Supporting Information

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SI Materials and Methods

Animals and Stab Wound Injury. In the experiments using type 2 inositol 1,4,5-trisphosphate (IP_3) receptor KO (IP_3R2KO; *Itpr2*$^{-}$/*Itpr2*$^{-}$) mice (1), heterozygote littermates (*Itpr2*$^{+}$/*Itpr2*$^{-}$) were used as controls. Astrocyte-specific N-cadherin knockout (*a*NcadKO mice) were generated by crossing homozygous N-cadherin floxed mice (2) and heterozygous tamoxifen (Tx)-inducible Cre recombinase-expressing mice that is driven by the astrocyte specific L-glutamate/L-aspartate transporter promoter (GLAST-CreERT2) mice (3). Littermates with a homozygous flox N-cadherin allele and no GLAST-CreERT2 allele were used as controls. Cre recombinase was activated by i.p. injection of Tx (20 mg mL^{-1} in corn oil; Sigma-Aldrich) in 2- to 10-mo-old mice for 5 d (2 mg/day) (3).

Stab wound injury (SWI) was performed at 7–14 d after the last Tx injection. The skulls of mice anesthetized with isoflurane [1.5–2% (vol/vol) in O_2] were exposed, and the somatosensory cortex was impaled with a scalpel (4) (Feather surgical blade #11) ∼1 mm posterior to and ∼1.5 mm lateral from the Bregma, at a depth of 0.5–0.8 mm. After 2–14 d, the animals were killed for analysis.

Ca^{2+} Imaging. For in vivo imaging of neocortical astrocytes, mice (>1 mo old) were anesthetized with i.p. injection of ketamine (75 mg kg^{-1}) and medetomidine (1 mg kg^{-1}). Body temperature was maintained using a heating pad. After the skull was exposed over the somatosensory cortex, a custom-made metal frame was attached to the skull using dental acrylic (Fuji BC or GC lute). A 2- to 3-mm-diameter craniotomy centered 1 mm posterior to the Bregma and 2.5 mm lateral to the midline was created using a dental drill, then covered with 1% wt/vol agarose and sealed with a glass coverslip. A solution containing 1 mM Oregon Green 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA)-1 acetoxymethyl (AM) ester (OGB1; Molecular Probes) and 25 μM sulforhodamine 101 (SR101; Sigma-Aldrich, for astrocyte identification) was injected for the experiments shown in Fig. S1 *A* and *B* (5, 6). A constant anesthesia level was maintained by a s.c. infusion of ketamine at 30 mg kg^{-1} h^{-1}.

Imaging was performed using a custom-built two-photon laser-scanning microscope based on a mode-locked Ti:sapphire laser system, operating at 800 nm with a laser-scanning system (TCS SP2; Leica) and water-immersion objective lens (25×, NA = 0.95; Leica). Intense laser illumination (80 mW under objective for 1.6 s) was applied 12 times to generate a laser ablation injury. For the analysis of long-term Ca^{2+} imaging (Fig. S1*B*), the ratio of the fluorescence intensity of OGB1 to that of SR101 was used to correct for artifacts derived from focal plane drift and animal movement.

For Ca^{2+} imaging in slice preparations, coronal slices of the somatosensory cortex (400 μm) were prepared from postnatal day (P) 12–15 IP_3R2KO and control littermate mice that had been anesthetized with isoflurane. Slices were cut in an ice-cold solution containing 185 mM sucrose, 2.5 mM KCl, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 25 mM D-glucose [oxygenated by 95% O_2 and 5% CO_2 , (vol/vol)]. Slices were incubated in oxygenated artificial cerebrospinal fluid [ACSF (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 25 D-glucose] for 45 min at 35 °C and then returned to room temperature. For dye loading, slices were incubated for 20 min at 35–37 °C in ACSF including 25 μM fluo-4 AM, 1 μM SR101, 0.005% Cremophor EL (Sigma-Aldrich), and 0.01% pluronic acid (Invitrogen). Imaging was performed at room temperature using a two-photon microscope (TSC MP5; Leica) equipped with a water-immersion objective (HCS IR APO, 25×, NA = 0.95; Leica) and a Ti:sapphire laser (MaiTai DeepSee; Spectra Physics), with excitation at 820 nm. Astrocytes were identified by SR101 staining (6), and the astrocytic fluo-4 signal was corrected for background fluorescence of an indicator-free area. Photobleaching was corrected for using a linear fit to the fluorescence intensity change. Ca^{2+} signals were evoked by perfusing ACSF containing 100 μM ATP for 3–5 min.

Histochemistry and Western Blot Analysis. After being anesthetized with pentobarbital, mice were fixed by perfusion of PBS containing 4% paraformaldehyde (PFA). The isolated brain was postfixed with 4% PFA for 1 h at 4 °C and cryoprotected with 20% sucrose overnight. Brain sections (20 μm) prepared with a cryomicrotome (CM1200; Leica) were permeabilized and blocked with PBS containing 0.2% Triton X-100, 10% FBS, and 1% BSA for 1 h at room temperature. After incubation with antibodies for N-cadherin [monoclonal, 1:1,000; BD Bioscience; killed at 2–14 d post injury (dpi)], GFAP (polyclonal, 1:200; Sigma-Aldrich; killed at 2–14 dpi), ionized calcium-binding adapter molecule 1 (Iba1; polyclonal, 1:1,000; Wako; killed at 4–5 dpi), CD45 (NCL-LCA, mouse; Novocastra; killed at 7 dpi), or Pum2 (polyclonal, 1:1,000; Aproscience; killed at 2–14 dpi) overnight at 4 °C, the sections were stained with Alexa Fluor 488- or 546-labeled goat anti-mouse or rabbit IgG antibody (1:500; Invitrogen) for 1 h at room temperature. Nissl staining was performed using NeuroTrace (Invitrogen). Sections were imaged using a confocal microscope (IX70 with FV 300; Olympus) equipped with an oil-immersion objective (60×, NA = 1.2) and an Ar or a green He-Ne laser.

For Western blot analysis, hemisphere-like tissue samples centering at the SWI core site with a diameter of 0.5–1.0 mm were dissected. Samples boiled in SDS-containing buffer were electrophoresed in polyacrylamide gels (SuperSep Ace; Wako) and transferred to PVDF membranes using a Mini Trans-Blot Cell and PowerPac300 (Bio-Rad). Proteins were probed with antibodies against Pum2 (1:1,000; Aproscience), N-cadherin (1:1,000; BD Bioscience), or GAPDH (1:2,500; Chemicon) for 1 h at room temperature and then with HRP-conjugated goat anti-mouse or rabbit antibody (MBL) for 1 h at room temperature. HRP was detected by Western Lightning ECL (PerkinElmer).

DNA Constructs and Virus Injection. The red fluorescent protein (RFP)-Pum2 expression vector was constructed by inserting rat Pum2 cDNA into pTagRFP-C (Evrogen). Pum2Δ was produced by truncating the RNA-binding domain of Pum2 (7). Pum2-activity reporter was constructed by subcloning the 3′ UTR of the N-cadherin (NM_031333) or the E-cadherin (NM_031334) gene fused to D2EGFP gene into pcDNA3.1(+). Pum2-insensitive reporter was produced by deleting the Pum2-binding sequence (TGTACATAA) in 3′ UTR of the N-cadherin gene. Adenovirus encoding IP_3 5-phosphatase (5ppase)-internal ribosome entry sites (IRES)-GFP, RFP-Pum2, RFP-IRES-Cre, GFP, or RFP was produced using cosmid vector (pAxCAwtit for 5ppase-IRES-EGFP and GFP; pAdenoX for RFP-Pum2, RFP-IRES-Cre, and RFP), and purified as described previously (8).

For adenovirus injection, mice were anesthetized with isoflurane, and craniotomy was performed on the somatosensory cortex (∼1 mm posterior, ∼1.5 mm lateral from the Bregma). The glass pipette filled with virus-containing solution was in-

serted to a depth of ~500 μm from the pia. Virus-containing solution was delivered at a rate of 20 nL min^{-1} using a micropump (Muromachi Kikai). The pipette was left in place for 10 min to prevent leakage of the solution. After 36 h, animals were killed for histochemical analysis or subjected to SWI.

Astrocyte Culture. Astrocytes were prepared from the neocortices of embryonic day 18–19 Sprague–Dawley rat fetuses or P1–3 mice as described previously (9). In brief, a neocortical cell suspension obtained by 2.5% trypsin treatment and gentle trituration was plated on a collagen-coated dish and cultured at 37 °C in DMEM containing 10% FBS. After several replatings, an astrocytic culture with >95% purity was obtained. Lipofectamine 2000 (Invitrogen) reagent was used for transient transfection. For immunocytochemistry, astrocytes were fixed with 4% PFA for 10 min and then permeabilized with 0.2% Triton X-100 for 5 min at room temperature. After blocking with 10% BSA for 30 min at room temperature, cells were incubated with mouse anti–N-cadherin antibody (1:1,000; BD Biosciences) for 16 h at 4 °C and then with Alexa Fluor 488-labeled goat anti-mouse IgG antibody (1:2,000–5,000; Invitrogen) for 1 h at room temperature. Images were acquired with an Olympus IX81-ZDC inverted microscope equipped with a 20× objective (NA = 0.4) and a Hamamatsu C9100-02 EM-CCD camera.

For the in vitro scratch assay, an astrocyte culture obtained from flox N-cadherin mice neocortex was infected by adenovirus vector encoding RFP-IRES-Cre or RFP. After 48 h, the culture was scratched with a 200-μL plastic tip to produce a <700-μm-wide cell-free gap. Then time-lapse fluorescence images of RFP were captured every 10 min for 24 h at 37 °C using the IX81-ZDC microscope. The distance advanced from the wound edge was quantified.

For Ca^{2+} imaging, cortical astrocytes prepared from P1–2 IP_3R2KO or control littermate mice (9) were loaded with 5 μM fluo-4 AM (Invitrogen) for 30 min at room temperature. Ca^{2+} signals were imaged every 1–3 s at room temperature using the IX81-ZDC microscope.

For DNA microarray analysis, 5ppase-expressing astrocytes served as "Ca^{2+} signal-silent" astrocytes and RFP-expressing astrocytes, which generate spontaneous Ca^{2+} oscillations, served as "Ca^{2+} signal-active" astrocytes (9). Total RNA of these cultures was purified using the Qiagen RNeasy Kit, followed by amplification and biotinylation with Affymetrix GeneChip 3′ IVT Express. Gene expression profiles were determined by GeneChip Rat Genome 230 2.0 microarrays (Affymetrix). Data have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (accession no. GSE39979). Confirmation with quantitative PCR analysis was performed using a LightCycler (Roche), SYBR premix Ex Taq (Takara), and primers for rat Pum2 (forward, gatcctcatccagtttgcattt; reverse, cgctccggtactttgctt). The relative level of Pum2 transcript compared with the housekeeping gene GAPDH was determined. Western blot analysis with culture lysates was performed as described above.

Data Analysis. All data were analyzed using MetaMorph 6 (Molecular Devices) and ImageJ (National Institutes of Health). Statistical analyses were performed using ORIGIN (OriginLab). Significance was determined using the Student *t* test or one-way ANOVA with Tukey's post hoc test.

Analyses of the number and morphology of GFAP-positive reactive astrocytes were performed using the *neurite outgrowth* module in MetaMorph. The numbers of reactive astrocytes and processes per cell in IP_3R2KO mice were normalized based on the average values obtained in control littermate mice. The orientation of each process was determined using binary images of individual astrocytes. The angle between the process orientation and a line passing through both the center of the injury site and the cell body was then determined. The fraction of injury site-oriented processes (angles between –90 and 90 degrees) was analyzed.

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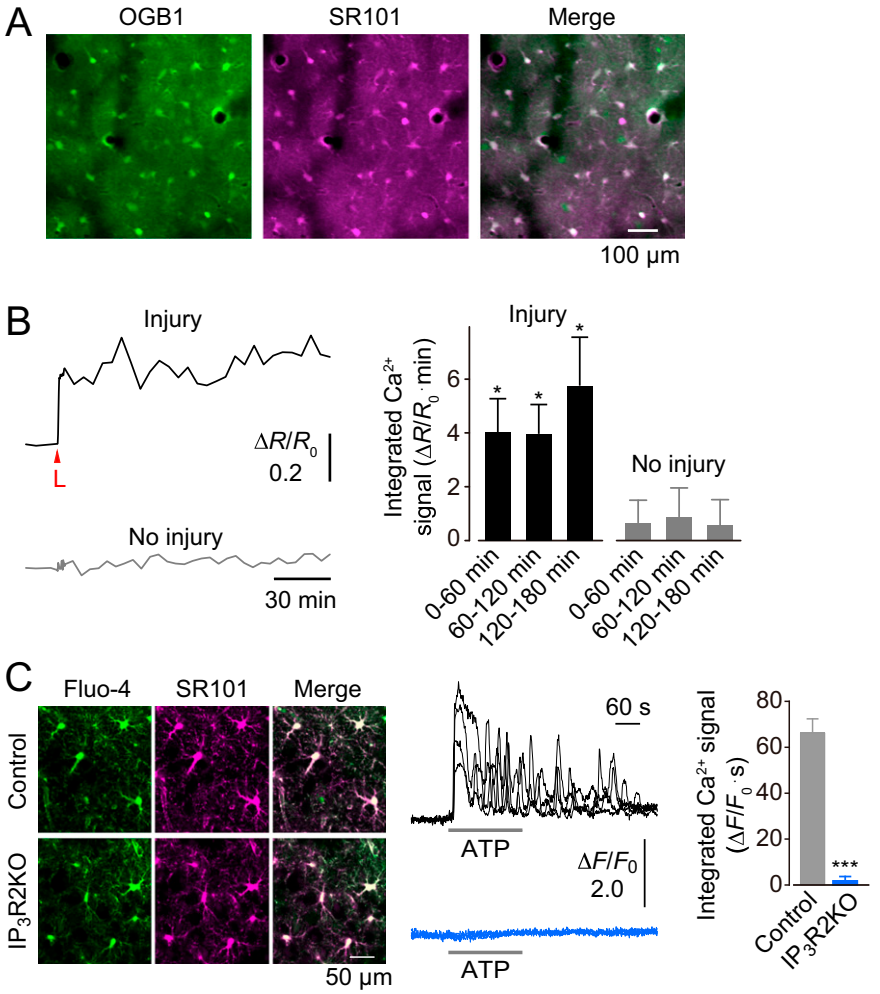

Fig. S1. Ca^{2+} imaging in neocortical astrocytes in vivo and in acute slice preparations from IP_3R2KO and littermate control mice. (*A*) Mouse cerebral cortex was loaded with the Ca^{2+} indicator Oregon Green BAPTA-1 (OGB1) in combination with the astrocytic marker sulforhodamine 101 (SR101). In vivo observation with a two-photon microscope showed that ~90% of cells loaded with OGB1 were stained with SR101 as well. (*B*) Extended time course of astrocytic Ca^{2+} signals in vivo. Astrocytic Ca^{2+} signals were evoked by laser-induced cortical injury as in Fig. 1*A*. Fractional changes in the ratio of OGB1 fluorescence to SR101 fluorescence are shown. (*Left*) Representative Ca^{2+} signals from individual cells. (*Right*) Time integral of Ca^{2+} signals. $n = 18$ cells for injury-applied animals, $n = 14$ cells for controls without injury, from two animals each. $^*P < 0.05$. (*C*) (*Left*) Fluo-4 Ca^{2+} imaging in neocortical astrocytes using acute slice preparations from IP_3R2KO and littermate control mice. Astrocytes were identified by SR101 staining. (*Center*) Ca^{2+} signals from individual cells. $n = 4$ each. (*Right*) Time integral of Ca^{2+} signals for 90 s after injury. $n = 79$ for control and $n = 62$ for IP_3R2KO, from three animals each. $^{***}P < 0.0001$.

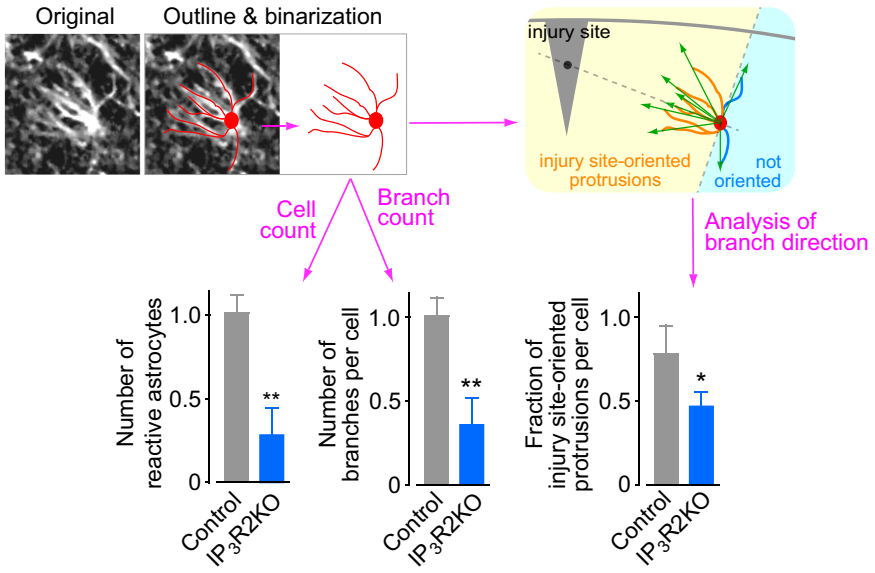

Fig. S2. Quantitative analysis of GFAP immunofluorescence in individual astrocytes. The number of astrocytes (*Left*), the number of branches per cell (*Center*), and fraction of injury site-oriented protrusions per cell (*Right*) were analyzed from the binarized image of GFAP immunofluorescence. In *Left* and *Center*, the values are normalized to those in control littermate animals. $n = 26–45$. $*P < 0.05$; $**P < 0.01$.

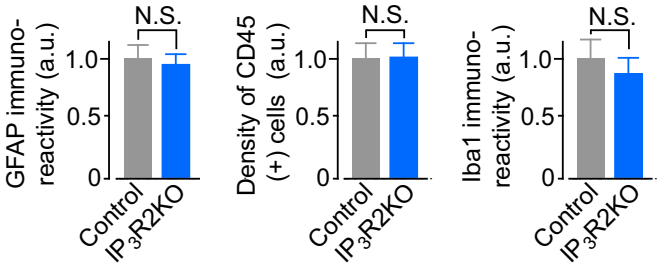

Fig. S3. Examination of IP_3R2KO mouse brains before injury. Levels of GFAP (*Left*), CD45 (*Center*), and Iba1 (*Right*) immunoreactivity in the cerebral cortex were compared between $IP3R_2KO$ mice and littermate control mice before SWI. The values were normalized by the average in control mice. $n = 4$. N.S., not significant.

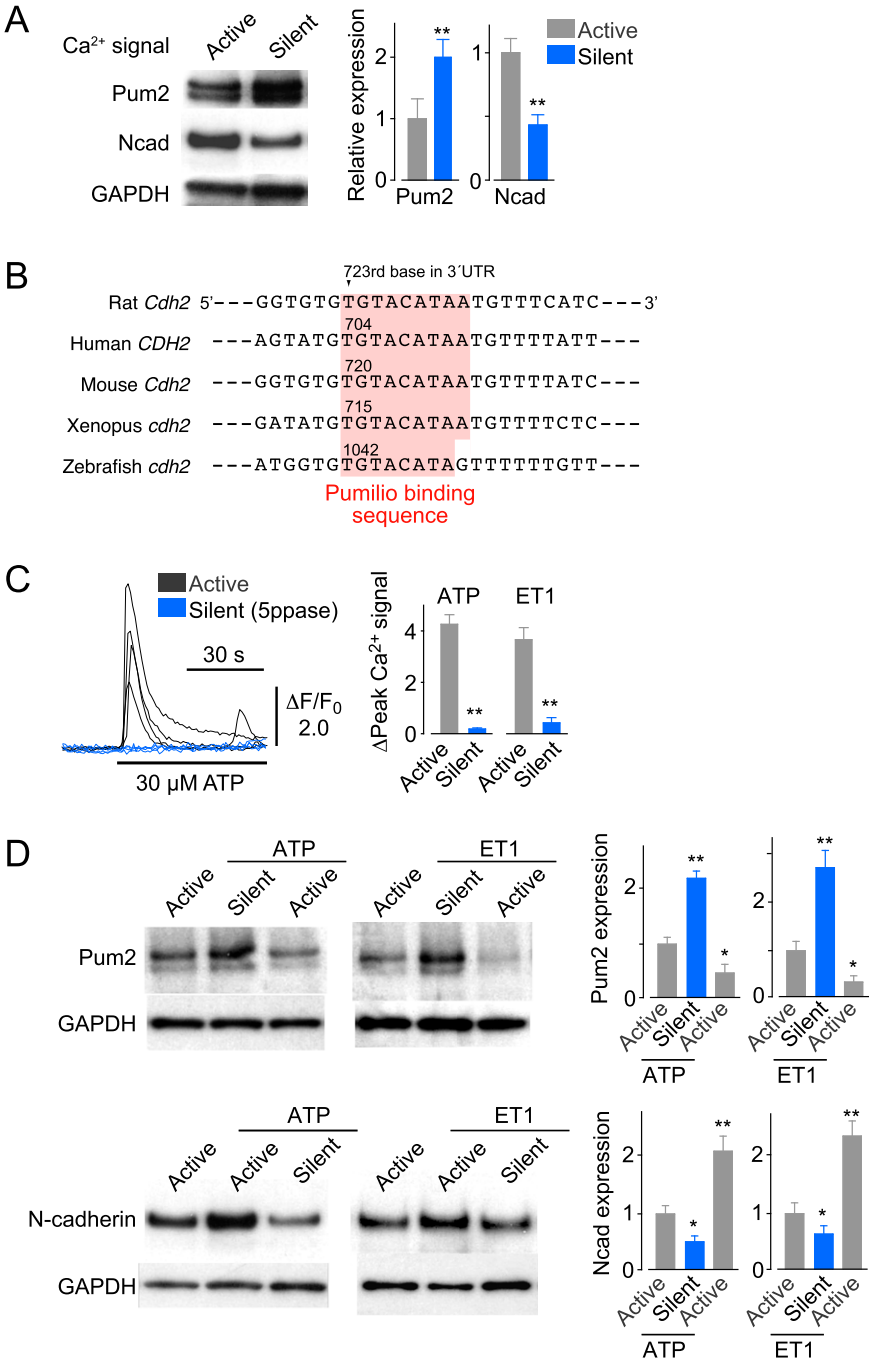

Fig. S4. Identification of N-cadherin and Pum2 as downstream signaling components of astrocytic Ca^{2+} signals. (*A*) Ca^{2+}-dependent regulation of Pum2 and N-cadherin (Ncad) protein expression levels. "Active" and "silent" indicate Ca^{2+} signal-positive normal astrocyte and Ca^{2+} signal-deficient astrocytes (expressing 5ppase), respectively (See *Astrocyte Culture* in *SI Materials and Methods*). $^{**}P < 0.01$. (*B*) Putative Pum2-binding sequence in the 3′ UTR of the vertebrate N-cadherin genes of various species. (*C*) 5ppase-mediated suppression of agonist-induced Ca^{2+} signals in cultured astrocytes. Representative traces (*Left*) and quantification (*Right*) of ATP-induced Ca^{2+} signals with and without 5ppase are shown. $n = 31–35$. $^{**}P < 0.0001$. (*D*) Effect of Ca^{2+} signal suppression on Pum2 and N-cadherin protein expression level. $n = 4$. Adenoviral vectors (silent, 5ppase-IRES-GFP; active, GFP) were used to obtain a high transduction rate (~99%). (*Left*) Western blot analyses performed after overnight incubation with 30 μM ATP or 100 nM endothelin 1 (ET1). (*Right*) Graphs showing signal intensity normalized to that of GAPDH. $^{*}P < 0.05$; $^{**}P < 0.01$.

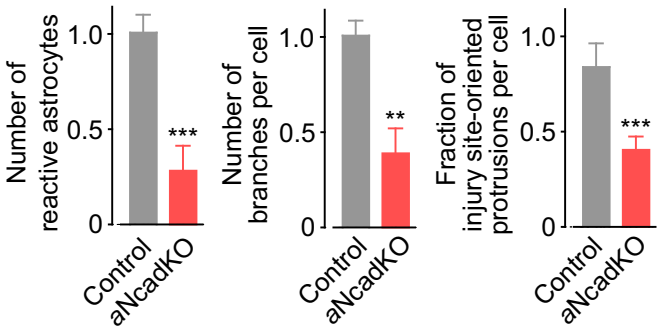

Fig. S5. Quantitative analysis of GFAP immunofluorescence in individual astrocytes in *a*NcadKO mice. The number of astrocytes (*Left*), number of branches per cell (*Center*), and fraction of injury site-oriented protrusions per cell (*Right*) were analyzed following the same procedure as in Fig. S3. $n = 36–60$. $^{**}P < 0.01$; $^{***}P < 0.001$.

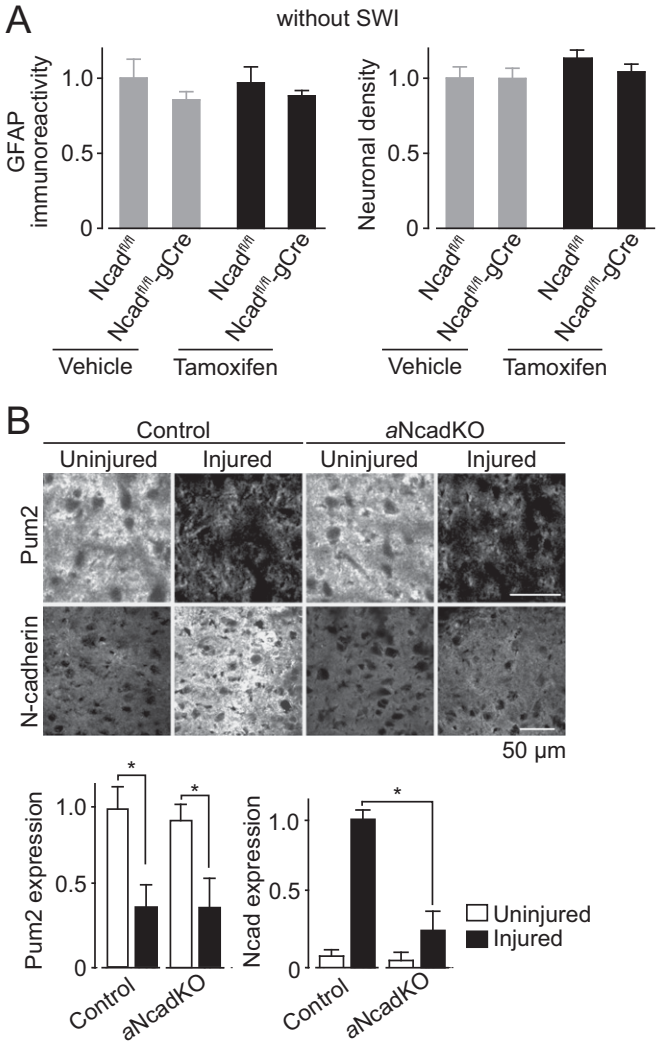

Fig. S6. Analyses using Tx-induced astrocyte-specific N-cadherin KO mice. (*A*) Comparison of the effects of Tx and N-cadherin gene disruption on GFAP expression levels and neuronal density before SWI. In the absence of SWI, GFAP immunoreactivity and neuronal density in the cerebral cortex were compared in N-cadherin floxed mice with the GLAST-CreERT2 gene and those without this gene (designated $Ncad^{fl/fl}$-gCre and $Ncad^{fl/fl}$, respectively) treated with corn oil with or without Tx. $n = 3–6$. $P > 0.08$ for all combinations (Tukey's test). Note that the mice designated as control and *a*NcadKO in Fig. 4 and Figs. S5, S6*B*, and S8 correspond to Tx-treated $Ncad^{fl/fl}$ and Tx-treated $Ncad^{fl/fl}$-gCre mice, respectively. (*B*) (*Upper*) Injury-induced changes in Pum2 and N-cadherin expression in control and *a*NcadKO mice. (*Lower*) Graphs showing immunofluorescence intensity normalized to the average value at the uninjured sites in control mice. $n = 6–14$. $^{*}P < 0.05$.

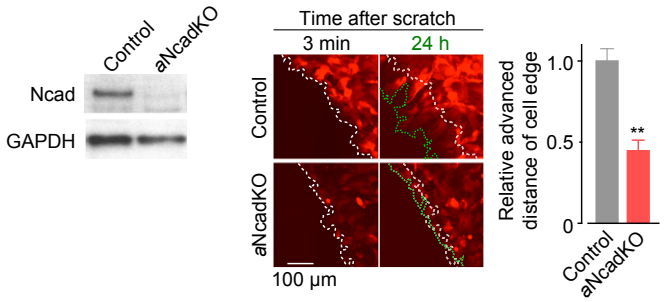

Fig. S7. Scratch wound assay using *a*NcadKO and control astrocytes. (*Left*) Western blot analysis showing the depletion of N-cadherin protein in cultured *a*NcadKO astrocytes produced by adenoviral transduction of N-cadherin floxed astrocytes with Cre recombinase (and infection marker RFP). Control cells were transduced with RFP. (*Center*) Images of astrocytes at 3 min or 24 h after the scratch wound. Dotted lines indicate the edges of astrocytes (white, 3 min; green, 24 h). (*Right*) Distance of cell edge advancement, normalized to the value in control cultures. $n = 15–19$. $^{**}P < 0.01$.

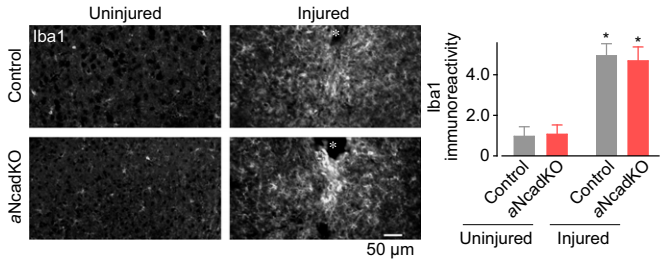

Fig. S8. Microglial activation in *a*NcadKO mice after SWI. (*Left*) Representative images of Iba 1 immunostaining. (*Right*) Graph showing quantification of Iba 1 immunoreactivity normalized to the value on the contralateral uninjured side. $n = 4–6$. $^{*}P < 0.05$.

Table S1. Down-regulated genes in Ca^{2+} signal-active astrocytes

Down-regulated genes in Ca^{2+} signal-active astrocytes	Probe ID	Gene name	Change log2 ratio
Gap junction membrane channel protein alpha 5	1387207_at	*Gja5*	5.4
Similar to OCTN3	1391516_at	*LOC303140*	5.4
Chondroitin sulfate proteoglycan 2	1388142_at	*RGD:619940*	5.4
Pumilio 2	1380604_at	*Pum2*	5.1
Chondroitin sulfate proteoglycan 2	1388054_a_at	*RGD:619940*	4.9
Solute carrier family 8 (sodium/calcium exchanger), member 3	1379701_at	*Slc8a3*	4.8
Similar to Urinary protein 3 precursor (RUP-3)	1370350_x_at	*Atpi /// LOC300504*	4.5
Myeloid leukemia factor 1	1377659_at	*RGD:1306854*	4.4
Similar to B lymphoid kinase	1385962_x_at	—	4.4
Mitogen-activated protein kinase 8	1392418_at	*Mapk8*	4.3
Mitogen-activated protein kinase kinase kinase 8	1369393_at	*Map3k8*	4.2
Similar to Trypsin V-A precursor	1392496_at	*LOC312273*	4.2
Vanin 1	1389253_at	*RGD:1310075*	4.1
Gene model 253	1376675_at	*RGD:1307951*	4.0
Similar to endolyn	1393466_at	*LOC362616*	4.0
Ig heavy chain 1a (serum IgG2a)	1388272_at	*RGD:1305720*	3.8
3-α-hydroxysteroid dehydrogenase	1370708_a_at	*RGD:708361*	3.8
Uridine phosphorylase 1	1372691_at	*RGD:1305566*	3.7
Similar to Igh-6 protein	1388166_at	*LOC299357*	3.7
Inositol polyphosphate-5-phosphatase A	1390774_at	*Inpp5a*	3.6
MAS-related GPR, member F	1382190_at	*Mrgprf*	3.6
Chondroitin sulfate proteoglycan 2	1371232_a_at	*RGD:619940*	3.6
Aldo-keto reductase family 1, member C-like 1	1394681_at	*RGD:1307514*	3.5
Lipopolysaccharide binding protein	1387868_at	*Lbp*	3.5
Glutamate receptor, ionotropic, kainate 5	1375783_at	*Grik5*	3.4
3-α-hydroxysteroid dehydrogenase	1396933_s_at	*RGD:708361*	3.4
Odorant binding protein 2B	1370632_at	*Obp2b*	3.4
Strongly similar to prohibitin	1375159_at	—	3.3
Similar to hypothetical protein MGC34760	1390801_at	*MGC94799*	3.3
Similar to FLJ00414 protein	1393435_at	—	3.2
Aquaporin 1	1369625_at	*Aqp1*	3.2
Selenoprotein N, 1	1395380_at	*RGD:1304574*	3.2
Similar to ataxin 2 related protein isoform A	1395607_at	—	3.1
Alkaline phosphatase, tissue-nonspecific	1368139_s_at	*Alpl*	3.0

DNA microarray analysis using GeneChip Rat Genome 230 2.0 microarrays (Affymetrix) was carried out to evaluate Ca^{2+}-dependent changes in gene expression profile using an homogenous culture of Ca^{2+} signal-silent and -active astrocytes produced by retroviral transduction of 5ppase. The signal intensity of each gene in the Ca^{2+} signal-active astrocytes was normalized to that in the Ca^{2+} signal-silent astrocytes. Highly down-regulated (greater than eightfold decrease in ratio) genes are listed.