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Isolation and Characterization of Living Primary Astroglial Cells Using the New GLAST-Specific Monoclonal Antibody ACSA-1

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KEY WORDS
astroglial cells; GLAST (EAAT1); ACSA-1 (astrocyte cell surface antigen-1); antibody; cell separation

ABSTRACT
Astrocytes show large morphological and functional heterogeneity and are involved in many aspects of neural function. Progress in defining astrocyte subpopulations has been hampered by the lack of a suitable antibody for their direct detection and isolation. Here, we describe a new monoclonal antibody, ACSA-1, which was generated by immunization of GLAST1 knockout mice. The antibody specifically detects an extracellular epitope of the astrocyte-specific L-glutamate/L-aspartate transporter GLAST (EAAT1, Slc1a3). As shown by immunohistochemistry, immunocytochemistry, and flow cytometry, ACSA-1 was cross-reactive for mouse, human, and rat. It labeled virtually all astrocytes positive for GFAP, GS, BLBP, RC2, and Nestin, including protoplastic, fibrous, and reactive astrocytes as well as Bergmann glia, Müller glia, and radial glia. Oligodendrocytes, microglia, neurons, and neuronal progenitors were negative for ACSA-1. Using an immunomagnetic approach, we established a method for the isolation of GLAST-positive cells with high purity. Binding of the antibody to GLAST and subsequent sorting of GLAST-positive cells neither interfered with cellular glutamate transport nor compromised astrocyte viability *in vitro*. The ACSA-1 antibody is not only a valuable tool to identify and track astrocytes by immunostaining, but also provides the possibility of separation and further analysis of pure astrocytes.

INTRODUCTION

Astrocytes are the most abundant class of cells in mammalian brain, fulfill a broad range of functions and show diverse phenotypes. They regulate ion and glutamate homeostasis, contribute to wound healing, form the blood–brain-barrier (BBB), and modulate the cerebral blood flow (Barres, 2008; Maragakis and Rothstein, 2006; Takano et al., 2006). Moreover astrocytes control the number, function, and plasticity of synapses (Christopherson et al., 2005; Fellin et al., 2004; Halassa et al., 2007; Ma et al., 2005; Mauch et al., 2001; Mori et al., 2005; Ullian et al., 2001). Most intriguingly, astroglial cells have also been shown to act as adult neural stem cells in the subependymal zone of the lateral ventricle's (LVs) lateral wall, as well as in the subgranular layer (SGL) of the dentate gyrus (Doetsch et al., 1999; Merkle and Alvarez-Buylla, 2006; Pastrana et al., 2009; Seri et al., 2001). Despite the importance of these cells, up to now no antibody has been available, which would allow labeling of living cells, and thus the purification of functional astrocytes. Most of the commonly used markers for astroglial cells are not located on the cell surface, including the glial fibrillary acidic protein (GFAP), brain lipid binding protein (BLBP), S100beta, aldehyde dehydrogenase 1 family member L1 (Aldh1L1), vimentin, glycogen granules, nestin, radial glia marker 2 (RC2), and glutamine synthetase (GS). A variety of astrocyte markers is expressed on the cell surface, such as the L-glutamate/L-aspartate transporter (GLAST, EAAT1, Slc1a3), L-glutamate transporter-1 (GLT-1, EAAT2, Slc1a2), Aquaporin-4 (Aqp4), or the extracellular matrix molecule Tenascin-C (TN-C). However, no antibody reacting with the respective extracellular domain has been reported. Accordingly, transgenic fluorescent pro-

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teins expressed under the control of the promoter for S100beta (Vives et al., 2003; Zuo et al., 2004), GFAP (Nolte et al., 2001, Zhuo et al., 1997), Glt-1 (Regan et al., 2007), Aldh1L1 (Cahoy et al. 2008), or the BLBP-Cre (Anthony et al., 2004), GLAST-CreER^{T2} (Mori et al., 2006; Slezak et al., 2007) as well as the Cx30-CreER^{T2} line (Slezak et al., 2007) have been used for imaging, flow cytometry–based cell sorting, or lineage tracing. However, transgenic lineage tracing should be interpreted with caution due to a number of critical aspects: (i) insufficient understanding of the transgenes' behavior, (ii) lack of correlation between inserted transgenes and endogenous expression of the corresponding gene, (iii) early-onset constitutive expression of markers in progenitor cells, and (iv) prolonged stability of fluorescent proteins (Nolte et al., 2001).

Among astrocyte markers, GLAST shows the most widespread expression in astrocyte subsets, i.e., most, if not all, quiescent and reactive astrocyte subpopulations, including Bergmann glia cells in the cerebellum, radial glia cells in the dentate gyrus and subventricular zone, and the retinal Müller glia cells (Barry and McDermott, 2005; Hartfuss et al., 2001; Shibata et al., 1997; Storck et al., 1992; Williams et al., 2005). GLAST-1 (EAAT1, Slc1a3) is one of five members of the excitatory neurotransmitter transporters in the central nervous system (Storck et al., 1992). It is a highly conserved integral membrane glycoprotein, which is located in the plasma membrane of glial cells regulating the concentration of the excitatory neurotransmitter L-glutamate.

Here, we report the use of a new monoclonal antibody, which recognizes an extracellular domain of GLAST. We show that the new antibody can be used in flow cytometry, immunohistochemistry, immunocytochemistry, and western blot analysis and is cross-reactive with the human, mouse, and rat GLAST orthologs. Using the new antibody, we could delineate the expression of GLAST on different subsets of astrocytes in different regions of the brain. Finally, we established a new method to enrich astrocytes using a magnetic cell sorting approach based on MACS® Technology. We show that the isolated astrocytes can be cultured for downstream applications. In summary, the new antibody is a widely applicable tool to study different astrocyte subpopulations, including progenitor cells.

MATERIALS AND METHODS

Animals

GLAST1 knockout mice (female, 6–8 week-old) were used for immunization (Stoffel et al., 2004). For screening and evaluation of the Anti-GLAST antibody CD1 wild-type mice and rats of different ages (P1–P10) were used. The animals were maintained under specific pathogen-free conditions according to the recommendations of the Federation of European Laboratory Animal Science Association. All experiments were performed in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Generation of Stable Transfectants Expressing GLAST

Total RNA was isolated from P1 mouse brain and cDNA was produced by reverse transcription. Mouse GLAST (EAAT1, Slc1a3) (Accession No. NM_148938) was generated by polymerase chain reaction (PCR). A cDNA fragment coding for GLAST was prepared, inserted into an expression vector, and introduced into 1881 cells by electroporation. Cells were grown in the presence of antibiotics to obtain resistant clones. Stable subclones were isolated by limiting dilution.

Generation of a Monoclonal Anti-GLAST Antibody

Four female GLAST1 knockout mice were immunized six times with 5×10^6 cells by contralateral footpad immunization. Transfected 1881 cells expressing GLAST served as immunogen, whereas untransfected 1881 cells were used as decoy.

Lymph node cells isolated from the immunized animals were fused with Sp2/0 myeloma cells in the presence of polyethylene glycol. The fusion resulted in ~1,000 hybridoma cell clones, which were screened for specific GLAST antigen recognition. Positive clones were further cultivated, expanded, and subcloned by limiting dilution.

The antibody is now available at Miltenyi Biotec (Anti-GLAST (ACSA-1)).

Tissue Dissociation

Brains from P1–P10 CD1 mice or P7 rats were removed. For flow cytometric analysis of GLAST expression in different brain regions, cortical hemispheres or cerebellum were dissociated separately. Retinae were obtained from P6–8 rats.

Tissue was dissociated using the MACS® Neural Tissue Dissociation Kit (T) (Miltenyi Biotec) in combination with the gentleMACS™ Dissociator (Miltenyi Biotec) according to the manufacturer's instructions. As the GLAST epitope detected by ACSA-1 shows papain sensitivity, a trypsin-based enzymatic tissue dissociation was crucial. Dissociated retinae were incubated for 35 min on a panning plate coated with a goat anti-rabbit IgG to remove microglia.

Flow Cytometric Analysis

Single-cell suspensions obtained from different regions of CD1 mouse brain tissue were co-stained with the generated Anti-GLAST antibody conjugated to APC and Anti-CD11b-PE, Anti-A2B5-PE, Anti-AN2-PE, Anti-O4-PE, and Anti-PSA-NCAM-PE (all antibodies from Miltenyi Biotec) for flow cytometric analysis. To avoid false-positive staining due to Fc receptor interactions, the FcR Blocking Reagent, mouse (Miltenyi Biotec) was

always applied before antibody labeling. Cell debris and dead cells (identified by propidium iodide) were excluded from the analysis. Data was acquired on a MACSQuant® Analyzer (Miltenyi Biotec) or a FACSCalibur™ flow cytometer (BD Biosciences).

Magnetic Labeling and Cell Isolation

Cell suspensions containing $\sim 1 \times 10^7$ cells were first labeled with the Anti-GLAST antibody (ACSA-1) conjugated to biotin. Then, superparamagnetic MicroBeads coupled to an Anti-Biotin antibody were applied. Before antibody labeling, nonspecific binding to the Fc receptor was blocked using the FcR Blocking Reagent, mouse (Miltenyi Biotec). Cells were resuspended in PBS with 0.5% BSA and the cell suspension was loaded onto an MS Column (Miltenyi Biotec), which was placed in the magnetic field of a MiniMACS™ Separator (Miltenyi Biotec). The magnetically labeled GLAST-positive cells were retained within the column and eluted as the positively selected cell fraction after removing the column from the magnet.

For cultivation, isolated GLAST-positive cells were seeded onto poly-D-lysine–coated glass coverslips and maintained in a humidified atmosphere (5% CO_2, 95% air) at 37°C for 1 week using MACS® Neuro Medium (Miltenyi Biotec) supplemented with MACS B27 PLUS (Miltenyi Biotec) and L-glutamine (0.5 mM, Invitrogen). Cultures were then fixed with 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 20 min at 4°C.

GLAST-positive cells isolated from rat retinae were cultured for the indicated periods of time onto PDL-coated 96-well imaging microplates (BD Biosciences) in MACS Neuro Medium (Miltenyi Biotec) supplemented with L-glutamine (2 mM, Invitrogen) and fetal calf serum (10%, Sigma).

In vivo Electroporation

In vivo electroporation was carried out in P1 mouse pups as described by Boutin et al. (2008). The pups were injected with a pCAAGS-derived vector, pCX (Morin et al., 2007), carrying tandem-Tomato (tdTomato) (Shaner et al., 2004). The animals were perfused one day post-electroporation and brains were treated for immunohistochemistry.

Immunohistochemistry

Immunohistochemistry was performed either on cryosections or floating sections. Cryosections of human cerebellum and rat brain were obtained from BioChain and Zyagen, respectively. Animals were euthanized and transcardially perfused with 4% PFA in PBS. Brains were postfixed in 4% PFA in PBS overnight and were either directly sectioned at 50μm with a vibratome (Leica) or cryoprotected in PBS-sucrose, frozen and sectioned with a cryostat.

Sections were first incubated overnight at 4°C with the following primary antibodies against GLAST (ACSA-1, mouse IgG2a), GFAP (rabbit polyclonal, DAKO or mouse IgG1, Millipore), and PSA-NCAM (mouse IgM, Miltenyi Biotec), NG2 (rabbit polyclonal, Chemicon), and carbonic anhydrase II (CAII) (rabbit polyclonal, Abcam). After several rinses, sections were incubated for 2–3 hours with the corresponding fluorescently labeled secondary antibody and viewed under a fluorescence microscope (Axioplan2, Apotome System or Z510 Confocal microscope, Zeiss).

Preparation of Primary Neural Cell Culture and Immunocytochemistry

Neural cells were obtained from 1- to 3-day-old neonatal mice. Mice were decapitated, the brain was removed and transferred to HBSS (w/o Ca^{2+}, Mg^{2+}) (Lonza) with 10 mM HEPES (Lonza). After peeling off the meninges, tissue was dissociated by using the MACS Neural Tissue Dissociation Kit (P) (Miltenyi Biotec) in combination with the gentleMACS Dissociator (Miltenyi Biotec). Cells were seeded onto poly-D-lysine– or poly-L-lysine–coated glass coverslips and cultivated at 37°C for 1 or 2 weeks *in vitro*. For cultivation MACS Neuro Medium (Miltenyi Biotec) supplemented with MACS B27 PLUS (Miltenyi Biotec) and L-glutamine (Invitrogen) was used.

For immunostaining primary antibodies against GLAST (ACSA-1, mouse IgG2a), GFAP (mouse IgG1, Millipore), Brain Lipid-Binding Protein (BLBP) (rabbit polyclonal, Abcam), RC2 (mouse IgM, Millipore), Glutamine Synthetase (GS) (mouse IgG1, Abcam, or rabbit polyclonal, Sigma), Myelin Oligodendrocyte Glycoprotein (MOG) (mouse IgG1, Millipore), O4 (mouse IgM, Miltenyi Biotec), Nestin (mouse IgG1, Gentaur), PSA-NCAM (mouse IgM, Miltenyi Biotec), NeuN (mouse IgG1, Millipore), Microtubuli-Associated Protein 2 (MAP2) (rabbit polyclonal, Millipore), and Neurofilament 160 (mouse IgG1, Sigma) were applied overnight at 4°C or for 3 h at room temperature. After rinsing with PBS, samples were incubated for 3 h at 4°C or 1 h at room temperature with the corresponding secondary antibodies (Invitrogen). Cover slips were mounted onto glass slides using fluorescence mounting medium (Dako) and samples were analyzed by confocal fluorescence microscopy (Leica TCS SP2).

[^{3}H]-D-Asp Uptake Assay

To explore functional effects of ACSA-1, we used HEK293 cells stable expressing EAAT1, EAAT2 or EAAT3 and the [^{3}H]-D-Asp uptake assay (Jensen and Brauner-Osborne, 2004). Cells were split into 96-well plates (Greiner-Bio-One GmbH) in DMEM-GlutaMAX-1 (Invitrogen) supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL), 5% dialyzed fetal bovine se-

rum (Invitrogen), and 1 mg/mL G-418 (Invitrogen). After 16–24 h the medium was aspirated, and cells were washed twice with assay buffer (75 μL/well), Hanks Buffered Saline Solution supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (Invitrogen). Then 50 μL assay buffer supplemented with radioligand and various concentrations of ACSA-1 or L-glutamate was added to each well, and the plate was incubated at 37°C for 6 min. Scintillation fluid (Perkin–Elmer) was added to each well. The plate was shaken for at least 1 h before corrected counts per minute (CCPM) were quantified using a Wallac 1450 MicroBeta® TriLux Liquid Scintillation Counter (Perkin–Elmer). All experiments were performed twice in duplicate. [^{3}H]-D-Asp (Amersham) was used at a 30 nM tracer concentration. L-Glutamate was used as control. Concentration/inhibition curves were generated by nonweighted least-squares fits using GraphPad Prism® 5.0.

RESULTS

Generation of a Monoclonal Anti-GLAST Antibody

Mouse GLAST1 shares a high overall amino acid sequence identity with human and rat GLAST1 (96.5% and 98.7%, respectively). This makes it difficult to obtain high-affinity antibodies, as the immunologic tolerance to similar "self" components leads to only weak immune responses. One way to overcome this limitation is to immunize a genetically modified host such as knockout mice, which do not recognize the respective knocked out antigen as self, and thus are able to produce a strong antibody-mediated immune response. Following this strategy, we immunized GLAST1 knockout mice (Stoffel et al., 2004). Hybridoma cell clones were screened by flow cytometry for specific GLAST antigen recognition using dissociated brain tissue obtained from P7–P10 mice and GLAST-expressing 1881 cells. The mouse IgG2A clone MJu4-7D1 showed specific staining, was subcloned by limiting dilution, and renamed ACSA-1 (astrocyte cell surface antigen-1).

Specificity of ACSA-1

We first assessed the specificity of the antibody by immunohistochemical staining of sagittal brain sections derived from P1 wild-type and GLAST1 knockout mice. In case of wild-type mice, GLAST immunoreactivity was detected throughout the brain (Fig. 1A,B). In contrast, no staining was observed in brain sections derived from GLAST1 knockout mice (Fig. 1C).

To investigate whether ACSA-1 could be used for labeling live astrocytes, we performed flow cytometric analysis of dissociated brain tissue from wild-type and GLAST1 knockout mice (P6). Cells were neither fixed nor permeabilized before analysis to restrict antibody binding to extracellular epitopes. Unequivocal staining could be detected in wild-type mice (Fig. 1D), whereas no staining of ACSA-1 was observed on brain cells derived from GLAST1 knockout mice (Fig. 1E). Finally,

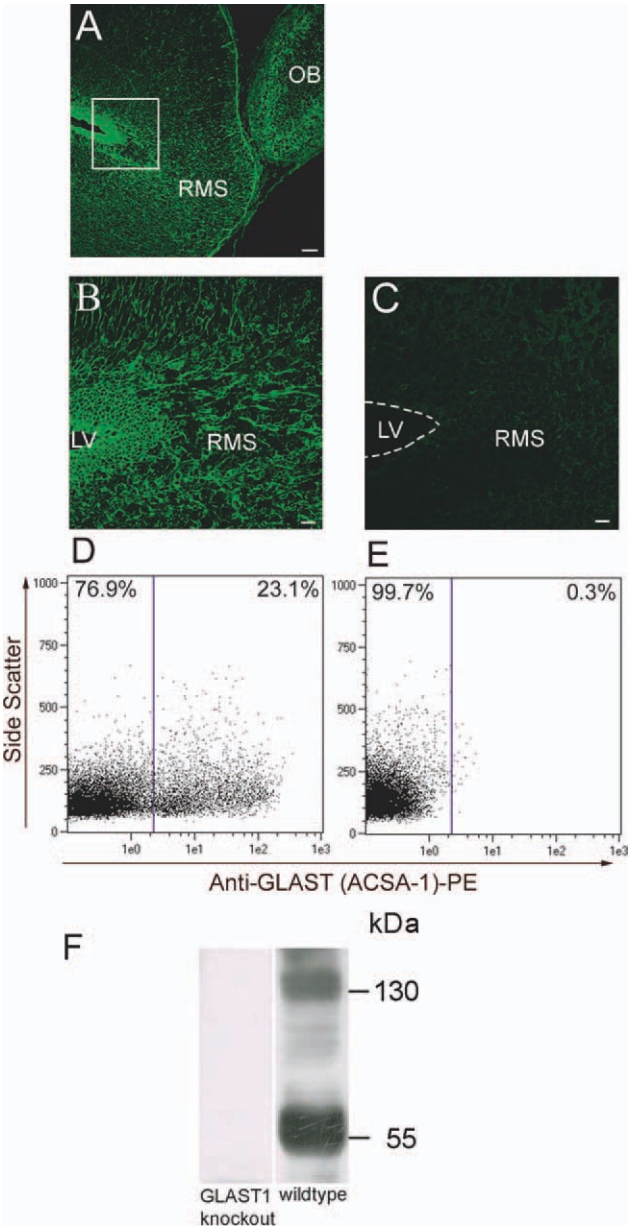

Fig. 1. Specificity of the Anti-GLAST (ACSA-1) antibody. Sagittal cryosections from 1-day-old wild-type and GLAST1 knockout mice were stained with the Anti-GLAST (ACSA-1) antibody. Bright staining was visible in case of wild-type mice (**A** and **B**) and a dense layer of GLAST-positive cells lined the LV. Details (box in A) are shown in B. No GLAST immunoreactivity was detected in brain sections from GLAST1 knockout mice (**C**). Scale bars represent 100 μm (A) or 20 μm (B, C). Flow cytometry of dissociated brain tissue derived from wild-type or GLAST1 knockout mice revealed 23% GLAST-positive cells in wild-type mice (**D**) and no positive cells in GLAST1 knockout mice (**E**). Western blot analysis showed two bands corresponding to the GLAST-monomer and dimer in case of wild-type mice. No corresponding signal was detected in GLAST1 knockout mice (**F**). LV: lateral ventricle, RMS: rostral migratory stream, OB: olfactory bulb. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

we tested the specificity of ACSA-1 by western blot analysis of total brain protein derived from wild-type and GLAST1 knockout mice. Two bands corresponding to the GLAST-monomer and to the dimer were detected in wild-type mice, whereas no corresponding signal was

observed in case of GLAST1 knockout mice (Fig. 1F, Supp. Info. Fig. 1). Taken together, these results demonstrate that ACSA-1 specifically detects an extracellular domain of the native as well as denaturated GLAST protein.

GLAST Expression in Different Mouse Brain Regions

We analyzed GLAST expression in the adult mouse brain. In general, GLAST-positive cells could be detected in all brain areas, with highest expression levels in the cerebellum (Fig. 2A–F). Strong ACSA-1 immunoreactivity was visible in the molecular layer of the cerebellum, extending into the Purkinje cell layer (Fig. 2A,D). In both areas GLAST immunoreactivity was in close proximity to GFAP-positive processes (Fig. 2B). Altogether, this pattern was in agreement with a strong expression of GLAST in Bergmann glia located in the Purkinje cell layer and extending processes through the molecular layer to the pial surface. However, a distinct astrocytic morphology of the GLAST-positive cells was not evident due to the dense network of processes and the membrane localization of the protein. In contrast to the peripheral cerebellar layers, the centrally positioned granule cell layer and the white matter tracts, containing climbing and mossy fibers, expressed lower amounts of GLAST while showing stronger GFAP expression (Fig. 2 A,B,D–F).

Next, we investigated GLAST expression in the forebrain (Fig. 2G–I). In the cerebral cortex, punctate GLAST expression was homogeneously distributed throughout all cortical layers (Fig. 2G). A clear correlation with the individual GFAP-positive astrocytes that are sparsely present throughout the cortical layers was not obvious. (Fig. 2H,I; arrows).

In the corpus collosum (Fig. 2J–O), GLAST was neither expressed by oligodendrocyte progenitors expressing NG2 (Fig. 2J–L), nor by mature oligodendrocytes as identified by expression of carbonic anhydrase II (CAII, Fig. 2M–O). Moreover, CAII positive oligodendrocytes in the cortex were also negative for GLAST (Fig. 2P–R). Since GLAST is expressed in radial glia type neuronal progenitors in the adult rodent brain (Gubert et al., 2009), we investigated ACSA-1 immunoreactivity in two neurogenic brain regions, the periventricular zone (PVZ) of the LV that generates inhibitory neurons for the olfactory bulb, and the hippocampal dentate gyrus that produces glutamatergic neurons.

In the wall of the LVs, GLAST and GFAP were widely expressed and showed a partly overlapping distribution (Fig. 3A–C). We used postnatal *in vivo* brain electroporation to label neurogenic radial glia cells in the mouse brain via expression of the fluorescent protein tdTomato (Boutin et al., 2008). One day postelectroporation tdTomato positive radial glia cells (arrow, Fig. 3B,C) expressed both GLAST and GFAP. Altogether, these data show that ACSA-1 identifies neural stem cells in the periventricular region of the postnatal forebrain.

After their generation along the walls of the LVs, neuronal precursors perform chain migration via the rostral migratory stream (RMS) into the OB, where they differentiate into local interneurons (for review, see: Whitman and Greer, 2009). During this migration they travel through a scaffold of astroglial tunnels that regulate their migration and survival (Platel et al., 2010). In agreement with this, tunnel astrocytes were strongly labeled by ACSA-1 throughout the RMS (Fig. 3D). In contrast, the chains of PSA-NCAM–expressing, migrating neuroblasts (Fig. 3E,F) were negative for GLAST, despite the fact that they derived from GLAST-positive neural stem cells in the PVZ.

In the second adult neurogenic region, the dentate gyrus of the hippocampus, GLAST was strongly expressed throughout the granule cell layer (GCL) (Fig. 3G–I) and was loosely associated to the fibrous, radially orientated GFAP-positive processes (Fig. 3G) in agreement with previous observations (Brunne et al., 2010). However, in addition to this glial localization, individual newly generated neurons, identified by the expression of the immature neuronal marker PSA-NCAM (Fig. 3H, arrow), were also GLAST-positive. This is in agreement with the glial nature of hippocampal neuronal progenitors (Liu et al., 2010).

In conclusion, detailed analyses of GLAST expression in the brain shows that the transporter is expressed by a wide phenotypic spectrum of astrocytes, but absent from mature neurons and oligodendrocytes.

Expression of GLAST on Primary Cultured Neural Cells

To monitor GLAST expression in cultured astrocytes, 1- or 2-week-old primary P1 mixed cell cultures were stained with antibodies reactive for GLAST, GFAP, BLBP, RC2, GS, MOG, O4, PSA-NCAM, NeuN, MAP2, and Nestin. Co-staining for GLAST and GFAP demonstrated that all GFAP-positive astrocytes were recognized by ACSA-1 in 7-day-old cell cultures (Fig. 4A). ACSA-1 immunoreactivity was clearly located to the cell surface and surrounded the intracellular GFAP staining. It nicely revealed the typical branched astrocyte morphology including very fine processes that are devoid of GFAP. We could not detect a gradient of ACSA-1 reactivity on distinct parts of the cell membrane pointing to an evenly distributed expression of GLAST. Notably, while all cultured astrocytes were positive for GLAST and GFAP after 7 days, we observed $GFAP^{-}/GLAST^{+}$ cells in 14-day-old cultures (Fig. 4B). It has been previously reported that primary cultures of astrocytes from neonatal rodent brain represent a GFAP-positive subpopulation of proliferating glial precursors with an immature or reactive phenotype (Bachoo et al., 2004). The appearance of $GFAP^{-}/GLAST^{+}$ cells after 14 days in culture raised the possibility that these astrocytes were no longer reactive due to stable culture conditions for a longer period of time. To further delineate the nature of the cultured astro-

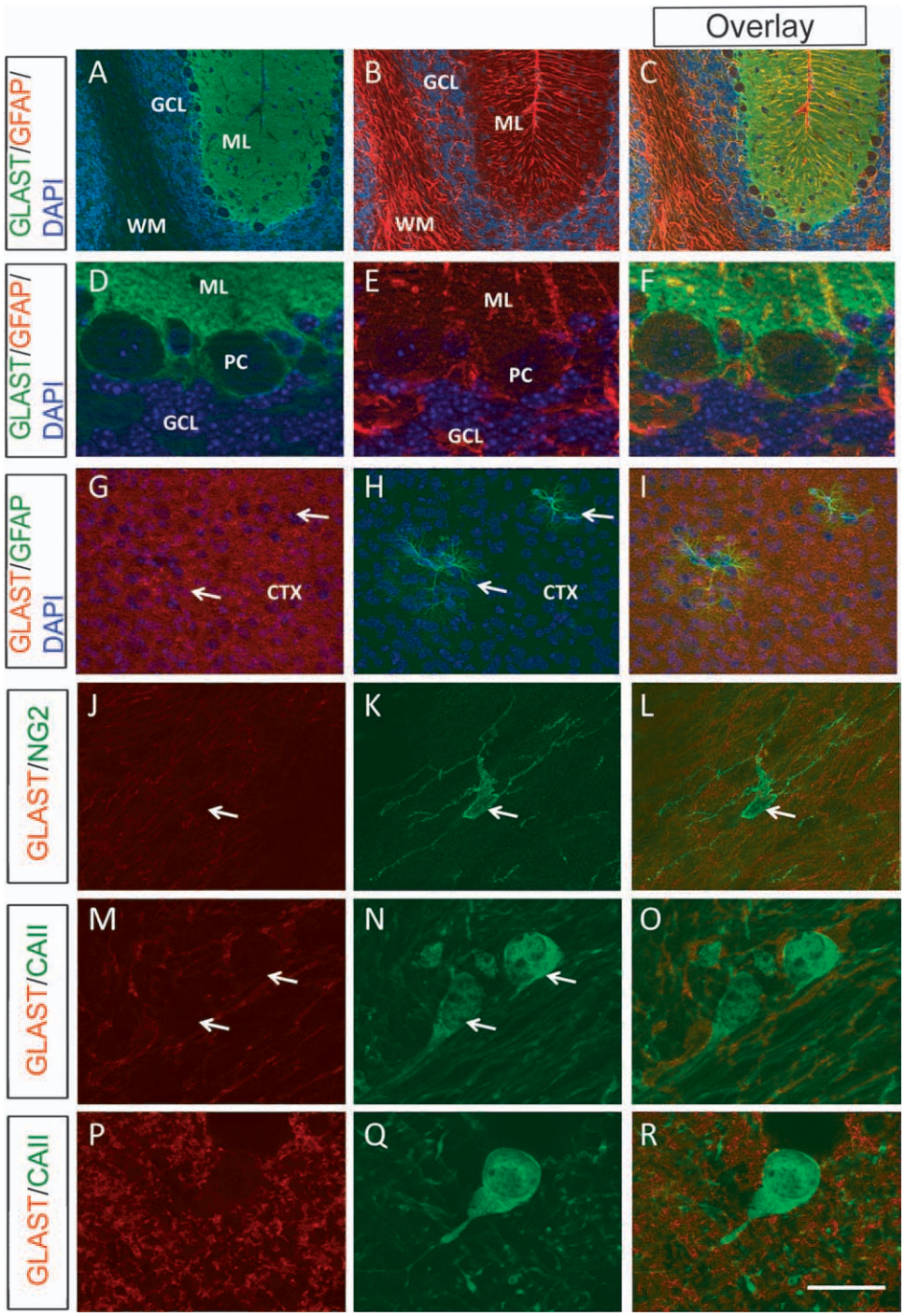

Fig. 2. Expression of GLAST in the cerebellum and forebrain. In the cerebellum (**A**–**F**, high magnification of A–**C** shown in **D**–F), strongest expression of GLAST was detected in the molecular layer (ML) (A, D) overlaying the radially organized GFAP-positive fibers of Bergman glia (**B**, C). Weaker expression of GLAST was found in the granule cell layer (GCL) and the centrally positioned white matter tracts (WM) (A). Purkinje cell (PC) bodies were embedded in GLAST immunoreactive structures, but appeared to be negative for the glutamate transporter (D). In the cortex (CTX) (**G**–**I**) GLAST expression was found throughout all layers with no particular preference (G). Individual GFAP positive protoplasmic astrocytes (H) showed no particular correlation with the local presence of GLAST. Oligodendrocytes precursors labeled with anti-NG2 in the corpus callosum (**K**) did not show any staining with anti-GLAST (**J**–**L**) nor the mature oligodendrocytes labeled with anti-CAII (**N**, **Q**) in the corpus callosum (**M**–**O**) or in the cortex (**P**–**R**). Scale bars represent 150 μm (A–C), 10 μm (D–F and M–R), 100 μm (G–I), or 25 μm (J–L). WM: white matter, ML: molecular layer, GCL: granule cell layer, PC: Purkinje cell, CTX: cortex. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

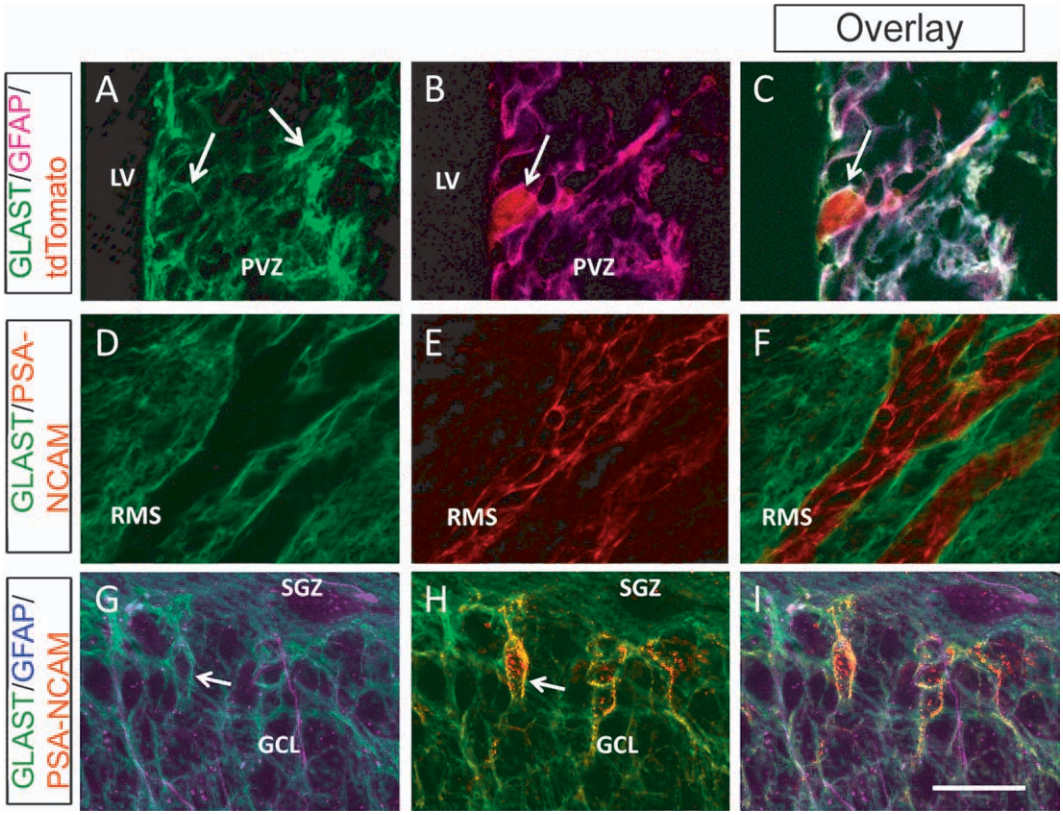

Fig. 3. Expression of GLAST in neuronal progenitor populations. Radial Glia type neural stem cells labeled by *in vivo* brain electroporation with the fluorescent protein tdTomato (red cells) showed strong expression of both GLAST (**A**) and GFAP (**B**). Along the lateral ventricles (LV, A–**C**) GLAST was expressed throughout the periventricular zone (PVZ), thereby correlating with the expression of GFAP (see merge in C). In the rostral migratory stream (RMS) (**D**–**F**) GLAST expression was confined to tunnel astrocytes (D), but was totally excluded from PSA-NCAM-positive, chain-migrating neuronal precursors (E, F). In the hippocampal dentate gyrus (**G**–**I**) GLAST was strongly expressed throughout the granule cell layer (GCL) and closely associated with radially oriented GFAP-positive fibers (G). Individual newly generated neurons, expressing the early neuronal marker PSA-NCAM (H), showed also co-expression of GLAST (H, I). Scale bar represents 15 μm (A–C), 25 μm (D–I). LV: lateral ventricle, GCL: granule cell layer, RMS: rostral migratory stream, SGZ: subgranular zone. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cytes, we performed co-stainings with additional astrocytic markers, such as BLBP, RC2, GS, and Nestin. All BLBP-, RC2-, and GS-positive cells with astrocyte morphology were detected by the ACSA-1 antibody (Fig. 4C–H). Most of the GLAST-positive cells showed expression of Nestin after 7 days *in vitro* (*div*). However, after 14 days only a minority of GLAST-positive cells expressed Nestin (Fig. 4I,J), which is consistent with the reduced GFAP expression described above. We found no co-localization with the oligodendrocyte specific markers MOG and O4 (Fig. 4K–N). Furthermore, we could not detect any double immunoreactivity for GLAST and markers for neuronal progenitor cells (PSA-NCAM) as well as young (NeuN) and adult neurons (MAP2) (Fig. 4O–T).

Taken together, in cultured neural cells GLAST expression, as revealed by ACSA-1, was detected in virtually all cells showing the typical astrocyte morphology and/or expression of any of the common astrocyte markers. It showed a broader expression than the classic astrocyte marker GFAP and was not found on other major brain cell types.

Flow Cytometric Characterization of GLAST-Positive Cells in the Postnatal Mouse Brain

Flow cytometry allowed us to quantify the proportion of GLAST-expressing cells in the different neural cell populations. Cortical hemispheres or cerebellum of P1, P3, and P7 mice were dissociated and the resulting cells were co-stained with ACSA-1 and markers specific for glial precursor cells (A2B5), oligodendrocyte progenitor cells (AN2), oligodendrocytes (O4), microglia (CD11b), and neuronal precursor cells (PSA-NCAM). Percentages of single as well as double-positive cells are indicated in the dot plots in Fig. 5A,B.

The total percentage of GLAST-positive cells ranged from 13 to 26%, depending on the brain region and the age of mice ($n = 3$) (Fig. 5A–C). A subpopulation of GLAST showed coexpression of the glial progenitor marker A2B5, resulting in a percentage of 3.5–4.5% GLAST/A2B5 double-positive cells in cortical hemispheres and cerebellum of P1 mice, while 9–22% GLAST single-positive and 8–14.5% A2B5 single-positive were detected. The percentage of GLAST/A2B5 double-posi-

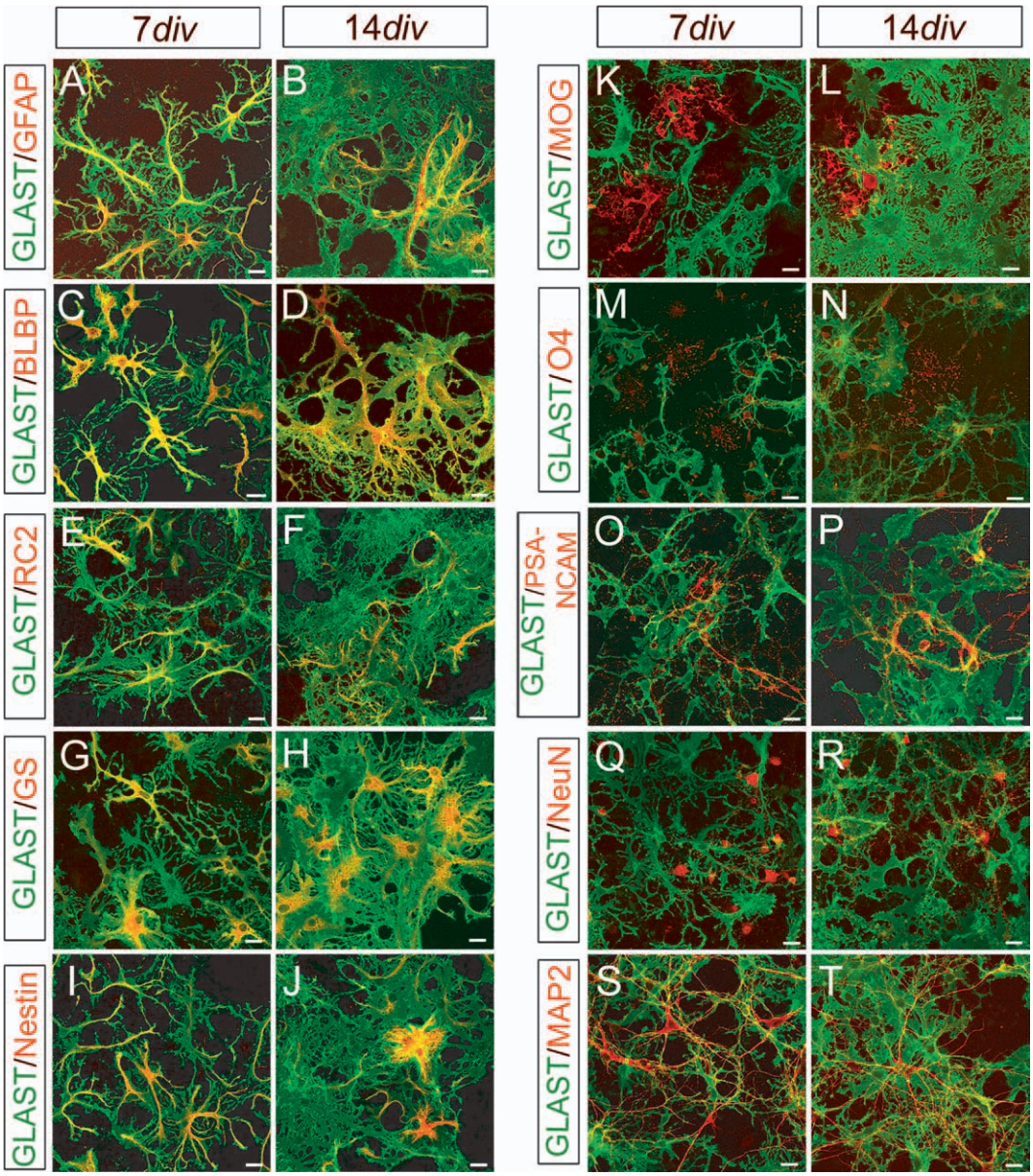

Fig. 4. GLAST expression in 7- or 14-day-old P1 neural cell cultures. Fluorescence images showing double staining of GLAST (green) and different neural cell markers (red). All GFAP-positive (**A**) cells were stained with the Anti-GLAST (ACSA-1) antibody in 7-day-old cell cultures. In contrast to GLAST, GFAP expression decreased after 14 *div* and only a minority of GLAST-positive cells were found to express GFAP (**B**). All BLBP, RC2, and GS-positive cells were detected by the ACSA-1 antibody (**C**–**H**). The majority of the GLAST-positive cells showed expression of Nestin in 7-day-old cell cultures (**I**), but only some astrocytes were Nestin-positive after 14 *div* (**J**). No GLAST labeling was observed in cells that were positive for MOG (**K**, **L**), O4 (**M**, **N**), PSA-NCAM (**O**, **P**), NeuN (**Q**, **R**), or MAP2 (**S**, **T**). Scale bars represent 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

tive cells decreased to 0.5–2% at 7 days of age in agreement with the decrease of A2B5-positive progenitor cells ($n = 3$) (Fig. 5A–C). In addition, A2B5 expression partly overlaps with AN2, a marker for oligodendrocyte precursors, whereas no GLAST immunoreactivity was detected in AN2 positive cells (Fig. 5A). This proves that ACSA-1 does not detect A2B5/AN2 double-positive oligodendrocyte precursor cells. The existence of cells showing co-expression of GLAST and A2B5 indicates that a subpopulation of A2B5 positive cells are astrocyte precursors and A2B5 expression is not only restricted to the oligodendrocyte precursors. This hypothesis is further supported by the fact, that only a certain proportion of A2B5 positive cells shows AN2 expression.

Furthermore, no significant ACSA-1 immunoreactivity was detected in O4-positive oligodendrocytes, PSA-NCAM-positive neuronal progenitor cells or CD11b-positive microglia (Fig. 5A,B), confirming that GLAST expression is restricted to the astrocyte lineage.

In addition, we analyzed the presence of GLAST-positive cells in various dissociated mouse organs from adult animals, including spleen, heart, kidney, and skin, and in

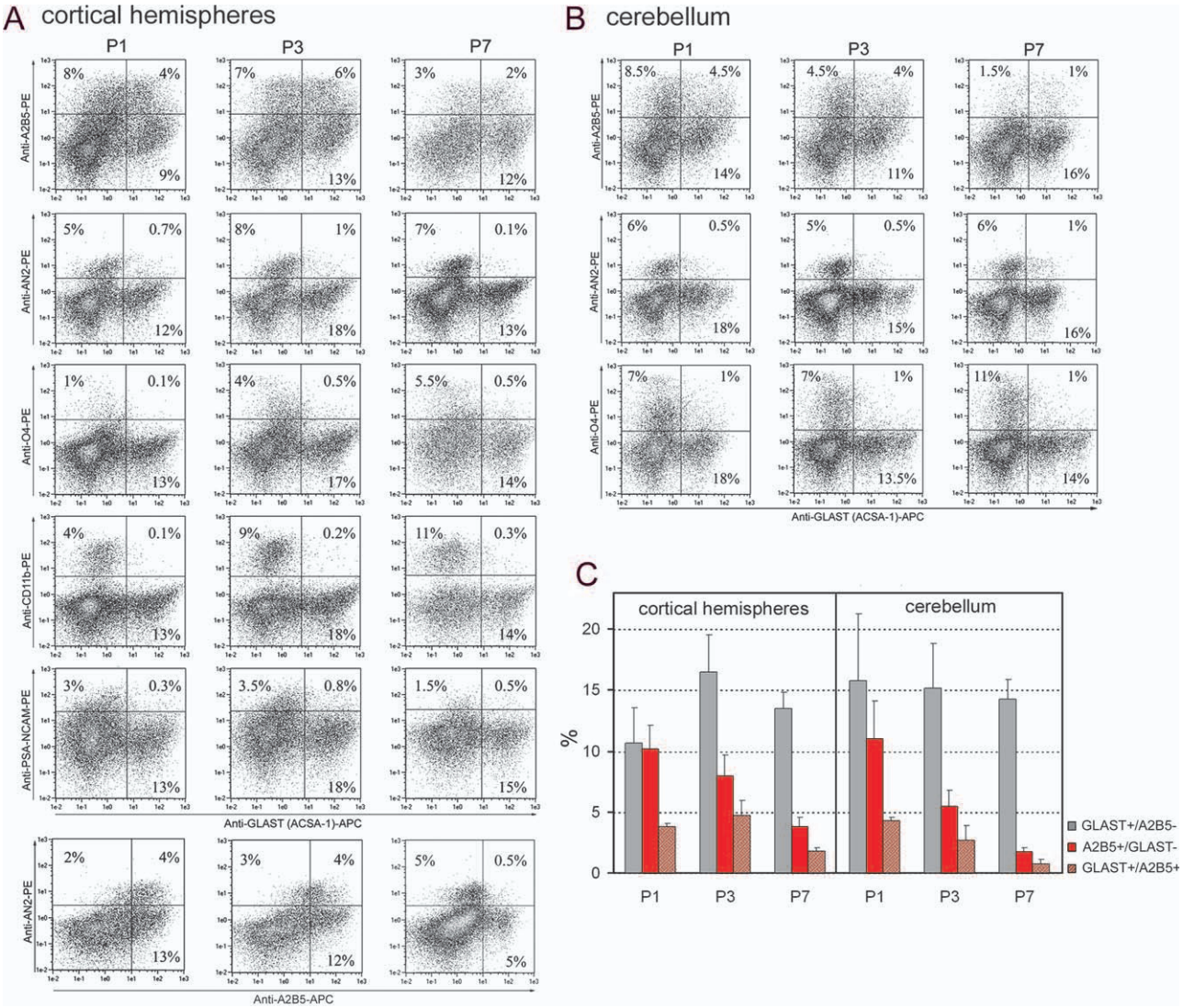

Fig. 5. Expression of different neural cell markers in P1, P3, P7 cortical hemispheres and cerebellum. Coexpression of GLAST and A2B5, AN2, or O4 in cells from dissociated cortical hemispheres (**A**) or cerebellar tissue (**B**) from P1, P3, and P7 mice was determined by flow cytometry. Only viable cells were included in the analysis and total percentages of positive cells are indicated in the dot plots. Co-staining of GLAST and A2B5 clearly showed a double-positive cell population as well as GLAST and A2B5 single-positive cells. Co-staining was also observed for A2B5 and AN2, but the percentage of double-positive cells clearly decreased in older mice. Flow cytometry showed no significant GLAST/AN2, GLAST/O4, GLAST/CD11b, and GLAST/PSA-NCAM double-positive cell populations. A very small percentage of double-positive cells was always detected, presumably due to weak nonspecific antibody binding. Based on flow cytometric analysis, percentages of GLAST single-positive, A2B5 single-positive, and GLAST/A2B5 double-positive cells were determined (**C**). Percentages of cells differed depending on the age of mice. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

human peripheral blood mononuclear cells (PBMC). Flow cytometric analysis did not indicate GLAST expression on any of these organs or cell types (data not shown).

ACSA-1 Cross-Reacts with Human and Rat GLAST

Based on the high amino acid sequence identity of mouse, rat, and human GLAST we hypothesized that ACSA-1 was cross-reactive for all three species. To test this hypothesis, cerebellar section of the adult human and rat brain were analyzed by immunohistochemical staining. Strong staining was observed in the molecular layer, especially in the Purkinje cell layer, which contains Bergmann glia. The staining intensity was much weaker in the granule cell layer and the white matter (Fig. 6A). In comparison to GLAST, GFAP staining was more evenly distributed without obvious differences in staining intensities among the different layers. Western blot analysis of total rat and human brain protein revealed different bands, that corresponded to the monomer and dimer (Fig. 6B) and flow cytometric analysis of dissociated P7 rat brain tissue showed 19.5% ACSA-1 positive cells (Fig. 6C).

Furthermore, human induced pluripotent stem (iPS) cells that were previously differentiated into neural stem cells (NSCs) (Koch et al., 2009), were found to sporadically

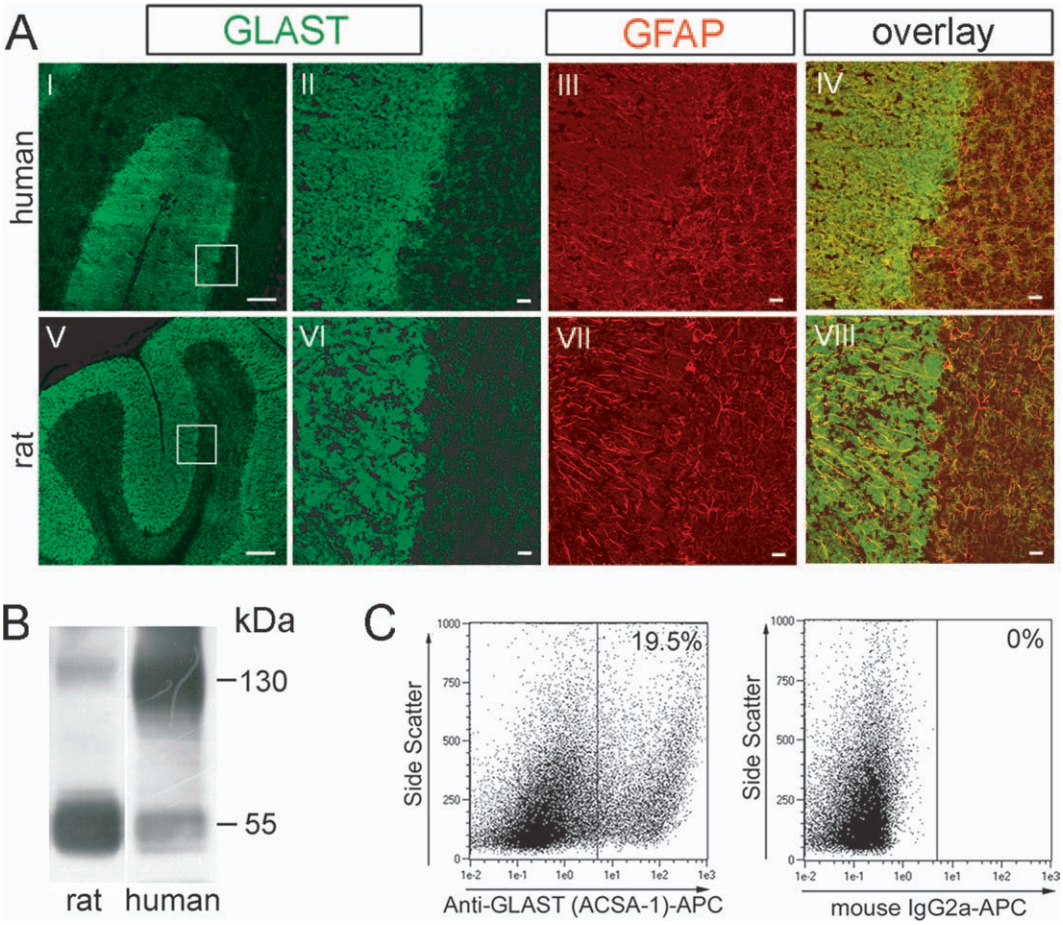

Fig. 6. Cross-reactivity of the Anti-GLAST (ACSA-1) antibody for rat and human GLAST. Staining of human and rat cerebellar frozen sections with the ACSA-1 and Anti-GFAP antibodies resulted in specific labeling of GLAST-positive cells (**A**). GLAST immunoreactivity is depicted in green (I,II,V,VI) and, for comparison, GFAP in red (III,VII). IV, and VIII show the overlay of GFAP and GLAST staining. Details (boxes in I and V) are shown in II-IV and VI-VIII, respectively. Strongest GLAST immunoreactivity was detected in the molecular layer. Western Blot analysis for rat and human brain tissue revealed different bands, corresponding to the GLAST-monomer and dimer (**B**). Flow cytometry of dissociated rat brain showed 19.5% GLAST-positive cells and no staining in the isotype control (**C**). Scale bars represent 200 μm (I,V) or 20 μm (II-IV, VI-VIII). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

acquire a GLAST-positive phenotype under conditions promoting spontaneous differentiation (Supp. Info. Fig. 2). These results demonstrate that the ACSA-1 antibody detects GLAST orthologs from rat and human.

Pharmacological Profile of ACSA-1

The [^{3}H]-D-Asp uptake assay was used to test a potential pharmacological effect of the antibody ACSA-1 on the glutamate/aspartate transporter GLAST (EAAT1). HEK293 cells stably expressing the glutamate transporters EAAT1, EAAT2, or EAAT3 were treated with the radioligand and increasing concentrations of the ACSA-1 antibody or L-glutamate as a control. L-Glutamate application inhibited [^{3}H]-D-Asp uptake and resulted in a dose-dependent decrease of the measured CCPM/well in case of all EAAT subtypes, with different maximal responses and IC50 values between 8 and 62 μM (Fig. 7A). Exposure of nontransfected wild-type HEK293 cells to L-glutamate showed a slight response due to endogeneous EAAT expression in HEK293 cells (IC50 values between 7 and 13 μM).

In contrast, ACSA-1, which was applied at concentrations ranging from 0.1 to 100 μg/mL, had no significant effect on the [^{3}H]-D-Asp uptake, indicating that the antibody does not affect the function of the glutamate/aspartate transporter EAAT1 (Fig. 7B).

ACSA-1 Allows Enrichment of Viable GLAST-Positive Cells by Magnetic Cell Sorting

To purify GLAST-positive astrocytes we devised a procedure where astrocytes are labeled with superparamagnetic MicroBeads and enriched in a magnetic field. Cells were stained with an Anti-GLAST (ACSA-1)-APC conjugate before and after separation to determine the percentage of GLAST-positive cells by flow cytometry.

The percentage of GLAST-positive cells could be increased from 13–20% to 90–95% (n = 5) by magnetic cell separation (Fig. 8A,B). If two consecutive MS

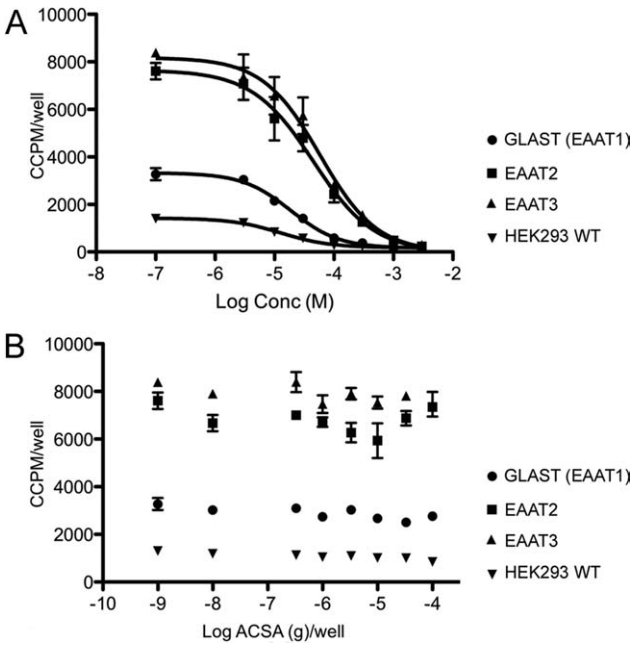

Fig. 7. Pharmacological profile of ACSA-1 in the [^{3}H]-D-Asp uptake assay. Concentration-inhibition curves for the effect of L-glutamate on HEK293 wild-type cells and HEK293 cells stably transfected with EAAT1, EAAT2, or EAAT3 showed a dose-dependent decrease of the CCPM/well with increasing L-glutamate concentrations (**A**). IC50 values ranged between 7 and 62 μM. The presence of ACSA-1 had no significant effect on EAAT1-, EAAT2-, or EAAT3-mediated aspartate uptake (**B**). One representative experiment is depicted.

Columns were used, the purity reached 96–98% (n = 5) (data not shown). Between 300,000 and 600,000 GLAST-positive cells were obtained per P7 CD1 mouse brain (n = 5). Approximately 90% of isolated astrocytes were identified as propidium iodide negative viable cells.

To test whether labeling of cells with ACSA-1 has any influence on astrocytes we cultured GLAST-positive cells for 7 days. Cultured cells showed proliferation and formed a dense layer of GLAST/GFAP double-positive cells (Fig. 8C). Astrocytes were successfully isolated from P1-P10 CD1 mice. For older animals, the presence of cell debris after tissue dissociation negatively influenced the separation performance, reducing the purity of isolated cells. Furthermore, viability of isolated astrocytes decreased by at least 20%.

We next tested whether the GLAST antibody could be used to isolate retinal Müller cells, which express GLAST on their surface (Derouiche and Rauen, 1995). We labeled retinal cell suspensions from 1-week-old rats with ACSA-1. Magnetic cell separation yielded 1.2 ± 0.6 × 10^6 GLAST-positive cells per retina (mean ± SD), which represented 6% ± 3% of the total number of retinal cells (n = 5). Our preparation contained essentially two categories of cells, namely postmitotic cells and cells that divide (Fig. 8D,E). The presence of dividing cells was probably due to the fact that, at this postnatal age, the retina still contains progenitor cells, which give rise to neurons and glia (Rapaport et al., 2004), and which are GLAST-positive. Müller cells and astrocytes were

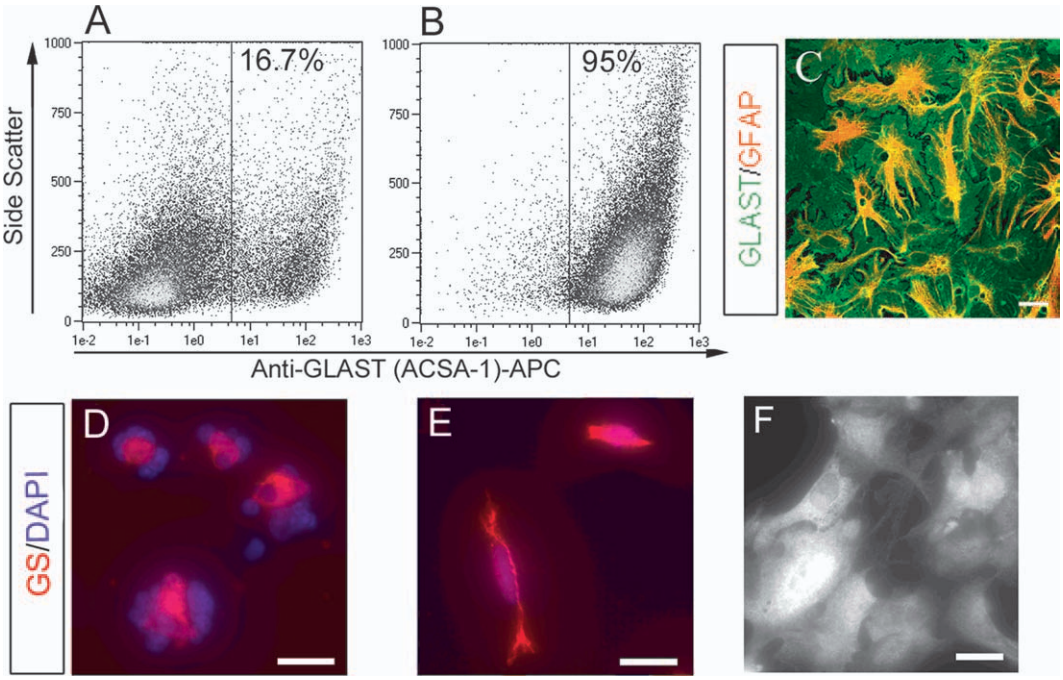

Fig. 8. Immunomagnetic isolation of GLAST-positive cells from dissociated mouse brain tissue and rat retina. GLAST-positive cells were isolated from dissociated P7 mouse brain (**A**–**C**) or retina (**D**–**F**) using the biotin-conjugated ACSA-1 antibody, Anti-Biotin MicroBeads, and an MS Column for magnetic separation. The cell suspension obtained from mouse brain showed a frequency of 16.7% GLAST-positive cells before separation (A), which increased to 95% in the positively selected cells (**B**). Immunostaining of the separated cells after 7 *div* with Anti-GLAST (ACSA-1) (green) and a GFAP-specific antibody (red) revealed a dense layer of GLAST/GFAP-positive cells. The figure shows one representative experiment. Fluorescence micrographs of retinal cells 24 hours (D, **E**) and 5 days (F) after isolation. Cells were stained with the nuclear dye DAPI (blue) and with an antibody against glutamine synthetase (red or white). Scale bars represent 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

identified by glutamine synthase (GS) immunoreactivity (Derouiche and Rauen, 1995). After 24 hours of culture, the percentage of GS-positive cells averaged at 29% ± 1% (n = 3), and their percentage increased after 5 days in culture (Fig. 8F). Staining with an antibody against neurofilaments did not label any cell indicating the absence of neurons. Our results show that the GLAST antibody can be used to enrich retinal Müller cells and astrocytes.

DISCUSSION

In this study, we generated a monoclonal GLAST-specific antibody, which recognizes an extracellular epitope of the mouse, rat, and human astrocyte-specific L-glutamate/L-aspartate transporter. To our knowledge, this is the first report about a monoclonal antibody directed against the extracellular domain of GLAST or any other astrocyte-specific surface marker. Therefore, this antibody opens up new perspectives for the characterization of GLAST-expressing cells. The antibody did not impair transporter function and the cultivation of astrocytes after sorting, thus allowing detailed analysis of astrocytes *in vitro*.

So far, analysis of GLAST localization and properties was based on different polyclonal antipeptide antibodies and RNA expression analysis, giving partly contradictory results. While Lehre et al. (1995) and Chaudhry et al. (1995) described that GLAST expression in the brain is restricted to astrocytes, Rothstein et al. (1994) stated that GLAST is also expressed on neurons, and others found it also expressed on activated microglia (Beschorner et al., 2007; van Landeghem et al., 2001; Xin et al., 2009). Furthermore, several reports emphasize that GLAST is expressed by oligodendrocytes (DeSilva et al., 2009; Domercq et al., 1999, 2005). Using the new antibody ACSA-1, we show that GLAST-positive cells co-express most markers described for astrocytes. In contrast, we did not find any evidence for GLAST expression in neurons, oligodendrocyte progenitor cells, oligodendrocytes, or microglia, neither by immunostaining of primary neural cell cultures or brain sections nor by flow cytometric analysis of dissociated brain tissue.

We also assessed GLAST expression in other organs, and again found no expression on any of the tested organs and cell types. This is in contrast to reports, mentioning GLAST expression on fibroblasts and platelets (Cooper et al., 1998; Zoia et al., 2004, 2005) or the expression of EAAT1 mRNA in different human organs (Arriza et al.,1994).

GLAST is among the best markers for astrocytes and radial glia cells in the developing and adult brain (Barry and Mc Dermott, 2005; Chaudhry et al., 1995; Hartfuss et al., 2001; Shibata et al. 1997; Williams et al., 2005). Nevertheless, the question arises whether GLAST is expressed on all astrocytes or whether it marks a subpopulation of functional distinct cells. Chaudhry et al. (1995) and Lehre et al. (1995) performed careful quantitative immunocytochemistry to analyze the distribution of GLAST in the brain and stated that no astrocytic plasma membrane appeared devoid of labeling. Also quiescent astrocytes in the adult cerebral cortex, which do not proliferate and lack expression of the astrocyte markers GFAP, vimentin, or nestin, are known to be GLAST-positive (Mori et al., 2005). We performed co-labeling with most of the known intracellular astrocyte markers and analyzed their expression by immunohistochemistry and immunocytochemistry. We showed that cells with astrocyte morphology detected by GFAP, GS, BLBP, or RC2 antibodies were GLAST-positive. To our knowledge, ACSA-1 labeling allowed the most comprehensive detection of astrocytes described so far, even exceeding the one found in GLAST::Cre-IRES-hrGFP knock-in mice, where 60–80% of all astrocytes can be labeled due to Cre expression from the GLAST locus. Importantly, our experiments showed that radial glia can be detected by the new antibody. Rodent radial glia, but not neuroepithelial cells, show expression of RC2, BLBP, and GLAST (Feng et al., 1994; Hartfuss et al., 2001; Shibata et al., 1997). Co-expression of tdTomato and GLAST in cells lining the LV after electroporation using a plasmid coding for tdTomato clearly proved that radial glia in the subventricular zone of early postnatal mice were identified by ACSA-1. In addition, flow cytometric data presented in this report provide new insight into the relationship between different glial cell types. The overlap of GLAST and A2B5 expression suggests that a subpopulation of A2B5-positive cells serves as astrocyte precursors, and that A2B5 expression is not restricted to cells committed to the oligodendrocyte lineage. We did not find a specific co-expression of GLAST and AN2, the mouse homolog of the rat NG2 proteoglycan, which labels cells of the oligodendrocyte lineage (Nishiyama et al. 2009). As we also did not find any GLAST and PSA-NCAM double-positive cells in the subventricular zone, it seems that GLAST expression is very tightly down-regulated when cells enter the neuronal lineage.

In summary, addressing the question how an astrocyte can be identified (for review, see: Barres, 2008; Kimelberg, 2004, 2010), we propose ACSA-1 as a general, determining marker for astrocytes. Although traditionally described as a homogeneous cell population, it is now evident that different astrocyte subtypes exist (Lee et al., 2006). Previous investigations proved that astroglia show morphological (Emsley et al., 2006), molecular (Bachoo et al., 2004), as well as functional (Matthias et al., 2003; McKhann et al., 1997; Zhou and Kimelberg, 2000, 2001) diversity. But still relatively little is known about the heterogeneity of astrocytes, due to the lack of tools to identify these cells and separate them from others. So far, direct isolation of astrocytes was only possible using transgenic mice that express a fluorescent protein under the control of an astrocyte-specific promoter in combination with flow cytometry–based cell sorting. However, recombination and expression of the transgene is inherited to all derivative cells, leading to an erroneous detection of false-positive "astrocytes." Moreover, in some cases inconsistencies between the

promoter-linked expression and the endogenously expressed protein were found (Nolte et al., 2001).

In another approach, Foo et al. (2011) recently reported purification of rodent astrocytes based on immunopanning. However, this method takes one day and requires several depletion steps before astrocytes are enriched.

We used the ACSA-1 antibody in a magnetic cell sorting approach, which, for the first time, allowed the direct isolation of viable astrocytes without the need for transgenic animals. Consequently, this antibody is well suited for the separation of astrocytes from other neural cells to further analyze and reveal their various functions.

Taken together, this report provides a comprehensive analysis and description of a novel cell surface marker for astrocytes, suited for immunohistochemistry, immunocytochemistry, flow cytometry, western blot analysis, as well as cell sorting. It can be used to further clarify functions of new types of astrocyte, i.e., reactive astrocytes, neoplastic astrocytes in brain tumors, and astrocytes exerting stem cell functions (Robel et al., 2011). The marker will also facilitate the isolation of astrocytes derived from pluripotent cells as well as direct reprogramming of pure astrocytic cells to neurons (Heinrich et al., 2011). As the ACSA-1 antibody cross-reacts with human GLAST, protocols can potentially be adapted for use with human cells in translational research.

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REFERENCES

Anthony TE, Klein C, Fishell G, Heintz N. 2004. Radial glia serve as neuronal progenitors in all regions of the central nervous system. Neuron 41:881–890.

Arriza JL, Fairman WA, Wadiche JI, Murdoch GH, Kavanaugh MP, Amara SG. 1994. Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. J Neurosci 14:5559–5569.

Bachoo RM, Kim RS, Ligon KL, Maher EA, Brennan C, Billings N, Chan S, Li C, Rowitch DH, Wong WH, DePinho RA. 2004. Molecular diversity of astrocytes with implications for neurological disorders. Proc Natl Acad Sci USA 101:8384–8389.

Barres BA. 2008. The mystery and magic of glia: A perspective on their roles in health and disease. Neuron 60:430–440.

Barry D, McDermott K. 2005. Differentiation of radial glia from radial precursor cells and transformation in astrocytes in the developing rat spinal cord. Glia 50:187–197.

Beschorner R, Simon P, Schauer N, Mittelbronn M, Schluesener HJ, Trautmann K, Dietz K, Meyermann R. 2007. Reactive astrocytes and activated microglial cells express EAAT1, but not EAAT2, reflecting a neuroprotective potential following ischaemia. Histopathology 50:897–910.

Boutin C, Diestel S, Desoeuvre A, Tiveron MC, Cremer H. 2008. Efficient in vivo electroporation of the postnatal rodent forebrain. PLoS One 3:e1883.

Bramanti V, Tomassoni D, Avitabile M, Amenta F, Avola R. 2010. Biomarkers of glial cell proliferation and differentiation in culture. Front Biosci 2:558–570.

Brunne B, Zhao S, Derouiche A, Herz J, May P, Frotscher M, Bock HH. 2010. Origin, maturation, and astroglial transformation of secondary radial glial cells in the developing dentate gyrus. Glia 58:1553–1569.

Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, Xing Y, Lubischer JL, Krieg PA, Krupenko SA, Thompson WJ, Barres BA. 2008. A transcriptome database for astrocytes, neurons, and oligodendrocytes: A new resource for understanding brain development and function. J Neurosci 28:264–278.

Chaudhry FA, Lehre KP, van Lookeren Campagne M, Ottersen OP, Danbolt NC, Storm-Mathisen J. 1995. Glutamate transporters in glial plasma membranes: Highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. Neuron 15:711–720.

Christopherson KS, Ullian EM, Stokes CC, Mullowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA. 2005. Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell 120:421–433.

Cooper B, Chebib M, Shen J, King NJ, Darvey IG, Kuchel PW, Rothstein JD, Balcar VJ. 1998. Structural selectivity and molecular nature of L-glutamate transport in cultured human fibroblasts. Arch Biochem Biophys 353:356–364.

Derouiche A, Rauen T. 1995. Coincidence of L-glutamate/L-asparatate transporter (GLAST) and glutamine synthetase (GS) immunoreactions in retinal glia: Evidence for coupling of GLAST and GS in transmitter clearance. J Neurosci Res 42:131–143.

DeSilva TM, Kabakov AY, Goldhoff PE, Volpe JJ, Rosenberg PA. 2009. Regulation of glutamate transport in developing rat oligodendrocytes. Neurobiol Dis 29:7898–7908.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703–716.

Domercq M, Sánchez-Gómez MV, Areso P, Matute C. 1999. Expression of glutamate transporters in rat optic oligodendrocytes. Eur J Neurosci 11:2226–2236.

Domercq M, Etxebarria E, Pérez-Samertin A, Matute C. 2005. Excitotoxic oligodendrocyte death and axonal damage induced by glutamate transporter inhibition. Glia 52:36–46.

Emsley JG, Macklis JK. 2006. Astroglial heterogeneity closely reflects the neuronal-defined anatomy of the adult murine CNS. Neuron Glia Biol 2:175–186.

Fellin T, Pascual O, Gobbo S, Pozzan T, Haydon PG, Carmignoto G. 2004. Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. Neuron 43:729–743.

Feng L, Hatten ME, Heintz N. 1994. Brain lipid-binding protein (BLBP): A novel signaling system in the developing mammalian CNS. Neuron 12:895–908.

Foo LC, Allen NJ, Bushong EA, Britten Ventura PB, Chung W-S, Zhou L, Cahoy JD, Daneman R, Zong H, Ellisman MH, Barres BA. 2011. Development of a method for the purification and culture of rodent astrocytes. Neuron 71:799–811.

Gubert F, Zaverucha-do-Valle C, Pimentel-Coelho PM, Mendez-Otero R, Santiago MF. 2009. Radial glia-like cells persist in the adult rat brain. Brain Res 1258:43–52.

Halassa MM, Fellin T, Haydon PG. 2007. The tripartite synapse: Roles for gliotransmission in health and disease. Trends Mol Med 13:54–63.

Hartfuss E, Galli R, Heins N, Gotz M. 2001. Characterization of CNS precursor subtypes and radial glia. Dev Biol 229:15–30.

Heinrich C, Gascón S, Masserdotti G, Lepier A, Sanchez R, Simon-Ebert T, Schroeder T, Götz M, Berninger B. 2011. Generation of subtype-specific neurons from postnatal astroglia of the mouse cerebral cortex. Nat Protoc 6:214–228.

Jensen AA, Brauner-Osborne H. 2004. Pharmacological characterization of human excitatory amino acid transporters EAAT1, EAAT2 and EAAT3 in a fluorescence-based membrane potential assay. Biochem Pharmacol 67:2115–2127.

Kimelberg HK. 2004. The problem of astrocyte identity. Neurochem Int 45:191–202.

Kimelberg HK. 2010. Functions of mature mammalian astrocytes: A current view. Neuroscientist 16:79–106.

Koch P, Opitz T, Steinbeck JA, Ladewig J, Brüstle O. 2009. A rosette-type, self-renewing human ES cell-derived neural stem cell with potential for in vitro instruction and synaptic integration. Proc Natl Acad Sci USA 106:3225–2330.

van Landeghem FKH, Stover JF, Bechmann I, Brück W, Unterberg A, Bührer C, von Deimling A. 2001. Early expression of glutamate transporter proteins in ramified microglia after controlled cortical impact injury in the rat. Glia 35:167–179.

Lee Y, Su M, Messing A, Brenner M. 2006. Astrocyte heterogeneity revealed by expression of a GFAP-LacZ transgene. Glia 53:677–687.

Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC. 1995. Differential expression of two glial glutamate transporters in the rat brain: Quantitative and immunocytochemical obvervations. J Neurosci 15:1835–1853.

Liu Y, Namba T, Liu J, Suzuki R, Shioda S, Seki T. 2010. Glial fibrillary acidic protein-expressing neural progenitors give rise to immature neurons via early intermediate progenitors expressing both glial fibrillary acidic protein and neuronal markers in the adult hippocampus. Neuroscience 166:241–251.

Ma DK, Ming GL, Song H. 2005. Glial influences on neural stem cell development: Cellular niches for adult neurogenesis. Curr Opin Neurobiol 15:514–520.

Maragakis NJ, Rothstein JD. 2006. Mechanisms of disease: Astrocytes in neurodegenerative disease. Nat Clin Pract Neurol 12:679–689.

Matthias K, Kirchhoff F, Seifert G, Hüttmann K, Matyash M, Kettenmann H, Steinhäuser C. 2003. Segregated expression of AMPA-type glutamate receptors and glutamate transporters defines distinct astrocyte populations in the mouse hippocampus. J Neurosci 23:1750–1758.

Mauch DH, Nagler K, Schumacher S, Goritz C, Muller EC, Otto A, Pfrieger FW. 2001. CNS synaptogenesis promoted by glia-derived cholesterol. Science 294:1354–1357.

McKhann IIG, D'Ambrosio R, Janigro D. 1997. Heterogeneity of astrocyte resting membrane potentials and intercellular coupling revealed by whole-cell and gramicidin-perforated patch recordings from cultured neocortical and hippocampal slice astrocytes. J Neurosci 17:6850–6863.

Merkle FT, Alvarez-Buylla A. 2006. Neural stem cells in mammalian development. Curr Opin Cell Biol 18:704–709.

Mori T, Buffo A, Götz M. 2005. The novel roles of glial cells revisited: The contribution of radial glia and astrocytes to neurogenesis. Curr Top Dev Biol 69:67–99.

Mori T, Tanaka K, Buffo A, Wurst W, Kühn R, Götz M. 2006. Inducible gene deletion in astroglia and radial glia—A valuable tool for functional and lineage analysis. Glia 54:21–34.

Morin X, Jaouen F, Durbec P. 2007. Control of planar divisions by the G-protein regulator LGN maintains progenitors in the chick neuroepithelium. Nat Neurosci 10:1440–1448.

Nishiyama A, Komitova M, Suzuki Ryusuke, Zhu Xiaoyin. 2009. Polydendrocytes (NG2 cells): Multifunctional cells with lineare plasticity. Nat Rev Neurosci 10:9–22.

Nolte C, Matyash M, Pivneva T Schipke CG, Ohlemeyer C, Hanisch UK, Kirchhoff F, Kettenmann H. 2001. GFAP promoter-controlled EGFP-expressing transgenic mice: A tool to visualize astrocytes and astrogliosis in living brain tissue. Glia 33:72–86.

Pastrana E, Cheng LC, Doetsch F. 2009. Simultaneous prospective purification of adult subventricular zone neural stem cells and their progeny. Proc Natl Acad Sci USA 107:6387–6392.

Platel JC, Stamboulian S, Nguyen I, Bordey A. 2010. Neurotransmitter signalling in postnatal neurogenesis: The first leg. Brian Res Rev 63:60–71.

Rapaport DH, Wong LL, Wood ED, Yasamura D, LaVail MM. 2004. Timing and topography of cell genesis in the rat retina. Comp Neurol 21:304–324.

Regan MR, Huang YH, Kim YS, Dykes-Hoberg MI, Jin L, Watkins AM, Bergles DE, Rothstein JD. 2007. Variations in promoter activity reveal a differential expression and physiology of glutamate transporters by glia in the developing and mature CNS. J Neurosi 27:6607–6619.

Robel S, Berninger B, Götz M. 2011. The stem cell potential of glia: Lessons from reactive gliosis. Nat Rev Neurosci 12:88–104.

Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW. 1994. Localization of neuronal and glial glutamate transporters. Neuron 13:713–725.

Seri B, Garcia-Verdugo JM, McEwan BS, Alvarez-Buylla A. 2001. Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosi 21:7153–7160.

Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY. 2004. Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nat Biotechnol 22:1567–1572.

Shibata T, Yamada K, Watanabe M, Ikenaka K, Wada K, Tanaka K, Inoue Y. 1997. Glutamate transporter GLAST is expressed in the radial glia-astrocyte lineage of developing mouse spinal cord. J Neurosci 17:9212–9219.

Slezak M, Göritz C, Niemiec A, Frisén J, Chambon P, Metzger D, Pfrieger FW. 2007. Transgenic mice for conditional gene manipulation in astroglial cells. Glia 55:1565–1576.

Stoffel W, Körner R, Wachtmann D, Keller BU. 2004. Functional analysis of glutamate transporters in excitatory synaptic transmission of GLAST1 and GLAST1/EAAC1 deficient mice. Brain Res Mol Brain Res 128:170–181.

Storck T, Schulte S, Hofmann K, Stoffel W. 1992. Structure, expression, and functional analysis of a Na+-dependent glutamate/aspartate transporter from rat brain. Proc Natl Acad Sci USA 89:10955–10959.

Takano T, Tian GF, Peng W, Lou N, Libionka W, Han X, Nedergaard M. 2006. Astrocyte-mediated control of cerebral blood flow. Nat Neurosci 9:260–267.

Ullian EM, Sapperstein ,SK, Christopherson KS, Barres ,BA. 2001. Control of synapse number by glia. Science 291:657–661.

Vives V, Alonso G, Solal AC, Joubert D, Legraverend C. 2003. Visualization of S100B-positive neurons and glia in the central nervous system of EGFP transgenic mice. J Comp Neurol 457:404–419.

Whitman MC, Greer CA. 2009. Adult neurogenesis and the olfactory system. Prog Neurobiol 89:162–175.

Williams S, Sullivan RK, Scott HL, Finkelstein DI, Colditz PB, Lingwood BE, Dodd PR, Pow DV. 2005. Glial glutamate transporter expression patterns in brains from multiple mammalian species. Glia 49:520–541.

Xin W-J, Weng H-R, Dougherty PM. 2009. Plasticity in expression of the glutamate transporters GLT-1 and GLAST in spinal dorsal horn glial cells following partial sciatic nerve ligation. Mol Pain 5:15.

Zhou M, Kimelberg HK. 2000. Freshly isolated astrocytes from rat hippocampus show two distinct current patterns and different $[K+]_0$ uptake capabilities. J Neurophysiol 84:2746–2757.

Zhou M, Kimelberg HK. 2001. Freshly isolated hippocampal CA1 astrocytes comprise two populations differing in glutamate transporter and AMPA receptor expression. J Neurosci 21:7901–7908.

Zhuo L, Sun B, Zhang CL, Fine A, Chiu SY, Messing A. 1997. Live astrocytes visualized by green fluorescent protein in transgenic mice. Dev Biol 187:36–42.

Zoia C, Cogliati T, Tagliabue E, Cavaletti G, Sala G, Galimberti G, Rivolta I, Rossi V, Frattola L, Ferrarese C. 2004. Glutamate transporters in platelets: EAAT1 decrease in aging and in Alzheimer's disease. Neurobiol Aging 25:149–157.

Zoia CP, Tagliabue E, Isella V, Begni B, Fumagalli L, Brighina L, Appollonio I, Racchi M, Ferrarese C. 2005. Fibroblast glutamate transport in aging and in AD: Correlations with disease severity. Neurobiol Aging 26:825–832.

Zuo Y, Lubischer JL, Kang H, Tian L, Mikesh M, Marks A, Scofield VL, Maika S, Newman C, Krieg P, Thompson WJ. 2004. Fluorescent proteins expressed in mouse transgenic lines mark subsets of glia, neurons, macrophages, and dendritic cells for vital examination. J Neurosci 24:10999–11009.