I was then added to the cell pellet, and the tube was vortexed and incubated on ice for 15 min. Further, ice cold cytoplasmic extraction reagent II was added and the tube was incubated for an additional 3 min. After 3 min centrifugation at maximum speed, supernatant containing cytoplasmic extract was carefully transferred to a clean pre-chilled tube. Nuclear extraction reagent was then added to the pellet and the tube was incubated on ice for 40 min. After centrifugation at maximum speed, the supernatant containing nuclear extract was transferred to a clean pre-chilled tube.

2.7. Western blotting

Western blot analysis was performed according to standard procedures. Protein concentration was measured using a Bradford assay (Bio-Rad). The same concentration of proteins (50 μg) from different fractions was separated on 12% SDS-PAGE and transferred to PVDF (polyvinyl difluoride) membrane (Sigma-Aldrich). The membrane was blocked with 5% non-fat milk in PBS containing 0.05% Tween-20 for 45 min at room temperature and then incubated with primary antibodies against STAM2 (rabbit polyclonal, diluted 1:1000, Abcam, ab63372), HDAC2 (mouse monoclonal, diluted 1:500, Abcam, ab12169), and VEGF (rabbit polyclonal, diluted 1:5000, Abcam, ab46154) at 4 °C overnight. Immune complexes were revealed using appropriate secondary antibodies coupled with horseradish peroxidase: goat anti-mouse IgG, HRP (diluted 1:5000, Abcam, ab6789) and goat anti-rabbit IgG, HRP (diluted 1:5000, Abcam, ab6721) for 2 h at room temperature, followed by chemiluminescent detection (Immobilon Western Chemiluminescent HRP Substrate; Millipore) and imaged using ChemiDoc XRS+ imaging system analyzed by Image Lab 5.2 software.

2.8. Expression data analysis

The anatomical structures of the mouse brain were identified with the aid of a mouse brain atlas (Dong, 2008; Hof et al., 2000). The intensity of an X-gal-staining area, RNA *in situ* hybridization signal and STAM2 antibody intensity were evaluated semiquantitatively through a microscope using the following categories: no signal (−); low signal (+); moderate signal (++) and high signal (+++). The results were rated independently by two observers and in case of disagreement a third observer was engaged.

2.9. Quantification of the immunofluorescence signal

For the quantification of the STAM2 and NeuN immunofluorescent signal samples were taken from four animals. Five randomly chosen sections of the cortex, hippocampus and striatum per animal and three fields per section were analyzed on a confocal microscope (Zeiss LSM 510 Meta). The number of positive cells in a field was counted manually.

3. Results

3.1. Stam2 was highly expressed in the mouse brain including the neocortex, hippocampus, olfactory bulb and cerebellum

Brain *Stam2* expression was evaluated by *in situ* hybridization in wild type mice, X-gal staining, reflecting the activity of the *lacZ* reporter gene in $Stam2^{Gt1Gaj}$ mouse line and using STAM2 immunohistochemistry (Figs. 1–3). Regions of high-level expression were detected by all three techniques with high reproducibility. However, β-galactosidase activity resulting from *lacZ* expression allowed better delineation of different brain substructures, as previously observed for other

genes (Couegnas et al., 2007), being a convenient, sensitive and high-resolution assay to detect the overall pattern of endogenous gene expression (Burn, 2012).

The distribution and relative intensity of the mRNA transcript of *Stam2* gene, relative intensity of X-gal positive areas and STAM2 immunofluorescence in different brain regions were semiquantitatively scored (Table 1). All three methods revealed appreciably high levels of *Stam2* in layer II and layer III (*lamina granularis externa* and *lamina pyramidalis externa*) of the neocortex and in the hippocampus, within the pyramidal layer of the CA1 and CA2 fields of Ammon's horn and in *gyrus fasciolaris*. Strong *Stam2* expression was also found in the olfactory bulb throughout the glomerular layer of the main olfactory bulb and in the anterior olfactory nucleus (Čunko et al., 2008). It was also found in the medial habenula of the epithalamus, and in the ventromedial and dorsomedial hypothalamic nuclei. In the metencephalon the highest *Stam2* expression was found in the cerebellum, mostly in the granular layer (Figs. 1–3). Additionally, a very weak, ubiquitous signal was visible in every cell, which was in accordance with a previous northern blot and RT-PCR analysis (Ćurlin et al., 2012; Takata et al., 2000).

The accuracy with which the fused *lacZ-Stam2* reporter gene reflected the endogenous presence of STAM2 protein was determined by comparing STAM2 immunohistochemistry and β-galactosidase presence. Cryosections of heterozygous $Stam2^{+/Gt1Gaj}$ brain were doubly labeled with STAM2 and β-galactosidase antibody. Immunohistochemistry against both antigens revealed a strong fluorescent signal in the *Stam2*-expressing regions identified by *in situ* hybridization and X-gal staining (Figs. 1B, 3). The overlap in the distribution of STAM2 and β-galactosidase proteins confirmed the observed expression pattern. This served as an evidence that the gene trap mice in general, and $Stam2^{Gt1Gaj}$ mice in particular, are appropriate animal models for expression studies.

Still in some brain areas (corpus mamillare, hypothalamic nuclei and hippocampal dentate gyrus) the reporter gene expression showed lower signal strength as observed by RNA *in situ* hybridization and immunohistochemistry (Table 1). Such "mismatches" were expected due to different detection levels of the three methods used. Both, mRNA expression and immunohistochemistry showed the weak ubiquitous signal not visible by X-gal staining. It was shown previously that *lacZ* histochemistry might underestimate the extent of gene expression or protein distribution (Couegnas et al., 2007; Mahony et al., 2002; Pereira et al., 2006). The *lacZ* expression could not be considered as a disadvantage, as it revealed positive areas, corresponding to the high level of *Stam2* expression.

In order to verify whether *Stam2* expression in the brain changes with age we performed an immunostaining assay for STAM2 on 4-month-old and 12-month-old mice. The staining pattern of STAM2 remained the same in both age groups (Fig. 3, Supplementary Table 1).

3.2. Stam2 expressing cells were predominantly neurons

In order to identify cell types expressing *Stam2*, brain cryosections were co-immunostained with STAM2 antibody and markers specific for neurons and glial cell types. The majority of STAM2-positive cells were co-labeled for neuronal markers MAP2 (the neuron-specific cytoskeletal marker) and NeuN (the neuron-specific nuclear marker), indicating that STAM2-positive cells were predominantly neurons (Fig. 4A). The percentage of cells co-expressing STAM2 and NeuN in the region of the cortex, hippocampus and striatum was 94.3%, 94.3% and 88.4% of the total stained cells respectively (Fig. 5). In neurons, STAM2 was visible in the cytoplasm throughout the soma and nerve fibers, showing a punctuate appearance and high fluorescence intensity. Rather unexpectedly, STAM2 was also visible in neuronal nuclei, and the nuclear signal was homogenously distributed. Glial cells (astrocytes,

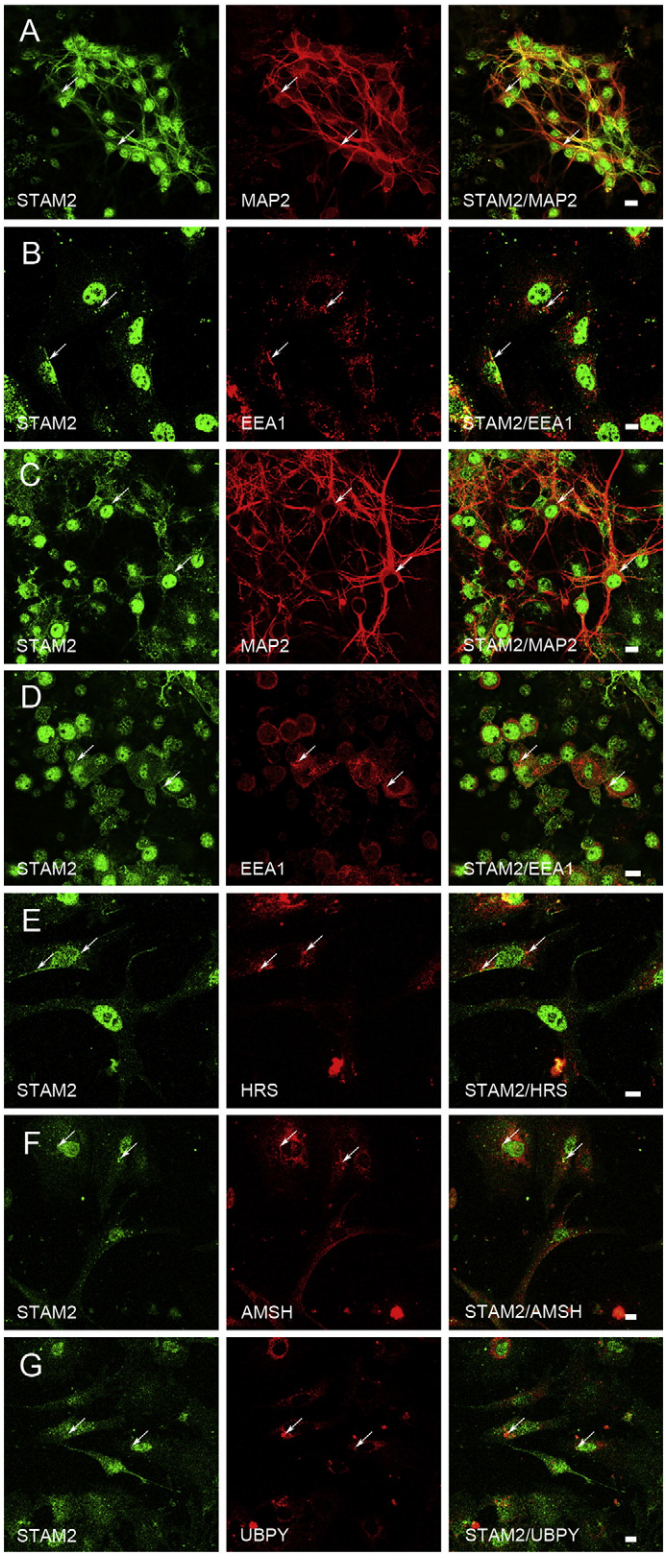


Fig. 6. Subcellular localization of endogenous STAM2 in primary neurons. (A) After 6 days in a culture, immunostaining with neuronal marker MAP2 demonstrated that the cells were predominantly neurons (arrows). (B) Primary neurons cultured for 6 days were co-stained with STAM2 and EEA1 antibody (arrows), indicating that STAM2 protein was localized mainly to the early endosome membrane. (C) After 14 days in a culture double-labeling results clearly showed that the cells in culture were positive for STAM2 and neuronal marker MAP2 (arrows). (D) Double staining with STAM2 and EEA1 antibody confirmed that STAM2 was localized in the punctuate structures positive for EEA1 in the mature neurons as well (arrows). (E) Arrows indicate co-localization of STAM2 with HRS, suggesting that the ESCRT-0 system is functional in neurons. (F, G) STAM2 partially co-localized (arrows) with AMSH (F) and UBPY (G), indicating an interaction between STAM2 and deubiquitinating enzymes in neurons. All bars are 10 μm.

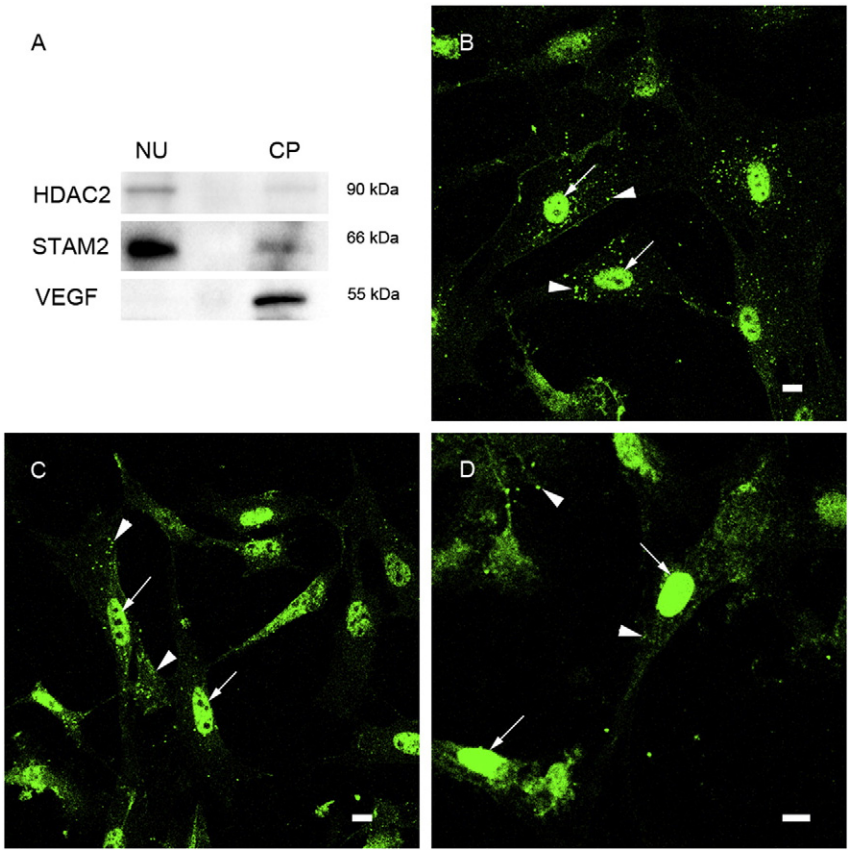


Fig. 7. Nuclear localization of STAM2 in neurons. (A) Primary neuronal cells obtained from the mice cortex were subjected to subcellular fractionation, as described in the Material and Methods section. Proteins from different cellular fractions (cytoplasmic and nuclear) were analyzed by SDS-PAGE and immunoblotted with STAM2 antibody. STAM2 protein was found in the cytoplasmic (NP) and nuclear (NU) fractions. VEGF and HDAC2 were used to confirm the integrity of each fraction. (B, C, D) Confocal microscopy images depicting intracellular nuclear localization of STAM2 in primary neurons. Neuronal cells were immunostained for endogenous STAM2 using three different antibodies obtained from Abcam (B), Bioworld Technology (C) and Santa Cruz Biotechnology (D). STAM2 was found to be partially localized in the nuclei (arrows), as well as in the cytoplasm (arrowheads) of neuronal cells. All bars are 10 μm.

microglia) visualized by GFAP and IBA1 antibody showed substantially weaker STAM2 signal than that in neurons. STAM2 exhibited a similar cytoplasmic staining pattern in both cell bodies and processes, but not in the nucleus (Fig. 4B, C). O4 antibody as a marker for oligodendrocytes showed a weak signal for STAM2 protein, similar to the weak ubiquitous staining observed in every cell (Fig. 4D).

3.3. In the cytoplasm of the neurons STAM2 was localized on the early endosomes as a part of the ESCRT-0 complex

Although STAM2 was previously suggested to be involved in endosomal trafficking, it remained unclear whether it was associated with the endosomes in the neurons. To examine subcellular localization of STAM2 protein, adult brain tissue and primary neurons cultured for 6 and 14 days were used for immunohistochemical analysis (Fig. 6A, C). In double-labeling experiments with EEA1 (a protein present in the early endosome membrane) an extensive but not a total overlap was observed (Fig. 6B, D).

Since STAM2 is an ESCRT-0 member, we next examined its co-localization with HRS, the other ESCRT-0 member, and ESCRT-0-associated deubiquitinating enzymes AMSH and UBPY. STAM2 was co-localized with HRS both *in vitro* and *in vivo* (Fig. 6E), and partially co-localized with AMSH and UBPY (Fig. 6F, G).

3.4. Stam2 was localized in the neuronal nuclei

In addition to cytoplasmic staining, STAM2 antibody showed a homogenous strong signal in the neuronal nuclei. This finding was novel and rather unexpected, as STAM2 was considered to be localized only on the cytoplasmic surface of early endosomes (Bache et al., 2003; Takata et al., 2000). To verify its nuclear localization, immunohistochemical analyses were performed with three different commercial antibodies obtained from three different companies (Abcam, Bioworld Technology and Santa Cruz Biotechnology), representing all commercially available antibodies at the time the experiments were performed. NeuN, a marker specific for neuronal nuclei was used to validate the STAM2 nuclear signal (Fig. 5). All three different STAM2 antibodies showed a homogenous signal in neuronal nuclei (Fig. 7B–D).

Another approach to analyze STAM2 intracellular localization was subcellular fractionation on neuronal cells obtained from the primary culture. Proteins from different cellular fractions (cytosolic and nuclear) were analyzed by SDS-PAGE followed by western blotting with a STAM2 antibody. Western blotting confirmed a considerable amount of STAM2 in the nuclear fraction as well as in the cytosolic fraction. VEGF and HDAC2, were detected in the cytoplasmic and nuclear fractions respectively, to confirm the integrity of each fraction (Fig. 7A). These results were consistent with the immunocytochemical staining, indicating that STAM2 was indeed present in the neuronal nuclei.

3.5. STAM2-associated proteins were not localized in the nucleus

The newly identified STAM2 neuronal nuclear expression prompted us to analyze whether STAM2-associated proteins, STAM1, HRS, AMSH and UBPY, would be in the nucleus as well. Immunohistochemistry to these proteins in the primary neuronal culture revealed two types

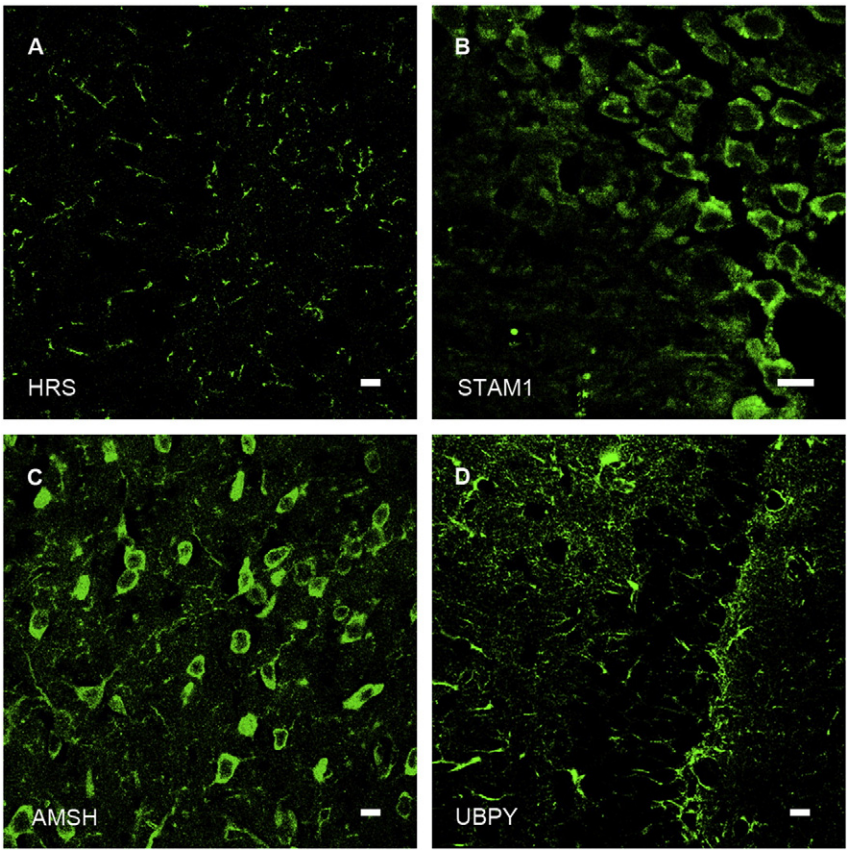


Fig. 8. Immunolocalization of STAM2-associated proteins in the brain. Confocal microscopy was employed to show localization of endogenous STAM1 (A), HRS (B), AMSH (C) and UBPY (D) at the cortex level. HRS and UBPY (A, D) antibodies were expressed in punctate patches throughout the cytoplasm, whereas the STAM1 and AMSH (B, C) staining was intense particularly in perinuclear regions of cells without detectable nuclear staining. All bars are 10 μm.

of cytoplasmic patterns: diffuse and punctate. Bright, punctate cytoplasmic staining was observed for HRS (Fig. 8A) and UBPY (Fig. 8D). In contrast, STAM1 (Fig. 8B) and AMSH (Fig. 8C) staining was diffuse throughout the cytoplasm, with a punctuate pattern surrounding the nuclear membrane. The nuclear localization of these STAM2-associated proteins was never observed, suggesting that these endosome-related proteins were not together with STAM2 in the nucleus of neurons.

4. Discussion

Spatial expression of *Stam2* in the mouse brain was similar to that reported for other ESCRT-0 members, *Stam1* and *Hrs*. The regions of high expression of *Stam2* mRNA coincided with the regions of *Stam1* and *Hrs* mRNA-expressing cells in the brain (Tamai et al., 2008; Yamada et al., 2001). All these genes are highly expressed in the cerebral cortex, hippocampus and cerebellar cortex, while high *Stam1* and *Stam2* expression overlapped in the olfactory bulb and caudate putamen region. When compared with other STAM2-associated proteins, there was an overlap between the regions of high *Stam2* expression and high *Amsh* expression in the cerebral cortex, olfactory bulb, hippocampus and cerebellum region (Ishii et al., 2001). All these genes together with *Stam2* were found to be highly expressed in the hippocampus. It was previously reported that STAM2-interacting proteins contributed to hippocampal pyramidal cell survival *in vivo*; loss of STAM1 or HRS caused significant disappearance of neurons in the CA3 region of the hippocampus and loss of AMSH led to neuronal loss in the CA1 subfield of the hippocampus (Ishii et al., 2001; Tamai et al., 2008; Yamada et al., 2001). There is no evidence of the loss of hippocampal neurons after the loss of STAM2 in the mutant mice with the function, but the loss of both STAM proteins is indeed embryonically lethal, implying that STAM1 and STAM2 compensate for each other (Yamada et al., 2002). STAM2 cannot compensate for all the functions mediated by STAM1, as shown by CA3 pyramidal cell loss in STAM1$^{-/-}$ mice. We showed that in the CA3 subfield the expression level of *Stam2* was lower than in the CA1 and CA2 subfields of the hippocampus.

High *Stam2* expression was found in neuronal cells in most regions of the mouse brain, while its expression in astrocytes, microglia and oligodendrocytes was low but detectable. This was in accordance with the distribution of STAM2-immunoreactive cells described in the enteric nervous system, where the majority of STAM2-positive cells co-stained with neuronal markers, while enteroglial cells showed a weak STAM2 signal (Kapuralin et al., 2012). A low level of STAM2 signal in various tissues has been previously described (Ćurlin et al., 2012; Takata et al., 2000), but here we demonstrated a different expression within different cell types of the nervous system.

STAM2 subcellular distribution was granular cytoplasmic, co-localized with early endosome marker EEA1, and members of the ESCRT-0 complex, STAM1 and HRS. This was related to the vesicular transport through early endosomes for down-regulation of the growth factor/receptor complexes (Bache et al., 2003; Takata et al., 2000). STAM2 was localized at axons and dendrites, indicating its possible role in endosomal trafficking at presynaptic and postsynaptic sites. Endosomes regulate a large number of processes in neurons, including formation and recycling of synaptic vesicles, retrograde axonal transport, growth factor-mediated cell signaling, degradation of proteins, axonal pathfinding during development, synaptic plasticity and more (Nixon and Cataldo, 1995; Schmieg et al., 2014; Steketee and Goldberg, 2012). Neurons are particularly sensitive to defects in the endosomal–lysosomal system, and its disruption is linked to several neurodegenerative diseases. Mutations in various proteins of the ESCRT complex are responsible for accumulation of ubiquitin-positive protein inclusions in the central nervous system, leading to

frontotemporal dementia and amyotrophic lateral sclerosis (Lee and Gao, 2012; Rusten and Simonsen, 2008; Saksena and Emr, 2009; Stuffers et al., 2009). The presence of STAM2 in neurons contributed to the evidence that ESCRT proteins play an important role in the brain.

The novel finding of nuclear localization of STAM2 in the neurons was confirmed by different approaches (immunolocalization and western blot on purified subcellular fractions). STAM2 lacks a nuclear localization sequence, and the previous studies showed endosomal and cytoplasmic STAM2 localization only (Bache et al., 2003; Takata et al., 2000). STAM2 *in vitro* studies were performed on non-neuronal cells only (Endo et al., 2000; Kanazawa et al., 2003; Mizuno et al., 2003; Rismanchi et al., 2009; Takata et al., 2000), and the data presented here pointed to the importance of further investigations of this protein in neuronal cells. The lack of nuclear co-localization of STAM2 with other ESCRT-0 and ESCRT-0-associated proteins, AMSH and UBPY, suggested that it had an additional function in neuronal nuclei independent of the ESCRT pathway. STAM2 is also involved in the regulation of intracellular signal transduction for DNA synthesis and induction of proto-oncogene c-MYC (Komada and Kitamura, 2005; Takeshita et al., 1997) being at the intersection of signaling pathways and membrane transport in the cell, which might well contribute to its nuclear expression.

Similarly to STAM2, TSG101, CHMP1, and CHMP3 are the ESCRT proteins also present in the nucleus (Tu et al., 2011). They share some features of the so-called "two-faced" proteins that have a dual role and regulate signaling pathways in seemingly opposite ways under different physiological conditions. TSG101, a member of the ESCRT-I complex binds the glucocorticoid receptor and inhibits transcriptional activation. CHMP1, a member of the ESCRT-II complex plays a role in chromatin remodeling, while CHMP3, a key component of ESCRT-III, is localized to the nucleus in addition to endosomes (Hittelman et al., 1999; Stauffer et al., 2001; Whitley et al., 2003). All these findings together suggest that some ESCRT proteins might serve as a link between endosomal sorting and transcriptional regulation.

5. Conclusion

Our study described for the first time the regional distribution of *Stam2* expression in the mouse brain and in the neurons. STAM2 was localized both in the cytoplasmic and nuclear compartment of the neurons. The newly identified neuron-specific nuclear localization of STAM2, together with its high expression in the brain indicated that STAM2 might have a specific function in the mouse nervous system.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mcn.2015.06.009.

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