Increased adenine nucleotide translocator 1 in reactive astrocytes facilitates glutamate transport

Charles R. Buck[a,*], **Michael J. Jurynec**[b], **Deepak K. Gupta**[c], **Alick K.T. Law**[c], **Johannes Bilger**[d], **Douglas C. Wallace**[d], and **Robert J. McKeon**[e]

[a] Oridis Biomed, Elisabethstrasse 84, A-8010, Graz, Austria

[b] Department of Human Genetics, University of Utah, Salt Lake City UT 84112, USA

[c] Department of Physiology, Emory University, Atlanta, GA 30322, USA

[d] Center for Molecular Medicine, Emory University, Atlanta, GA 30322, USA

[e] Department of Cell Biology, Emory University, Atlanta, GA 30322, USA

Abstract

A hallmark of central nervous system (CNS) pathology is reactive astrocyte production of the chronic glial scar that is inhibitory to neuronal regeneration. The reactive astrocyte response is complex; these cells also produce neurotrophic factors and are responsible for removal of extracellular glutamate, the excitatory neurotransmitter that rises to neurotoxic levels in injury and disease. To identify genes expressed by reactive astrocytes, we employed an in vivo model of the glial scar and differential display PCR and found an increase in the level of Ant1, a mitochondrial ATP/ADP exchanger that facilitates the flux of ATP out of the mitochondria. Ant1 expression in reactive astrocytes is regulated by transforming growth factor-β1, a pluripotent CNS injury-induced cytokine. The significance of increased Ant1 is evident from the observation that glutamate uptake is significantly decreased in astrocytes from Ant1 null mutant mice while a specific Ant inhibitor reduces glutamate uptake in wild-type astrocytes. Thus, the astrocytic response to CNS injury includes an apparent increase in energy mobilization capacity by Ant1 that contributes to neuroprotective, energy-dependent glutamate uptake.

Keywords

Glial scar; Neuroprotection; Mitochondria; Transforming growth factor-β1 (TGF-β1); CNS injury; Differential display PCR

Introduction

Reactive astrogliosis describes the response of astrocytes to central nervous system (CNS) injury. This response is defined by a dramatic increase in the number and size of glial fibrillary acidic protein (GFAP)-positive astrocytes and is one of the most consistent findings in neurologic disease and after traumatic CNS injury. Despite the prevalence of this response, the functional significance of reactive gliosis is poorly understood. Reactive astrocytes contribute to axonal regenerative failure at the site of injury by forming the glial scar, a dense network of processes and growth inhibitory extracellular matrix (ECM) molecules (Bovolenta et al., 1997; McKeon et al., 1995). These cells also protect vulnerable neurons after injury by

* Corresponding author. Fax: +43-316-325-776-22. *E-mail address:* buck@oridis-biomed.com (C.R. Buck)..

maintaining ion homeostasis in the brain (Takahashi et al., 1997) and by producing neurotrophic factors that support neuronal survival (Rudge et al., 1992). Astrocytes contribute to termination of excitatory synaptic activity by removing the neurotransmitter glutamate from the extracellular space via astrocyte-specific glutamate transporters (Lehre et al., 1995; Rothstein et al., 1996; Tanaka et al., 1997). Glutamate uptake is of particular importance following CNS injury because excessive glutamate released by damaged neurons can initiate a self-propagating excitotoxic cascade beginning with the overstimulation of glutamate receptors, excessive Ca^{2+} influx, oxidative stress, and ultimately neuronal cell death with concomitant additional glutamate release (Greene and Greenamyre, 1996). The capacity for glutamate uptake may be overwhelmed and glutamate excitotoxicity perpetuated (Eng et al., 1997; Ye and Sontheimer, 1998). The multifaceted nature of the astrocytic response to injury is evident following ablation of reactive astrocytes in ganciclovirtreated transgenic mice expressing the herpes simplex thymidine kinase gene under the control of the GFAP promoter. Exacerbated neuronal cell death in these mice following cerebral cortex stab wound injury was reduced by an NMDA glutamate receptor antagonist, indicating elevated (excitotoxic) extracellular glutamate levels in the absence of astrocytes. Moreover, these animals display increased neurite outgrowth throughout the injured area from intact neurons surrounding the lesion (Bush et al., 1999). Thus, reactive astrocytes play a role in both neuroprotection and inhibition of neurite outgrowth after CNS injury.

Several cytokines and growth factors that regulate the glial response to injury are elevated in areas of CNS damage (Lindholm et al., 1992; Logan et al., 1992; Mocchetti et al., 1996; Wang et al., 1997a). TGF-β1 is a particularly potent stimulator of reactive astrogliosis. Astrocytes exposed to TGF-β1 increase expression of GFAP (Krohn et al., 1999; Reilly et al., 1998) as well as putative axon growth inhibitory ECM proteins such as tenascin and neurocan (Asher et al., 2000; Smith and Hale, 1997). Intracerebral cortical injection of TGF-β1 increases astrocytic production of the ECM molecules laminin and fibronectin in areas of reactive gliosis, and in vivo administration of TGF-β neutralizing antibodies reduces ECM deposition following brain injury (Logan et al., 1994; 1999)

The molecular mechanisms of reactive gliosis, including the role of TGF-β1 in this process, are largely unexplored. Therefore, we employed differential display PCR (ddPCR) to identify genes specifically regulated by TGF-β1 in an in vivo model of reactive gliosis. We demonstrate that mRNA levels for the adenine nucleotide translocator isoform 1 (Ant1) are elevated in areas of reactive gliosis and regulated by TGF-β. Glutamate transport by Ant1$^{-/-}$ astrocytes is reduced by 70%, indicating an important role for Ant1 in this process. These data suggest that mobilization of mitochondrial ATP may be a key component of the astrocytic response to CNS injury.

Materials and methods

The in vivo glial scar model

A stab wound was made with a scalpel blade in the cortex of adult male rats (250 g) or mice (more than 30 days old) and a nitrocellulose filter inserted at the wound site to induce a glial scar characteristic of chronic CNS injury (McKeon et al., 1995, 1999; Rudge et al., 1989). A major advantage of this model is that the implant, complete with associated cells and ECM, can subsequently be removed for biochemical and molecular analysis. Filters were left in place for 14–30 days when the animals were sacrificed for removal and processing of the adherent chronic astroglial scar and astrocytic infiltration. In some cases, prior to implantation the filters were first soaked in antibodies to neutralize TGF-β signaling (pan-specific anti-TGFβ, 10 μg/ml, and anti-TGFβ receptor type II, 0.5 μg/ml) (R&D, Minneapolis, MN) and 5 μl of this antibody solution was injected into the lateral ventricles daily via indwelling cannulae. Control animals were implanted with vehicle-soaked filters and injected with vehicle. For each RNA

and protein extraction, 4–10 animals were subjected to bilateral cortical knife cuts into which the nitrocellulose filters were implanted. At least three independent groups of animals were generated for each RNA and protein study described except for the TGF-β1 neutralized and vehicle infused controls used for ddPCR in which one group of 5 rats were implanted for each treatment. The Institutional Animal Care and Usage Committee of Emory University approved all animal studies.

Differential display PCR

ddPCR (Liang and Pardee, 1992) was performed with RNA isolated from in vivo glial scar filter implants, with and without concurrent treatment with TGFβ neutralizing antibodies, using the RNAimage system (GenHunter). cDNA fragments that were upregulated in the control versus anti-TGFβ treated filter implants were selected for further characterization.

In situ hybridization

ISH was performed as we have recently described (McKeon et al., 1999). Briefly, an RT-PCR product corresponding to a unique 3′ untranslated sequence of the Ant1 mRNA was subcloned in pGEM-T (Promega) to enable synthesis of single-stranded digoxigenin-labeled RNA probes using dig-UTP RNA labeling mix (Boehringer Mannheim) and either T7 or SP6 RNA polymerase as described by the supplier.

Frozen brain coronal sections (10 μm) containing glial scar filter implants were prehybridized for 4 h at 65°C in hybridization buffer [50% formamide, 0.1 M NaCl, 20 mM Tris (pH 8.0), 5 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate, and 10 mM DTT] and hybridized at 65°C overnight in the same buffer with 1 μg/ml added dig-labeled probe. The sections were washed for 10 min in 1× SSC (0.15 M NaCl, 15 mM $NaC_6H_5O_7$, pH 7.0) and 10 min with 1.5× SSC at 60°C, followed by two washes at 37°C in 2× SSC for 20 min each. Sections were digested with 0.1 μg/ml RNAse A in 2× SSC at 37°C for 30 min, followed with two 10-min washes in 2× SSC at room temperature and two 30-min washes in 0.2× SSC at 60°C. Following equilibration in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5), sections were blocked for 1 h at room temperature and hybridization detected by incubation with anti-digoxigenin Fab fragments (1:5000) in blocking buffer (Boehringer Mannheim), washed for 1 h in maleic acid buffer, and developed 4 h to overnight in color development buffer (0.1 M Tris, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$, 0.1% Tween 20, 10% polyvinyl alcohol, 0.315 mg/ml nitroblue tetrozolium, 0.175 mg/ml bromochloroindoyl phosphate). The color development reaction was terminated in neutralizing buffer (10 mM Tris, pH 5, 1 mM EDTA), and slides were dehydrated through an alcohol series, coverslipped in Permount (Sigma), and viewed and photographed with a Leitz Orthoplan 2 microscope equipped with a Hammatsu Orca CCD camera. Digital images were obtained with OpenLab software and imported to Adobe Photoshop for preparation of figures.

Controls for ISH studies included RNase treatment of control sections prior to hybridization, omission of the cRNA probe, hybridization with an excess of unlabeled antisense probe, and hybridization with corresponding sense strand probe. Each of these controls eliminated the specific signal.

Immunohistochemistry

Glial scar filter implant-containing brain sections were blocked with 3% normal rabbit serum prior to exposure to the primary antibody (anti-GFAP monoclonal antibody diluted 1:2000 in PBS (pH 7.3) with 0.04% Triton X-100 (PBST) for 12–18 h at 4°C. Following thorough washes in PBST, sections were incubated for 1 h at room temperature with a biotin-conjugated secondary antibody (diluted 1:5000 in PBS) (Jackson ImmunoResearch), washed as above, and incubated with avidin–Texas Red (NEN; 1:200 in PBS). Sections were then washed again

and coverslipped in Vectashield (Vector Labs) and viewed and photographed as described above.

To demonstrate specificity, in separate control sections the primary or secondary antibodies were omitted and inappropriate secondary antibodies employed. These controls eliminated the GFAP signal.

RT-PCR

Semi-quantitative RT-PCR was performed as previously described (McKeon et al., 1999). Briefly, total RNA was prepared from uninjured cortical tissue or pooled nitrocellulose implants removed from the cortex of injured animals and from cultured astrocytes by acidic phenol extraction. RNA was digested with DNAse I to eliminate potential contamination by genomic DNA. cDNA synthesis was performed from 1 μg of RNA using random hexamer primers and SuperScript II reverse transcriptase (RT) (Gibco/BRL) for 1 h at 42°C. One-tenth to 1/100 of this reaction was directly added to the PCR reaction cocktail, depending upon the abundance of the mRNA in question and the amplification efficiency of the primer pair. For each primer pair, pilot PCR reactions were sampled between cycles 15 and 33 at every third cycle to insure that the reactions were assessed during the exponential phase of amplification. The levels of gene expression relative to the housekeeping gene GAPDH are thereby comparable. The primers employed in this study were GAPDH-1 forward ATCACCATCTTCCAGGAGCG, reverse TAGGAACACGGAAGGCCATG; GAPDH-2 forward AGAAGGTGGTGAAGCAGGCATC, reverse ATGTAGGCCATGAGGTC; Ant1 forward TGGATCTGTGAACCTGTGAACTTG, reverse GCATCATCATCTACAGAGCTGCC; Ant2 forward GTCCTAGGTGTTCTCCCCGA, reverse ATTGAGTCCCATCATTATTGTC; ATP8 forward GCCACAACTAGACACATGCAC, reverse GGGTAATGAAAGAGGCAAATAG; ATP β synthase forward GAATCCAGTCTCCACCCGGA, reverse GGTGGCGGTGCCTGCCTTCG; COIV forward TGATGTGGCCCACGTCAAGCTG, reverse TGTCCAGCATCCGCTTGGTCTG. The specificity of the PCR reaction was demonstrated by direct sequence analysis of the products. The primary data provided for these studies are representative of at least three RT-PCR experiments from independent RNA samples.

Immunoblot analysis

One month postsurgery, implanted filters were removed, rinsed free of adherent tissue, and homogenized with a Dounce homogenizer in 100 μl of buffer H [210 mM mannitol, 70 mM sucrose, 5 mM Hepes, 5 mM K-EGTA, 0.1% Triton X-100, (pH 7.2)] containing 5 μg/ml aprotinin, 0.5 μg/ml antipain, 0.1 μg/ml pepstatin A, and 0.2 mM phenylmethylsulfonyl fluoride, sonicated for 10 s, and clarified by centrifugation at 7000g for 60 s. Protein concentration was determined with the bicinchoninic acid assay (Pierce) and 30-μg aliquots were subjected to electrophoresis in a 4–15% gradient precast SDS–PAGE gel. Gels were electroblotted onto PVDF membrane (Millipore), blocked overnight in 8% dried milk in Tris-buffered saline, and probed with anti-peptide antisera specific for rodent Ant1 (1:500) or Ant2 (1:200) (Graham et al., 1997; Levy et al., 2000) or for unphosphorylated p42/44 mitogen activated protein kinase (MAPK) (New England Biolabs) as a loading control in milk diluent (Kiekegaard & Perry Labs) at 4°C overnight. Following washing and peroxidase-conjugated secondary antibody incubation, positive signal was visualized using chemiluminescence (Amersham) according to manufacturer's protocol. Immunoblot images were obtained on the Kodak Digital Science Image Station 440CF and imported into Adobe Photoshop v.5.5 for figure preparation. The level of expression of Ant1 relative to expression of p42/44 MAPK was determined densitometrically using Kodak 1 D image analysis software.

Primary astrocyte cultures and glutamate uptake

Primary neonatal astrocyte cell cultures were established according the method of McCarthy and de Vellis (McCarthy and de Vellis, 1980). Briefly, cerebral cortical tissue was microdissected from postnatal day 1 or 2 mouse brain, incubated in 0.25% trypsin/0.1% EDTA (Cellgro) for 5 min at 37°C, dissociated by gentle trituration through flame-polished Pasteur pipettes, dispersed into poly-lysine-coated tissue culture flasks, and incubated in DMEM/F12 with 10% FBS overnight at 37°C with 5% CO_2. The next morning cultures were shaken vigorously and washed in HBSS (Gibco), and nonadherent cells were removed. Remaining cells are highly enriched for astrocytes as determined by immunocytochemical detection of cell-type-specific markers. The astrocytes were maintained in DMEM/F12 with 10% FBS, fed twice weekly, and passaged when confluent, usually weekly. Only cell passages 3 – 10 were employed for these studies.

Glutamate uptake studies were performed as described by Blanc et al. (Blanc et al., 1998). Nearly confluent cultures of astrocytes were washed in Locke's buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5.0 mM Hepes, 10 mM glucose, 0.002% gentamycin, pH 7.2) and incubated in 0.1 μCi/ml [^{3}H]glutamate along with 50 μM cold glutamate for 3, 7, or 15 min. Over this time course, glutamate uptake continuously increased. The cells were washed four times with ice-cold Locke's buffer and lysed in 0.5 M NaOH, 0.05% SDS, for 15 min under constant agitation prior to determination of protein content and radioactive glutamate uptake. Uptake is expressed as counts per minute [^{3}H] glutamate per milligram of protein per minute.

Specific inhibition of Ant function was achieved by pre-incubating wild-type astrocytes with 50 μM carboxyatractyloside (CATR, Calbiochem) in the standard medium DMEM/F12 with 10% FBS for various periods prior to glutamate uptake. A dose-dependent inhibition of glutamate uptake was also observed with 5–100 μM CATR treatment for 24 h prior to glutamate uptake measurements (data not shown). All glutamate uptake studies were performed at least three times and on primary astrocyte cultures obtained from different mouse litters.

Transcription reporter assays

Primary astrocyte cultures in 12-well dishes were exposed to DNA cocktails consisting of 1.0 μg of Ant1 promoter firefly luciferase reporter (Promega, pGL3) and 0.5 μg of β-galactosidase reporter control plasmid (pSV-βgal) mixed with 3 μl Fugene 6 (Roche) diluted in 97 μl of OPTI-MEM (Life Technologies) per well. Cells were incubated with the DNA for 24 h at 37°C, rinsed once with 0.5 ml of serum-free medium, and cultured with an equal volume of N2-supplemented serum-free medium (Roche) for an additional 48 h. Parallel wells were treated with TGF-β1 (10 ng/ml, R&D Technologies) for the final 48 h. Cell harvest and luciferase assays were performed using the Dual-Light kit (Tropix) according to the manufacturer's protocol. Briefly, cells were washed twice with 350 μl of ice- cold PBS, lysed in 30 μl of the supplied lysis buffer containing 0.5 mM dithiothreitol, and centrifuged at 15,000g for 2 min to pellet debris. Supernatants were transferred to fresh microcentrifuge tubes and stored at −70°C. Firefly luciferase and β-galactosidase activities were assayed in 10 μl of extract with 25 μl of Buffer A and 100 μl of diluted Buffer B, respectively. Luciferase activities were normalized to β-galactosidase activity and expressed as relative light units (RLUs). Mean RLUs for TGF-β1-treated samples were compared to untreated controls using Student's t test ($n = 4$ wells for each condition). These data were replicated in at least three independent primary astrocyte cultures.

Results

To begin to characterize the molecular mechanisms involved in the astrocytic response to CNS injury, differential display PCR (Liang and Pardee, 1992) was used to identify genes that were upregulated in gliotic tissue compared with glial scars in which reactive astrocyte gene expression was modulated by immunoneutralization of TGF-β1. Differential display revealed that the mRNA encoding Ant1 was increased in astroglial scars versus tissue treated with TGF-β neutralizing antibodies. Ant1 is a major mitochondrial inner membrane protein that mobilizes mitochondrial energy by exchanging mitochondrial ATP for cytosolic ADP (Klingenberg, 1985).

Ant1 in situ hybridization verifies that this mRNA is strongly expressed in reactive astrocytes of the glial scar. The intensity of the Ant1 mRNA hybridization signal is greatest at the site of CNS injury, i.e., in and around the filter implant, and diminished to basal levels with increasing distance from the implant (Fig. 1A). This pattern of expression is reminiscent of GFAP immunoreactivity, the diagnostic marker of the glial scar (Bignami and Dahl, 1976) (Fig. 1B). At higher magnification, Ant1 mRNA expression is evident in reactive astrocytes, including cells that have migrated into, or elaborated processes into the implant. Ant1 mRNA coincides precisely with GFAP expression in these cells (Fig. 1C and D). When TGF-β neutralizing antibodies were administered intraventricularly each day following filter implantation, increased Ant1 mRNA in reactive astrocytes was eliminated (Fig. 1E). Thus, the induction of Ant1 mRNA in reactive astrocytes is mediated via TGF-β1, as suggested from the ddPCR experiment.

Differential expression of Ant1 mRNA after CNS injury was confirmed by semi-quantitative RT-PCR analysis in which parallel reactions are performed to amplify the relatively invariant housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) (Fig. 2A). Similar levels of GAPDH RT-PCR product reflect similar quantities of cDNA added to the reaction and demonstrate that, relative to GAPDH mRNA, Ant1 mRNA levels are higher in the in vivo glial scar than in uninjured cerebral cortex. Moreover, increased Ant1 mRNA levels were not seen in RNA isolated from filters exposed to TGF-β neutralizing antibodies following implantation (αTF lanes).

Primary cultures of neonatal astrocytes express elevated levels of GFAP in response to TGF-β and have been utilized as a model system for reactive astrogliosis (Yu et al., 1993). RT-PCR revealed that Ant1 mRNA is expressed in cultured rodent astrocytes, indicating that these cultures would be useful for characterization of the role of increased Ant1 mRNA in reactive astrocytes (Fig. 2A). To explore TGF-β1 regulation of Ant1 gene expression in primary astrocyte cultures, these cells were incubated in serum-free medium in the presence or absence of TGF-β1. TGF-β1 treatment for 72 h resulted in an increase in Ant1 mRNA, reminiscent of the increase seen in the in vivo glial scar (Fig. 2B). By contrast, Ant2 mRNA, encoding a protein closely related to Ant1 (Dorner et al., 1999) also expressed in astrocytes, was not apparently changed (Fig. 2B).

TGF-β1 regulation of Ant1 mRNA expression is further supported by transcription reporter assays. A 7 kb fragment of the mouse Ant1 gene promoter proximal to the transcription start site (Levy et al., 2000) was cloned into a promoter-less firefly luciferase reporter gene expression plasmid. The TGF-β1 response of this construct was tested by transfection into primary astrocyte cell cultures. The presence of TGF-β1 in the culture medium for 24 h stimulated 80% more luciferase expression than parallel transfections exposed to control serum-free culture medium (Fig. 2C). This result is consistent with the independent demonstrations by ddPCR, RT-PCR, and in situ hybridization that Ant1 mRNA is elevated in

reactive astrocytes of the in vivo glial scar. Further, the transcription reporter assay directly demonstrates that TGF-β1 can increase transcription from the Ant1 gene in astrocytes.

To further assess the effects of injury on astrocytic Ant expression, protein extracts were obtained from glial scar filter implants, uninjured cerebral cortex, and other tissues and used with isoform-specific affinity-purified antisera to either Ant1 or Ant2 to examine protein levels (Graham et al., 1997; Levy et al., 2000). Consistent with changes in mRNA levels, Ant1 protein was detected at higher levels in gliotic tissue than in uninjured cortex (Fig. 3A). Interestingly, Ant2 expression in the glial scar filter implants was decreased compared to uninjured cortex. This apparent decrease may reflect higher levels of Ant2 expression in cortical neurons than astrocytes as suggested by Ant2 in situ hybridization (data not shown). In any case, Ant2 protein levels are not elevated in gliotic tissue. From three independent protein extracts of glial scar filter implant and uninjured cortex, a more quantitative analysis of Ant1 protein levels was undertaken with simultaneous detection of the level of the relatively invariant unphosphorylated mitogen activated protein kinase 42/44 (MAPK) (Fig. 3B). Densitometric analysis of the Ant1 immunoblot signals in filter vs uninjured cortex again revealed that Ant1 levels are increased nearly two-fold in filter implant extracts compared with uninjured cortex (Fig. 3B). Thus, increased Ant1 mRNA that we have detected in reactive astrocytes of the in vivo glial scar results in increased Ant1 protein in these cells.

Increased Ant1 in reactive astrocytes suggests that mobilization of mitochondrial-derived energy may be an important feature of astrocyte activation. To determine whether genes involved in mitochondrial energy production were also induced in reactive astrogliosis, we examined mRNA expression levels of the nuclear DNA-encoded ATP synthase β subunit and COIV of complex IV and of the mitochondrial DNA-encoded gene ATP8. mRNA expression levels of all three of these OXPHOS-related mitochondrial genes were comparable in filter implant, uninjured cortex, and cultured astrocyte samples, whereas the level of expression of Ant1 mRNA in these independent RNA preparations again showed a clear increase in the filter implant (Fig. 4). Therefore, the increase in Ant1 mRNA in reactive astrocytes does not represent a global increase in expression of mRNAs encoding mitochondrial proteins, indicating that the level of Ant1 may regulate the capacity for mitochondrial energy mobilization in reactive astrocytes.

To further explore the role for Ant1 in reactive astrogliosis in vivo, we reexamined the astrocytic response to CNS injury in transgenic mice lacking Ant1 (Graham et al., 1997). In both wild-type and Ant1 null mutant mice, filter implantation stimulated robust astrogliosis, as assessed by GFAP immunoreactivity in sections from the implanted cerebral cortex (Fig. 5A), and the levels of GFAP protein were similar in both +/+ and −/− filter implant extracts (Fig. 5B). Ant1 was readily detected in +/+ but not in Ant1 −/− glial scars as expected, while the closely related Ant2 isoform remained at low but detectable levels in both wild-type and null mutant glial scars (Fig. 5B). This finding is consistent with previous studies of the Ant1^{−/−} mouse indicating that Ant2 does not compensate for the lack of Ant1 in tissues in which both Ant isoforms are normally expressed (Graham et al., 1997). These data demonstrate that Ant1 is not essential for a reactive astrocyte response to CNS injury; a glial scar is formed in Ant1 null mutant mice that is similar to the wild-type animal.

The similar regulation of Ant1 in vivo (reactive astrocytes) and in vitro indicated that primary astrocyte cultures would be useful for analysis of the functional significance of increased Ant1 mRNA in reactive gliosis. While astrocytes are highly glycolytic cells, capable of producing 25 – 30% of total ATP via glycolysis (Silver and Erecinska, 1997), the induction of Ant1 suggests that energy from oxidative phosphorylation is needed to respond to CNS injury. One possible requirement for additional mitochondrial ATP is glutamate uptake. A selective blockade of oxidative metabolism in astrocytes reduces ATP levels and decreases glutamate

transport (Swanson and Benington, 1996), suggesting that both glycolysis and oxidative phosphorylation are required to maintain ionic gradients in cultured astrocytes (Silver et al., 1997). To investigate the physiological function of the Ant1 induction in reactive astrocytes, we examined glutamate uptake, a critical energy-dependent neuroprotective astrocyte function (Blanc et al., 1998; Rothstein et al., 1996; Sonnewald et al., 1997; Tanaka et al., 1997). In mouse primary astrocyte cell culture, glutamate transport is reduced in the presence of the specific Ant inhibitor carboxyatractyloside (Table 1) (Streicher-Scott et al., 1993). Furthermore, glutamate uptake is reduced 70% in primary astrocyte cultures prepared from Ant1$^{-/-}$ mice relative to Ant1$^{+/+}$ controls (Fig. 6). Ant mobilization of mitochondrial ATP is, therefore, important for astrocyte-mediated neuroprotective mechanisms.

Not all energy-dependent cellular functions are compromised in Ant1$^{-/-}$ astrocytes, however. In the same culture medium used for the glutamate uptake studies, astrocyte proliferation was measured by seeding equal numbers of wild-type and Ant1 null mutant primary astrocytes on triplicate gridded multiwell dishes in parallel. After 24 h, an average of 1509 (±111.4) wild-type and 1511 (±60.9) Ant1 knockout cells were counted in 25 grids. Similarly, after 48 h, there was still no difference in the number of cells (wild-type astrocytes = 1949 ± 143.4; null mutant astrocytes = 2253 ± 178.8). Thus, Ant1$^{-/-}$ astrocytes can access sufficient energy for normal proliferation. Further, immunoblot analysis reveals that the levels of GFAP and the astrocytic glutamate transporters Glt-1 and GLAST are not different between astrocytes from each genotype (data not shown). Together these data support the suggestion that increased Ant1 is a specific mechanism whereby reactive astrocytes mobilize additional cellular energy after CNS injury for glutamate uptake and possibly other energy-dependent functions.

Discussion

Reactive gliosis

Reactive astrogliosis is particularly critical in modulating both axon outgrowth and neuronal survival/cell death (Bush et al., 1999). CNS damage stimulates astrocytic hypertrophy and these reactive astrocytes are identified by a dramatic increase in expression of the astroglial-specific intermediate filament protein, GFAP. Although the significance of increased GFAP expression remains unclear (Gomi et al., 1995; Pekny et al., 1995; Wang et al., 1997b), reactive astrocytes participate in axonal regenerative failure, potentially by secreting specific ECM molecules capable of inhibiting axonal growth (Asher et al., 2000; Davies et al., 1999; McKeon et al., 1995, 1999). In contrast, reactive astrocytes also protect vulnerable neurons from injury or death (Bush et al., 1999). Relative to neurons, astrocytes express high levels of antioxidant enzymes that may regulate levels of reactive oxygen species generated following CNS injury (Peuchen et al., 1997). Additionally, astrocytes have the important primary responsibility for removal of glutamate from the synaptic cleft (Lehre et al., 1995; Rothstein et al., 1996). Failure of glutamate reuptake increases neuronal Ca^{2+} influx via excessive stimulation of neuronal glutamate receptors resulting in a self-propagating and ultimately neurotoxic increase in extracellular glutamate (Sonnewald et al., 1997; Ye and Sontheimer, 1998).

Reactive astrocytes may access mitochondrial ATP via increased Ant1 expression

We demonstrate with numerous methods in independent experiments that expression of the gene encoding Ant1 is increased in GFAP-positive reactive astrocytes of the chronic glial scar in vivo. The Ant proteins are major inner mitochondrial membrane proteins that enable exchange of mitochondrial-generated ATP for cytosolic ADP (Klingenberg, 1985). The importance of Ant1 in linking energy consuming cytosolic processes with energy production in mitochondria is demonstrated in Ant1 null mutant mice that are viable and fertile but demonstrate progressive cardiac myopathy and severe exercise intolerance (Graham et al., 1997). Thus, Ant1 function is critical even for highly glycolytic tissues (such as skeletal and

cardiac muscle). Although coexpressed in many tissues (Dorner et al., 1999; Levy et al., 2000), the two rodent Ant isoforms are differentially regulated. Expression of the Ant2 isoform is not altered in our in vivo model of reactive gliosis and cannot fully compensate for loss of Ant1 function in cultured astrocytes.

Our demonstration of increased Ant1 in reactive astrocytes of the chronic glial scar suggests that these cells experience a long-term energy demand following traumatic brain injury. This demand is met, at least in part, by a TGF-β1-mediated increase in Ant1 that stimulates exchange of mitochondrial ATP for cytosolic ADP. The relationship between Ant1 and energy-consuming processes in reactive astrocytes is illustrated schematically in Fig. 7. We hypothesize that an increase in this mitochondrial ATP translocator in reactive astrocytes mobilizes mitochondrial ATP stores to increase cytosolic ATP levels, thereby meeting an increased bioenergetic demand. Although our data indicate that expression of a catalytic subunit of ATP synthase is not increased in reactive astrocytes, we cannot rule out the possibility that these cells may also increase ATP production.

TGF-β1 in CNS injury

Unlike the closely related family members TGF-β2 and -β3 that are expressed at high levels in the developing CNS and down regulated in the adult, high levels of TGF-β1 in the CNS are detected only following injury (Logan et al., 1992). TGF-β1 has been suggested to be neuroprotective via stimulation of astrocytic genes (Buisson et al., 1998). Additionally, TGF-β1 stimulates the expression ECM molecules that promote wound healing (Baghdassarian et al., 1993; Zambruno et al., 1995) and that regulate neurite outgrowth (Asher et al., 2000; Smith and Hale, 1997), indicating that the astrocytic response to this cytokine is significant following CNS injury.

Ant1 expression increases in reactive astrocytes after cortical injury and this increase is blocked by immunoneutralization of TGF-β and the type II TGF-β receptor in vivo. In addition, in cultured astrocytes TGF-β1 both induces transcription from the Ant1 promoter and increases expression of endogenous Ant1 mRNA. Together, these data provide strong evidence that Ant1 expression after cortical injury is regulated by TGF-β1. Moreover, TGF-β1 appears to specifically regulate Ant1 since expression of the closely related Ant2 is not elevated in gliotic tissue following CNS injury. Similarly, TGF-β1 induces expression from Ant1 promoter-containing transcription reporter constructs (Fig. 2C), but not from Ant2 promoter constructs (Law, A.K.T. et al., unpublished observations), consistent with the suggestion that these isoforms are differentially regulated after injury.

Functional significance of increased Ant1 expression in reactive astrocytes

The addition of TGF-β1 to primary astrocytes cultured in serum-free medium recapitulated the increase in Ant1, but not Ant2, observed in the glial scar in vivo. This observation encouraged us to test the functional significance of increased reactive astrocyte Ant1 in this culture system. $Ant1^{-/-}$ mouse primary astrocytes transport 70% less glutamate than do astrocytes from genetically matched control mice. Likewise, glutamate uptake by wild-type astrocytes is inhibited by the specific Ant inhibitor carboxyatractyloside (Table 1), supporting a direct role for increased Ant1 in this critical neuroprotective function and indicating that reduced glutamate uptake in $Ant1^{-/-}$ astrocytes is unlikely to be the result of indirect effects from the null mutation.

Importantly, these data suggest that reactive astrocytes, generally considered highly glycolytic cells, meet chronic increased bioenergetic demand through increased mobilization of mitochondrial energy stores. Since glutamate transport is a critical, energy-dependent astrocyte function in vivo, the profound effect of the absence of Ant1 on glutamate transport efficiency

suggests that increased Ant1 mRNA in the glial scar is required to facilitate astrocyte function, including glutamate transport in response to CNS injury.

Although the neuronal response to CNS injury has been a primary experimental focus, the glial scar generated by reactive astrocytes is one of the most consistent findings in CNS injury and disease and is considered the primary barrier to significant axonal regeneration following CNS injury (Fitch and Silver, 1997). Our analysis of gene expression in gliotic tissue has revealed for the first time the induction of a mitochondrial protein, Ant1, in astrocytes in response to CNS injury. These data demonstrate that a critical component of the astrocytic CNS injury response involves mitochondrial energy mobilization. Increased energy demand in reactive astrocytes is consistent with the need to reestablish ionic homeostasis within the injured brain, without which vulnerable neurons are more likely to suffer irreparable damage. In this regard, Ant1 is likely to be an important regulator of the multifaceted astrocytic response to chronic CNS injury.

Acknowledgments

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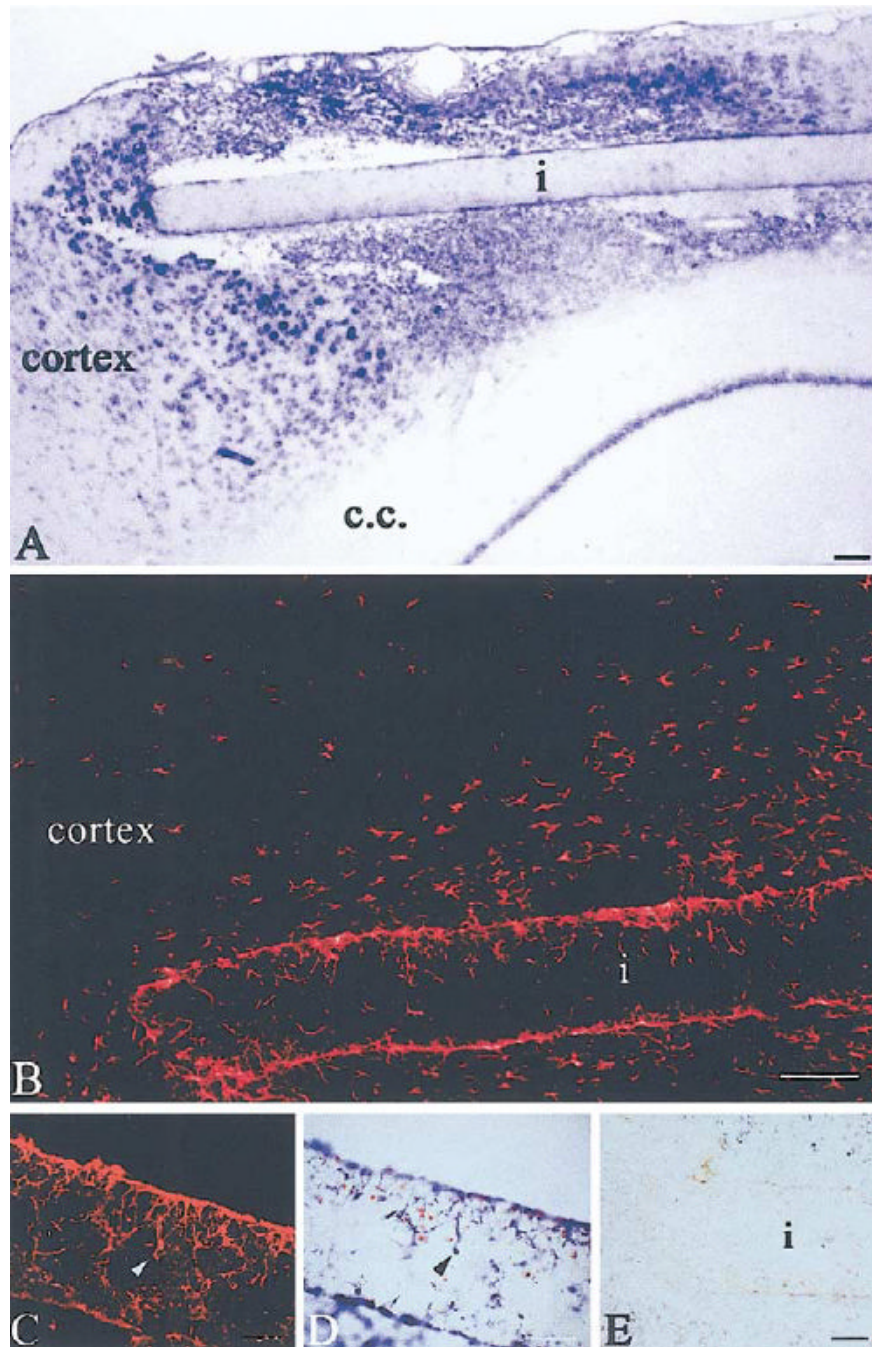
Fig. 1.
TGF-β1 stimulates Ant1 mRNA expression in reactive astrocytes of the glial scar (A) In situ hybridization to a brain tissue section containing a nitrocellulose filter glial scar implant (i) reveals strong Ant1 mRNA expression in and around the glial scar filter implant. The intensity of the hybridization signal is diminished with increasing distance from the glial scar filter implant (bottom left). Ant1 mRNA expression is also demonstrated in neurons of the CA1 region of the hippocampus (bottom right). c.c. = corpus callosum. (B) Immunohistochemistry for GFAP on a section from brain implanted with a nitrocellulose filter demonstrates the localized reactive astrogliosis that occurs within and surrounding the filter implant (i) in this in vivo model for the glial scar. (C) High magnification view of GFAP immunohistochemistry

in an in vivo glial scar filter implant illustrates reactive astrocyte cell bodies and processes (e.g., arrowhead) of the glial scar that have invaded the filter implant (i). The cortical tissue dorsal to this implant separated from the filter during tissue processing (top). (D) In situ hybridization for Ant1 mRNA on the same section as shown in (C) demonstrates Ant1 mRNA within GFAP-immunopositive reactive astrocyte cell bodies and processes (arrowhead) invading the filter. (E) In situ hybridization for Ant1 mRNA reveals that the intense Ant1 mRNA hybridization is eliminated in reactive astrocytes in and around the glial scar filter implant (i) formed in the presence of TGF-β-neutralizing antibodies. Ant1 mRNA hybridization distant from the implant and in other adjacent cells types is unaffected by TGF-β neutralization. Scale bars in A and B equal 100 μm; bars in C, D, and E equal 50 μm.
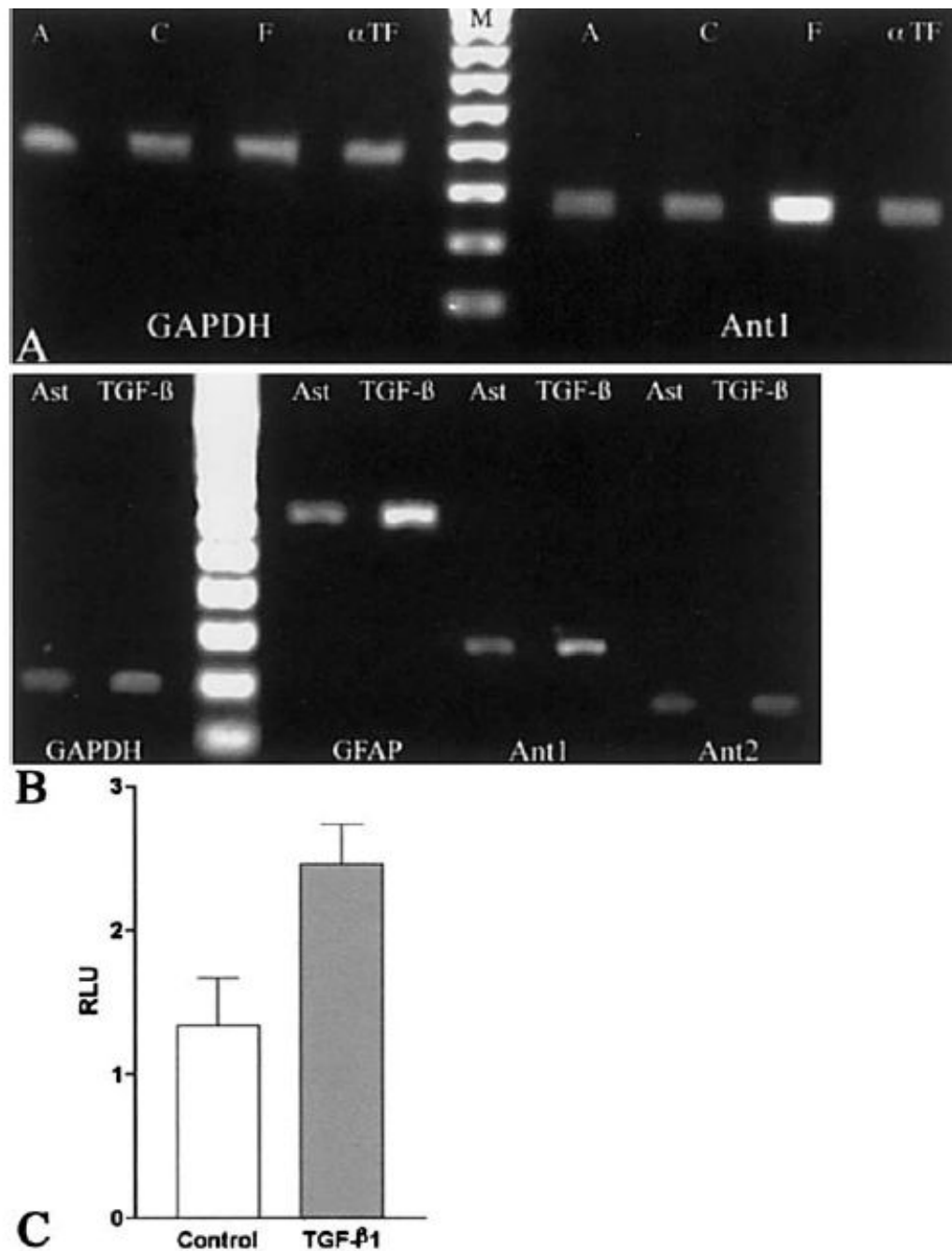
Fig. 2.
Increased Ant1 mRNA level in reactive astrocytes is regulated by TGF-β1. (A) Semi-quantitative RT-PCR analysis of GAPDH and Ant1 mRNA expression levels in astrocyte primary cultures (A), uninjured cerebral cortex (C), and glial scar filter implants (F) demonstrates that Ant1 mRNA levels are increased in the chronic glial scar filter implant. Neutralization of TGF-β1 in and around the filter implant in vivo (αTF) eliminates the increase in Ant1 mRNA. Equal amounts of cDNA, relative to GAPDH expression, were included for each reaction. (B) RT-PCR analysis demonstrates that TGF-β1 also regulates expression of Ant1 in primary astrocyte cell culture. GAPDH amplification (with primer set 2) again indicates that equivalent amounts of cDNA were analyzed. Three days of treatment with 10 ng/ml TGF-

β1 (TGF-β) increases Ant1 mRNA levels compared with untreated astrocytes (Ast). The level of mRNA encoding the closely related isoform Ant2 is not elevated in response to TGF-β1 treatment. TGF-β1 treatment increases GFAP mRNA levels, validating the relative quantitation of mRNA levels and the efficacy of the cytokine treatment. All RT-PCR reactions were sampled in the exponential phase of amplification. (C) A luciferase reporter construct driven by the full-length (7 kb) mouse Ant1 promoter is induced 1.8-fold by 24 h of TGF-β1 treatment following transfection into primary astrocyte cell cultures. Luciferase activity is given in relative light units (RLU) and corrected for transfection efficiency with a cotransfected β-galactosidase transcription reporter construct. Transfection with Ant1 promoter-containing constructs results in a significant 80% increase in TGF-β1 stimulated luciferase activity ($P =$ 0.0394, two-tailed Student's t test, error bar indicates SEM).

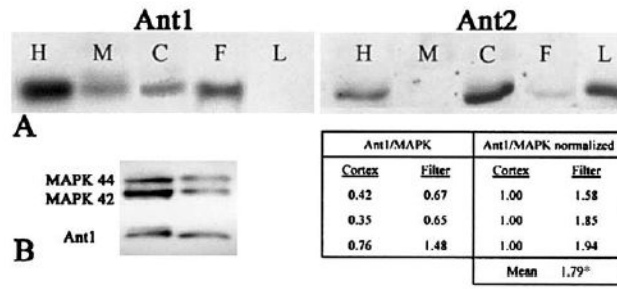| Ant1/MAPK		Ant1/MAPK normalized	
Cortex	Filter	Cortex	Filter
0.42	0.67	1.00	1.58
0.35	0.65	1.00	1.85
0.76	1.48	1.00	1.94
		Mean	1.79*

Fig. 3.

Ant1 protein levels are elevated in the in vivo glial scar. (A) Thirty micrograms of protein extracted from heart (H), skeletal muscle (M), uninjured cerebral cortex (C), glial scar filter implant (F), and liver (L) was subjected to immunoblot analysis with Ant1 and Ant2 specific antisera. Ant1-is readily detected in heart and skeletal muscle and expression is apparently increased in gliotic tissue compared with the uninjured cortex. Ant2 is detected in all tissues examined except skeletal muscle and is weakly expressed in reactive astrocytes of the glial scar filter implant, especially when compared to the robust, presumably neuronal expression in the uninjured cortex. Both Ant isoforms are ~31kDa. (B) Independent filter implant and uninjured cerebral cortex protein extracts were simultaneously immunoblotted for Ant1 and for p42/44 MAPK as a loading control. A representative blot from three independent analyses is shown. Densitometric analyses of these immunoblots (values given in rows of the table) reveals that, relative to MAPK expression levels, Ant1 protein is elevated by 79% in the in vivo glial scar filter implant. (*This difference is statistically significant, $P = 0.00095$, Student's t test).
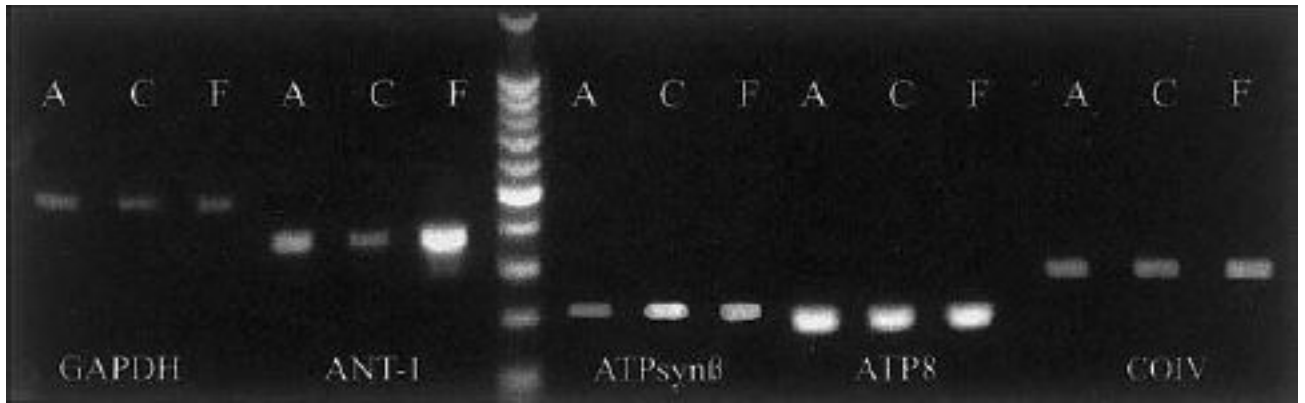
Fig. 4.
Ant1 mRNA increase in reactive gliosis. RT-PCR analysis of mitochondrial gene expression in primary astrocyte cultures (A), uninjured cerebral cortex (C), and glial scar filter implants (F). GAPDH amplification indicates that approximately equivalent amounts of mRNA were examined. Ant1 mRNA expression is elevated in glial scar. mRNA expression levels of the nuclear DNA-encoded ATP synthase β and COIV genes and of the mitochondrial DNA-encoded ATP8 gene are unchanged in the glial scar filter implant compared with uninjured cortex. RT-PCR reactions were analyzed in the exponential phase of amplification.
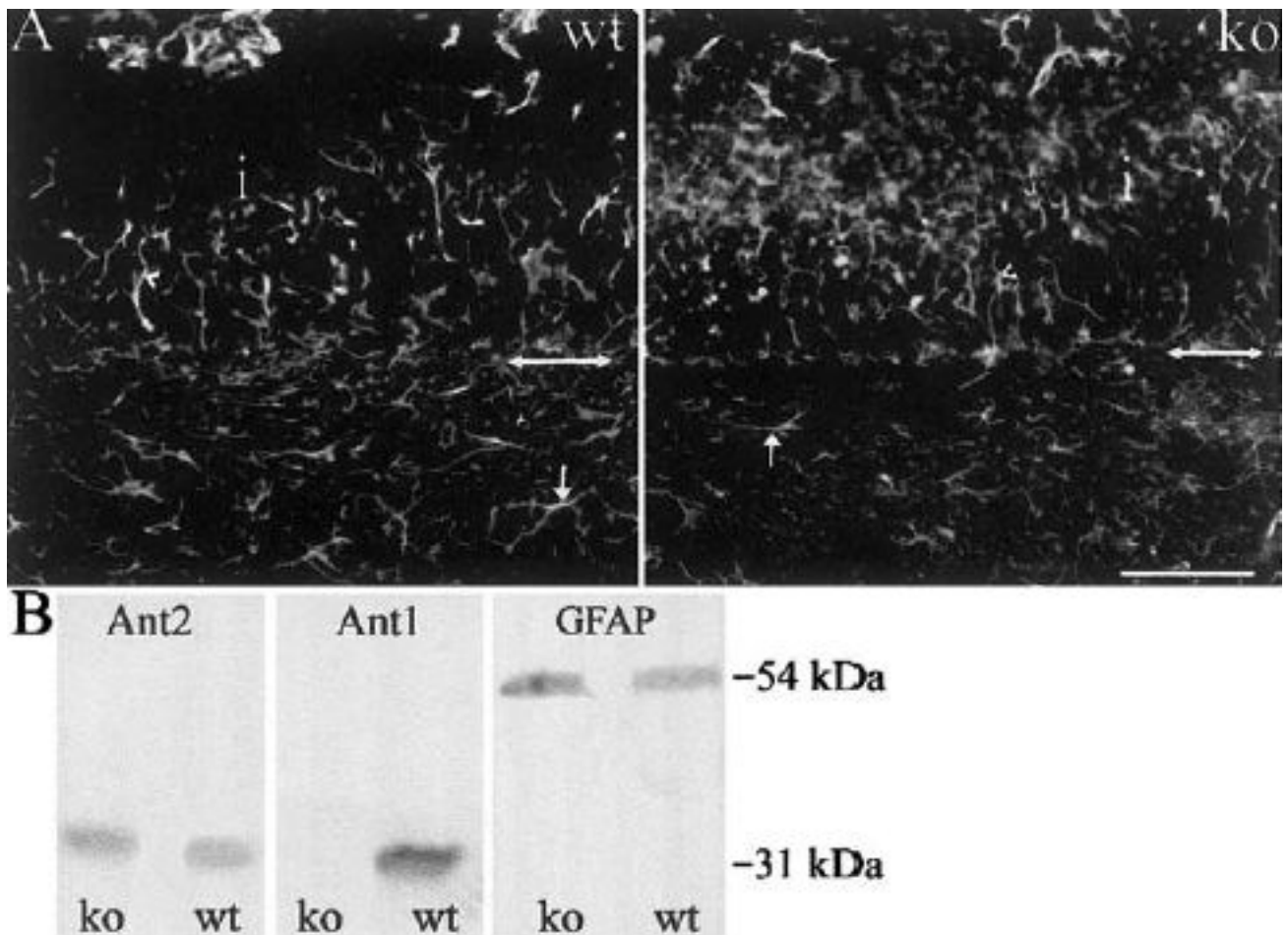
Fig. 5.
Glial scar formation in Ant1 null mutant mice. (A) Fourteen days following filter implantation robust reactive astrogliosis is evidenced by GFAP immunohistochemistry in brain tissue sections containing the filter implants (i) from wild-type (wt) and Ant1 null mutant "knockout" (ko) mice. Arrows indicate reactive astrocytes surrounding the implant and arrowheads denote astrocytic processes within the implant. The double-headed arrows indicate the (roughly horizontal) borders between the filter implant and cerebral cortex. Scale bar = 50 μm. (B) Glial scar filter implants from Ant1 null mutant (ko) and genetically matched wild-type (wt) control mice were retrieved for immunoblot analysis of equal amounts of protein 14 days following the injury and implantation. GFAP expression indicates that these mouse strains mount a similar astrogliotic response to the filter implant. Ant1 is expressed in the wild-type, but not in the Ant1 knockout glial scar, as expected. Ant2 is detected in the glial scar filter implant in both mouse strains but the level of this isoform is not apparently different in the null mutant compared with the wild-type strain.
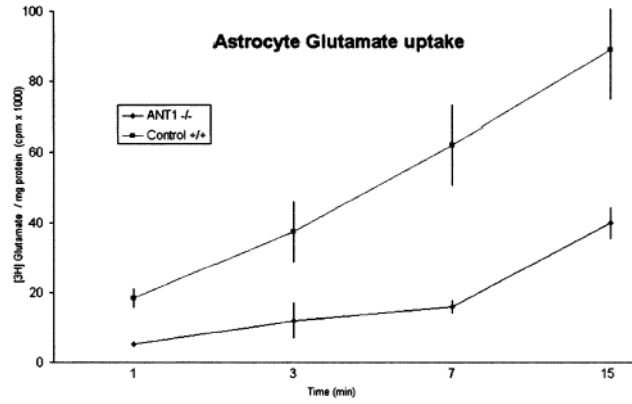
Fig. 6.
Ant1 expression is important for astrocytic glutamate uptake. Glutamate uptake was measured in primary astrocyte cultures from Ant1$^{-/-}$ and control (+/+) mice for 1, 3, 7, and 15 min. Astrocytes from Ant1$^{-/-}$ null mutant mice transport ~70% less glutamate than astrocytes from genetically matched controls. Data shown are the mean [^{3}H]glutamate uptake (±one standard deviation) of at least four independent assays from separate primary cultures. The differences in uptake are significant at each time point as determined with Student's t test ($P < 0.004$).
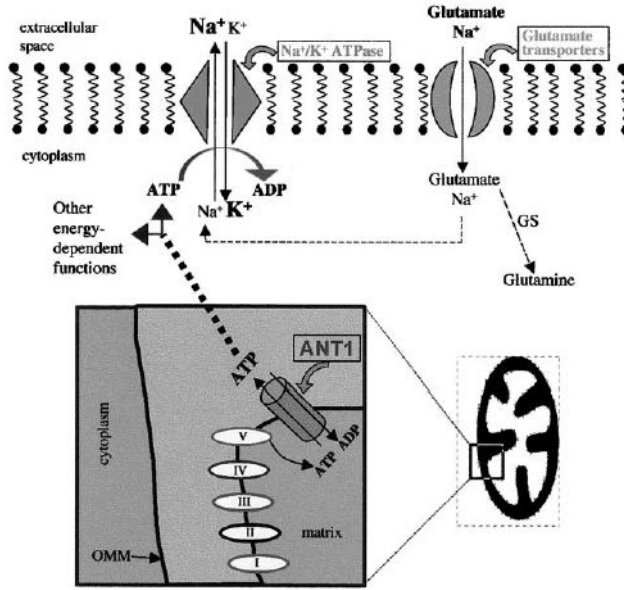
Fig. 7.
Reactive astrocytes of the CNS glial scar increase Ant1 to mobilize mitochondrial energy stores. Schematic illustration of Ant1 on the inner mitochondrial membrane (cylinder) distal to the ATP synthase activity of complex V. Ant1 transports ATP from the mitochondrial matrix, which then crosses the outer mitochondrial membrane (OMM) to the cytoplasm. Astrocyte-specific glutamate transporters (Glt-1/GLAST) cotransport extracellular glutamate with sodium, and Na^+/K^+ ATPase activity is required to maintain the sodium gradient necessary for transporter function. Ant1 may mobilize mitochondrial ATP to provide energy for this and other reactive astrocyte energy-consuming processes.

Table 1

Carboxyatractyloside (CATR) inhibits astrocytic glutamate uptake

Duration of 50 μM CATR treatment (h)	Glutamate uptake (cpm/mg protein)	Percentage of control
0 (vehicle only)	146,103	100
6	124,630	85.3
12	98,094	67.1
24	61,226	41.9