2 MATERIAL AND METHODS

2.1 Animals

10-14-day old NMRI mice were either obtained from the research group's own NMRI breeding in the local animal facility or were delivered by Tierzucht Schönwalde GmbH or by Charles River Laboratories.

2.2 Preparation of acute brain slices

2.2.1 Buffers and solutions

• **Bicarbonate buffer** (Artificial Cerebrospinal Fluid, ACSF):

For preparation, Ca^{2+} dye loading, storage and perfusion of acute mouse brain slices the following bicarbonate buffered salt solution mimicking the composition of the cerebrospinal fluid (therefore also named Artificial Cerebrospinal Fluid, ACSF) was used:

Sodium chloride (NaCl)	134 mM	(Roth)
Potassium chloride (KCl)	2.5 mM	(Roth)
Magnesium chloride ($MgCl_2$)	1.3 mM	(Roth)
Calcium chloride ($CaCl_2$)	2 mM	(Roth)
Dipotassium hydrogenphosphate (K_2HPO_4)	1.25 mM	(Merck)
Sodium hydrogencarbonate ($NaHCO_3$)	26 mM	(Roth)
D-Glucose	10 mM	(Merck)

The buffer was gassed with carbogen (95% O_2, 5% CO_2) for 20-30 minutes to ensure saturation with oxygen (O_2) and carbon dioxide (CO_2) before the pH was adjusted to 7.4 with additional sodium hydrogencarbonate. A share of of this buffer was cooled down on ice to below 6 °C and was used for the preparation procedure of acute brain slices. During all times, except during the preparation itself and the Ca^{2+} dye loading, the buffer was directly carbogen-gassed.

2.2.2 Preparation procedure

Experiments with acutely prepared brain slices in contrast to experiments with cell cultures allow the investigation of CNS cell types within their natural surrounding. For the accomplishment of relevant measurements it is important to retain an environment as close as possible to the situation *in vivo*, since cells in culture have been observed to express altered properties depending on culture conditions. However, the slicing procedure is damaging the tissue and may also have consequences, which cause artifacts. For slice preparation 10–14-day old NMRI mice were chosen to enable selective loading of astrocytes with a Ca^{2+}-sensitive dye [285]. In brain slices of younger animals, not only astrocytes but also neurons are loaded with Ca^{2+} dye. In older animals the cellular dye-uptake is very low [285].

The mice were decapitated and their brains were removed and transferred to ice-cold bicarbonate buffer for one minute to slow the metabolic rate and other cellular processes and to consequently minimize hypoxic and excitotoxic damage. The cerebellum was removed by a razor blade cut and the cerebrum was glued onto a metal plate holder with an acrylic glue (SuperAttack), the cutting site facing downwards. To ensure an upright position and stability during slicing, the brain was supported at its ventral site, facing away from the blade of the vibratome, by an agarose block (2% agarose; Roth). The metal plate was fixed in the slicing chamber of a vibratome and covered with ice-cold bicarbonate buffer until the brain was completely immersed. It was proceeded rapidly, without unnecessarily damaging the tissue or putting pressure on it. Coronal cortical slices of 250-µm thicknesses were cut in ice-cold bicarbonate buffer using either a vibratome (VT 1000 S; Leica) or a “Mikrotom mit vibrierender Klinge” (HM 650 V; Microm International) equipped with a razor blade (Wilkinson Sword). Slices were transferred to a store, containing bicarbonate buffer of room temperature, by a wide glass pipette and kept for at least 45 minutes prior to staining. Slices were not longer used than 8 hours after the preparation.

2.3 Cell culture

2.3.1 Solutions, buffers, culture media, and supplements

• HEPES buffer

For Ca^{2+} dye loading, storage, and perfusion of cell cultures the following HEPES buffered salt solution was used.

NaCl	150 mM	(Roth)
KCl	5.4 mM	(Roth)
$CaCl_2$	1.3 mM	(Roth)
Magnesium sulfate ($MgSO_4$)	0.83 mM	(Roth)
HEPES	10 mM	(Roth)
Glucose	5 mM	(Merck)

The pH was adjusted to 7.4 with 1 M NaOH.

· Dulbecco´s modified Eagle Medium (DMEM)

DMEM was used for cultivation of primary astrocytes and microglia and for preparation of oligodendrocytes.

DMEM		(Gibco)
Fetal calf serum (FCS)	10%	(Gibco)
L-Glutamin	2 mM	(Seromed)
Penicillin	100 units/ml	(Seromed)
Streptomycin	100 µg/ml	(Seromed)

· Basal Medium Eagle (BME)

BME was used for preparation of cerebellar granule cells.

BME		(Seromed)
FCS	10%	(Gibco)
KCl	25 mM	(Roth)
glucose	32 mM	(Merck)
L-Glutamin	2 mM	(Seromed)
Penicillin	100 units/ml	(Seromed)
Streptomycin	100 µg/ml	(Seromed)

· SATO medium

SATO medium [286] was used for cultivation of oligodendrocytes and cerebellar granule cells.

DMEM powder	10 g/l	(Serva)
Sodium hydrogen carbonate ($NaHCO_3$)	11.91 mM	(Merck)
D-(+)-Glucose	32 mM	(Sigma)
Transferrin	0.1 g/l	(Sigma)
Insulin	10 mg/l	(Sigma)
Putrescine	0,1 mM	(Sigma)
Progesterone	0,2 µM	(Sigma)
3,3´,5-Triiodo-L-Thyronine (T3)	0,5 µM	(Sigma)
Thyroxine (T4)	0,5 µM	(Sigma)
Sodium selenite (Na_2SeO_3)	222 nM	(Sigma)
Gentamycine	2.5 g/l	(Seromed)
Bovine serum albumine (BSA)	10 mg/l	(Sigma)
L-Glutamine	2 mM	(Serva)
Potassium chloride (KCl)	25 mM	(Roth)

2.3.2 Glial culture preparations

Cultures were prepared from the brains of newborn NMRI mice. All procedures were carried out under sterile conditions. Newborn NMRI mice were decapitated. Their skulls were cut open and the brains were carefully removed and collected in a petri dish filled with ice cold “Hank´s balanced salt solution” (HBSS; Seromed). For preparation of microglial and oligodendrocyte cultures the olfactory bulbs, the cerebellum, and the brainstem were removed, whereas for the preparation of cortical astrocyte cultures, only the cortex was further processed. The cortical hemispheres were separated and carefully freed from meninges to avoid contamination of the cultures by endothelial cells and fibroblasts. The brain tissue was then collected in a falcon tube filled with HBSS for enzymatic and mechanic dissociation. First, it was incubated with 1% trypsin (Boehringer) and 0.05% DNAse (Worthington) at room temperature. The DNAse was used to circumvent the formation of aggregates caused by released DNA. This enzymatic degradation reaction was stopped after 5 minutes by addition of serum-containing “Dulbecco´s modified Eagle Medium” (DMEM; for composition, see 2.3.1). The tissue was then carefully homogenized with a fire-polished glass pipette and it was filled up with more DMEM. Bubbling

air through the homogenate was avoided to circumvent detrimental oxidation processes. The homogenate was centrifuged at 800 rpm at 4°C for 10 minutes. The supernatant was removed; the pellet resuspended in DMEM and the centrifugation step was repeated. After removal of the supernatant the cells were resuspended in the appropriate amount of DMEM (5 ml per brain) and cultured in poly-L-lysin (PLL, 100 µg/ml; Sigma)-coated 75 cm^2 culture flasks (Nunc) in a volume of 10 ml. For astrocyte cultures, cells were also directly plated on poly-L-lysine-coated glass coverslips (∅ 15 mm) at densities of $3–5\times10^4$ cells per coverslip. 20 coverslips were kept in a Petri dish of 10-cm diameter (TPP). Cells were cultured under standard conditions (37°C, 5% CO_2, 95% air, 90% humidity) in a cell culture incubator in DMEM. One day later, cultures were washed twice with HBSS to remove cellular debris.

To obtain astrocytic cultures, the cells were maintained for 4 days and after reaching a subconfluent state, microglial cells and oligodendrocytes, as well as their early precursors, were dislodged by manual shaking and removed by washing with HBSS. The purity of the astrocytes was routinely determined by immunofluorescence using an antibody against glial fibrillary acidic protein (GFAP; Dako Cytomation), a specific astrocytic marker. The cultures typically showed more than 90% cells positive for GFAP.

To obtain microglial and oligodendrocytic cultures, mixed glial cells were maintained longer (9-12 days) with medium changes every third day until the astrocytes were confluent.

Microglial cells were then separated from the underlying astrocytic monolayer by gently shaking the bottles for one hour at 37°C in a shaker-incubator (100 rpm). The microglia were seeded on degreased but uncoated glass coverslips at a density of 5×10^4 cells per coverslip, kept in four-well culture plates (Nunc, Wiesbaden, Germany) in DMEM. Cultures usually contained more than 95% microglial cells, as revealed by staining with *Griffonia simplicifolia* isolectin B4-FITC (Sigma), which selectively binds to microglia. Cultures were used for experiments 1 to 5 days after plating.

Oligodendrocytes and their early precursors were then dislodged from the astrocytic monolayer by strong manual shaking and plated on PLL (20 µg/ml)-coated glass coverslips at densities of $3–5\times10^4$ cells per coverslip, of which 20 were kept in a Petri dish of 10 cm diameter in SATO medium (for composition, see 2.3.1) supplemented with 2% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin. Without additional growth factors most precursors developed into oligodendrocytes, which began to mature within a few days.

All glial cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

2.3.3 Cerebellar neuron preparation

Cerebellar neurons (granule cells) were prepared from 5-day-old NMRI mice as described by Schnitzer and Schachner [287] with some modifications. Mice were decapitated and their heads two times disinfected by briefly spraying ethanol on them. The skulls were cut open and the brains carefully taken out and transferred into a petri dish containing ice-cold HBSS. The cerebelli were cut off, freed from meninges, and collected in a 10-ml Falcon tube in ice-cold HBSS. The HBSS was then removed and replaced by 1% trypsin (Boehringer) and 0.05% DNAse (Worthington) and it was incubated for 3-4 minutes in a cell culture incubator (37°C, 5% CO_2, 95% air, 90% humidity). The trypsin reaction was then stopped by addition of Basal Medium Eagle (BME; for composition, see 2.3.1), the supernatant was removed and 0.05% DNAse was re-added. The brain tissue was carefully 3 times homogenized with a Pasteur pipette, it was filled up with BME and further homogenized. Cells were then spun down at 800 rpm at 4°C for 10 minutes. The supernatant was removed and the pellet resuspended in 1 ml BME per animal. The homogenate was filtered through a 40-µm sieve into a 50-ml Falcon tube. The tube was gently shaken to disperse the cells before determining the cell concentration. Afterwards, neurons were plated on PLL-coated (100 µg/ml) glass coverslips, kept in Petri dishes of 10-cm diameter at a density of 3×10^5 cells per coverslip in BME at 37°C in a humidified atmosphere of 95% air and 5% CO_2. After one day the culture medium was changed to SATO medium supplemented with 2% FCS and 100 units/ml penicillin and 100 µg/ml streptomycin. Cytosine arabinoside (10 µM; Sigma) was added to prevent proliferation of glial cells. Experiments were started a week after plating.

2.3.4 Cell lines

The mouse glioma cell line GL261 was purchased from the National Cancer Institute, NCI-Frederick (MD, USA) and the 1321N1 human astrocytoma cell line was a kind gift of Georg Reiser (Inst. für Neurobiochemie, Universität Magdeburg, Germany). Cell lines were kept in DMEM in 75 cm^2-flasks. The cell line GL261 was stably transfected with the red fluorescent

protein dsRedII (BD Bioscience) and geneticin (G-418 sulphate; Gibco) was added to their medium at a concentration of 0.15 mg/ml for selection of dsRed-positive cells. Medium was changed twice a week and cells were split after reaching confluence, usually once a week. Cells were plated on glass coverslips, which were kept in Petri dishes of 10 cm diameter at a density of 4×10^5 cells per coverslip and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. They were washed to remove cellular debris the day after plating.

2.3.5 PLL coating

Culture flasks or Petri dishes containing glass coverslips (∅ 15 mm) were incubated at least for half an hour with 0.01% PLL-solution (100 µg/ml or 20 µg/ml PLL for oligodendrocytes). The flasks were then washed three times with double distilled water (ddH_2O) and once with phosphate buffered saline (PBS; Gibco).

2.4 Ca^{2+} imaging

2.4.1 Solvents

- Dimethylsulfoxide (DMSO) (Roth)
- Ethanol (Roth)
- Methanol (Roth)

2.4.2 Agonist, antagonists, blockers

Substance (For experiments with cell cultures substances were applied in HEPES; for experiments with acute brain slices in bicarbonate buffer.)	**c [mM]**	**Manufacturer**
- 2-Aminoethoxydiphenylborane (2-APB) *[IP_3R blocker]*	100	(Tocris)
- Adenosine	10	(Sigma)
- Adenosine 5'-triphosphate (ATP)	100	(Sigma)
- Bicuculline *[GABA(A) receptor antagonist]*	10	

· Carbenoxolone *[gap junction and connexin hemichannel blocker]*	100	(Sigma)
· 9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS-15943) *[P1 adenosine receptor antagonist]*	10	(Sigma)
· Ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) *[extracellular Ca^{2+} chelator]*	1000	(Sigma)
· glycyl-L-phenylalanine 2-naphthylamide (GPN) *[cathepsin C substrate; leads to osmotic burst of lysosomes]*	200	(Sigma)
· Lanthanium Chloride ($LaCl_3$) *[CCE blocker]*	20	(Sigma)
· N_ω-nitro-L-arginine (L-NNA) *[NOS inhibitor]*	2000	(Sigma)
· Nicotinic acid adenine dinucleotide (NAAD)	5	(Sigma)
· Nicotinic acid adenine dinucleotide phosphate ($NAADP^+$)	5/10/25	(Sigma)
· Nicotinamid adenine dinucleotide phosphate ($NADP^+$)	5	(Sigma)
· (S)-3,5-Dihydroxyphenylglycine (DHPG) *[group 1 mGluR agonist]*	20	(Sigma)
· (S)-α-Methyl-4-carboxyphenylglycine (MCPG) *[non-selective group I and II mGluR antagonist]*	500	(Sigma)
· S-nitrosoglutathione (SNOG) *[NO donor]*	100	(Sigma)
· Reactive Blue *[P2Y purinoceptor antagonist]*	30	(Sigma)
· Suramine *[non-selective purinoreceptor antagonist]*	100	(Sigma)
· Trans-(1S,3R)-1-amino-1,3-cyclopentanedicarboxylic acid (t-ACPD) *[group 1 mGluR agonist]*	50	(Sigma)
· Tetrodotoxin (TTX) *[Na^+ channel blocker]*	1	(Tocris)

· Thapsigargin (tg) *[SERCA inhibitor]*	1 or 0.5	(Sigma)
· Verapamil *[VGCC blocker]*	100	(Tocris)

2.4.3 Fluo-4-loading of slices

Slices were bulk-loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-4 in its acetoxymethylester form (Fluo-4/AM; Molecular Probes or Invitrogen; Fig. 6*A*). An acetoxymethylester group renders the membrane impermeable dye membrane permeable. Once taken up by a cell, cytosolic esterases remove this goup and the dye remains within the cells [288]. This dye changes its spectral properties upon the binding of calcium ions. In the Ca^{2+}-free form, the dye is not fluorescent, whereas in the Ca^{2+}-bound form, it can be excited to green (520 nm) fluorescence by light of a wavelength of 488 nm (Fig. 6*B*). Since Fluo-4 is a non-ratiometric dye with a single excitation and a single emission wavelength, it is not possible to calibrate the recorded fluorescence intensities and to determine absolute Ca^{2+} concentrations in the cells. Only relative values are obtained. In this work fluorescence intensity values are nomalized by the average of the first 12 measured values (F_0) to obtain a relative unit F/F_0.

The slices were incubated with Fluo-4-acetoxymethylester (10 µM) in bicarbonate buffer containing 0.02% of the surfactant and detergent Pluronic-127 (Molecular Probes) at room temperature for 40-50 minutes followed by 10-15 minutes in a cell culture incubator at 37°C and 5% CO_2. During the period at room temperature the Fluo-4/AM-containing buffer was not gassed directly, but its environment was carbogen saturated. For the above described staining protocol used with aninmals of the age range between 10-14 days it was previously demonstrated that astrocytes are loaded almost exclusively [285]. After staining, slices were moved back to the store and used for experiments within two hours.

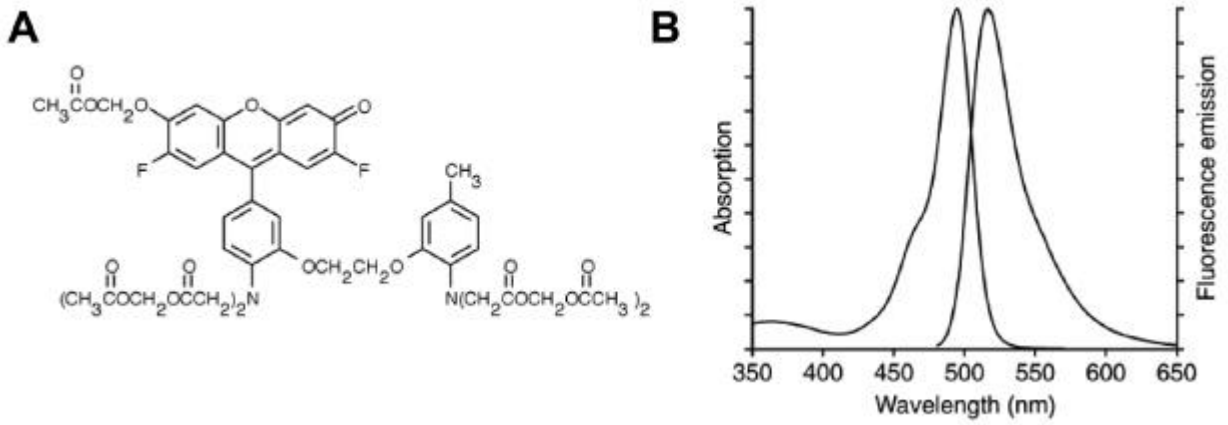

Fig. 6 Fluo-4

A, molecular structure of Fluo-4/AM. *B*, absorption and fluorescence emission spectra of Ca^{2+}-saturated Fluo-4 in pH 7.2 buffer. Figures are copied from www.invitrogen.com.

2.4.4 Fluo-4-loading of cells in culture

Cultured or acutely isolated cells were plated on glass coverslips, loaded with Fluo-4/AM (5 µM) for 30 minutes at room temperature in HEPES containing 0.01% Pluronic-127 (Molecular Probes). Subsequently cells were washed and kept in bath solution (HEPES) for at least 10 minutes prior to the experiments. The following procedures were identical with the description for confocal- and videomicroscopy for brain slices apart from the utilisation of HEPES instead of bicarbonate buffer.

2.4.5 Confocal- and Videomicroscopy

For the imaging procedure the brain slices were fixed on a coverslip using a U-shaped platinum wire stringed with nylon threads and then transferred to a custom-made plexiglass perfusion chamber on an upright microscope (Axioskop FS or Axioplan, Zeiss) equipped with a 20 times magnifying water immersion objective (UMPlanFl, numeric aperture: 0.5; Olympus). Slices were continuously superfused with gassed bicarbonate buffer at a flow rate of around 3-4 ml per minute. Inflow was kept up solely by gravity and surface tension; outflow was achieved by a pump (model 1B./003-304MC2/65; Petro Gas Ausrüstung). Both were adjusted so the buffer volume in the measuring chamber remained constant over time. Substances were applied by changing the perfusate through plastic switches built in in the inflow perfusion tube close to the

various solution containers or simply by transferring the inflow tube from one solution container to another.

Intracellular Ca^{2+} changes were detected using either a confocal laser-scanning microscope (Sarastro 2000, Molecular Dynamics) with an open pinhole or a conventional imaging system. As a light source for fluorophore excitation for the laser-scanning microscope served the 488 nm band of an Ar^{+}/Kr^{+}-laser at an intensity of 30%. The fluorescence light emission was controlled by a 510 nm secondary dichroic beam splitter, followed by a 515-545 nm band pass filter and detected by a photomultiplier tube. The light source for the imaging system was the Xenon lamp of a monochromator (Polychrome 4; Till Photonics) set to 488 nm in combination with the XF100-2 filter set of Omega Optical inc. for Fluo-4 detection. Images were taken with a cooled charge coupled device (CCD) camera (SensiCam, PCO). Both, the monochromator and the CCD camera were operated via an EPC9 patch clamp amplifier (Heka) by the TIDA programme (Heka), used in combination with the 'Easily Imaging Cells' software (Heka). Due to the scanning rate of the confocal microscope the sampling rate was limited to 0.25 Hz, whereas with the CCD-camera 0.5 Hz was achieved. The exposure time for the conventional imaging setup was 180 milliseconds combined with a binning of 2×2 pixels. Pixel intensities were recorded within the range of 0-255. Images were stored on a PC and further processed and analysed with conventional software (ImagePro, Media Cybernetics).

The changes in emitted fluorescence light intensity (F) were determined by drawing regions of interest (ROIs) around all single cells within the field of view showing a change in fluorescence, followed by temporal analysis. The obtained F values of single cells were expressed as fluorescence intensity ratios F/F_0 and plotted over time, resulting in F/F_0 traces. The resting fluorescence value F_0 was determined at the beginning of each experiment as an average of the first 12 measured values. For the experiments in which the perfusion or heating were switched off as a stimulus, background subtraction was carried out, since the fluorescence of the areas, in which no cells could be identified, increased relatively strongly at times and masked the spiking patterns of single cells. For background subtraction, three ROIs, not including cells, were placed and their F/F_0 values were calculated. The three F/F_0 traces were averaged and subtracted from the F/F_0 traces of the cells of the respective experiment. Average traces were calculated by averaging the F/F_0 traces of all cells within one experiment.

To study the effect of pharmaceuticals on $NAADP^+$- or stop of perfusion-induced Ca^{2+} signaling, $NAADP^+$ was applied twice or the perfusion was switched off twice respectively, separated by a washout and/or drug preincubation interval of at least 10 minutes. The response of a given slice during the second $NAADP^+$ application or stop of perfusion in the presence of a drug, was then compared to the response during the first "internal control" $NAADP^+$ application or stop of perfusion, respectively, with regard to the responding cell population and the "average amplitude change", a measure for amplitude and frequency of the Ca^{2+} signals. All experiments with $NAADP^+$ were carried out at room temperature to minimize dye leakage. Experiments, in which the perfusion was switched off, were conducted at the temperatures stated in the results section.

2.5 Immunostaining

2.5.1 Buffers

• Phosphate buffer (PB) (0,1 M)

NaH_2PO_4	19 mM	(Merck)
Na_2HPO_4	81 mM	(Merck)

The pH was adjusted to 7.4 using 1 M NaOH or 1 M HCl.

• Tris-buffered saline (TBS)

Tris	100 mM	(Sigma)
NaCl	150 mM	(Roth)

The pH was adjusted to 7.4 using 1 M NaOH or 1 M HCl.

• Blocking buffer for slices

in PB

BSA	0.5%	(Fluka)
Horse serum	1%	(Gibco)
Normal goat serum	4%	(Gibco)
TX100	0.01%	(Merck)

• **Blocking buffer for culture**

in TBS

BSA	5%	(Fluka)
Normal goat serum	5%	(Gibco)

2.5.2 Antibodies

Antibody	Dilution (in the respective blocking buffer)	Manufacturer
• purified rat anti mouse CD38 monoclonal antibody	1:20	(Cedarlane)
• rabbit anti S-100β antibodies	1:500	(Swant)
• rabbit anti GFAP antibodies	1:1000	(Dako)
• *Griffonia simplicifolia* isolectin B4 (ILB4)-FITC	1:50 (in PB)	(Sigma)
• secondary antibody Cy2-conjugated goat anti rabbit IgG (H+L)	1:100	(Jackson/Dianova)
• secondary antibody Alexa Fluor 594 goat anti-rat IgG (H+L)	1:200	(Molecular Probes)
• secondary antibody Alexa-568-conjugated goat anti rabbit IgG (H+L)	1:1000	(Molecular Probes)

2.5.3 Perfusion fixation

To preserve the structure of brain tissue during deep-freezing for the preparation of cryosections or for storage, it has to be fixed by the fixative paraformaldehyde (PA). To obtain an adequate fixation of the brain by also reaching the deep layers, infusion of the fixative through the vascular system was necessary. For this purpose mice were deeply anaesthetised with sodium pentobarbital (100 mg per kg body weight; Sanofi). For perfusion, an animal's thorax was opened to reveal the heart. The pericardium was carefully opened, without injuring the heart more severely and interfering with its functionality. To permit the drainage of the blood and later of the perfusion solution, a cava leading into the right half of the brain was severed. The left ventricle was injected with a cannula connected to a tube and a pump by which the respective solutions were pumped though the animals vascular system. Afterwards it was first perfused intracardially

with a physiological salt solution (0.9% NaCl) to wash out the blood to prevent its coagulation by the fixative. Then it was perfused with a solution of 4% PA in 0.1 M phosphate buffer (PB, pH 7.4, 100 ml per animal). When the animal stiffened and contracted, brains were dissected out and postfixed for 24 hours at 4°C in the same fixative. Subsequently they were transferred to a 30% sucrose (Merck)-containing PB and incubated for several days to osmotically withdraw water from the tissue to prevent damage by developing ice crystals, until the tissue's density was higher than that of the sucrose solution. Brains were quickly frozen in isopentane (methylbutane, Roth) cooled by dry ice and kept at –80°C.

2.5.4 Cryosections

The fixed brain tissue was embedded in Tissue Tek (Sakura Finetek) and fixed on a metal plate, which served as a holder, in the cooled workspace of a kryotome (Leica CM3000). It was cut coronally into 16 µm-thick slices at –20°C. Cryosections were mounted on gelatine-coated slides, allowed to dry for at least 30 minutes at room temperature and then stored at –20°C.

2.5.5 Immunostaining of slices

A specific antibody staining for the $NAADP^+$-generating enzyme CD38 was conducted combined with fluorescence secondary antibody detection. This was carried out in parallel with an antibody staining for S-100β, a specific marker for astrocytes in the cortex, to assess whether cortical astrocytes express CD38.

After thawing, cryosections were postfixed for 15 minutes with 4% PA solution, then permeabilized with a 0.1% solution of the surfactant and detergent Triton X-100 (TX100, Merck) in PB for 15 minutes and incubated in "blocking buffer for slices" (for composition, see 2.5.1) for 45 minutes at room temperature to saturate unspecific binding sites. Rabbit anti S-100β antibodies were diluted 1:500 and incubated with the sections for 5 hours at room temperature. Sections were washed with PB and then incubated with 1:20 diluted purified rat anti mouse CD38 monoclonal antibody over night. Sections were again washed with PB followed by incubation with the two secondary antibodies Cy2-conjugated goat anti rabbit IgG (H+L) (1:100 dilution) for αS-100 antibody and Alexa Fluor 594 goat anti-rat IgG (H+L) (1:200 dilution) for αCD38 antibody detection for 5 hours at room temperature. After three washes, sections were

mounted with CitiFluor (Agar Scientific) and inspected with the confocal microscope described in the Ca^{2+} imaging section with a 63 times magnifying oil immersion objective. Green fluorescence of the Cy2-conjugated secondary antibody was excited by 488 nm (30% intensity) and detected through the filters described in the Ca^{2+} imaging section by a photomultiplier tube. Red fluorescence of the Alexa Fluor 594 secondary antibody was stimulated by 514 nm (100% intensity) and detected through a 535 nm secondary dichroic beamsplitter followed by a 570 nm long pass filter. Specificity of immunoreactivity was controlled by incubation of tissue sections in dilution buffer instead of primary antibody solution. These control experiments were always negative.

2.5.6 Immunostaining of cultures

Astrocytes grown on glass coverslips as described above were fixed with 4% PA for 15 minutes at room temperature, washed with TBS, permeabilised with 0.05% TX100 in TBS, and incubated in a "culture blocking buffer" (for composition, see 2.5.1) for one hour at room temperature. Cells were incubated at 4^0C with 1:1000-diluted rabbit anti-GFAP antibodies (Dako) for one hour. Then the cells were washed with the surfactant and detergent Tween 20 (0.5%; Merck) in TBS and incubated with the secondary antibody Alexa-568-conjugated goat anti rabbit IgG (H+L) (1:1000 dilution; Molecular Probes) for one hour and washed again. As a control, cells were incubated in buffer without primary antibodies followed by incubation in secondary antibody solution. Cells were mounted by the ProLong Antifade Kit (Molecular Probes).

For the isolectin B4-FITC staining, microglial cultures grown on glass coverslips, were fixed with 2% PA for 10 minutes, washed with 0.1 M PB and then incubated with 1:50 diluted ILB4 coupled to the fluorophor FITC for 30 minutes at room temperature. After washing with 0.1 M PB, cells were mounted by the ProLong Antifade Kit.

2.6 Statistical analysis

2.6.1 Reacting cell number

The population of cells within a slice, which responded during application of $NAADP^+$ or during switched off perfusion was determined for both, the first "internal control" recording and the second recording in the presence of a drug, and both were compared. The number of cells responding the second time was expressed as percentage of the number of cells responding the first time. It was distinguished between cells, which reacted both times or only the second. The results obtained from different slices for a given condition were averaged.

2.6.2 "Ca^{2+} signaling activity" and "(overall) average amplitude change"

As a second measure, an activity parameter was evaluated and compared for the first and the second response: the "Ca^{2+} signaling activity". It was defined as the average change in the F/F_0 value of single cells between two consecutive images within a set time frame of about two minutes during a $NAADP^+$ application. Consequently, it is equal to the average change in the F/F_0 value of single cells within 2 or 4.5 seconds – the time interval between two consecutive images, depending on the used setup. The 'Ca^{2+} signaling activity' during the first internal control and the second $NAADP^+$ application was then compared for individual cells, which reacted *both* times. Each cell's activity during the second $NAADP^+$ application was expressed as percentage of its activity during the first. Then it was averaged for cells within one slice and subsequently results from the different slices were averaged for a given condition. Therefore, the measure of this Ca^{2+} signaling activity was termed "% average amplitude change" with which the respective bar graphs with black bars were labelled.

It was proceeded similarly with the experiments in which the perfusion or heating was switched off as a stimulus. In those experiments the "Ca^{2+} signaling activity" was determined and compared for *all* cells, which were active during at least *one* of the time frames chosen for comparison and not only time frames during the stimulus were analyzed, but also frames before and afterwards. Furthermore, the values obtained for all cells within a given time frame were averaged first, and only afterwards the averages were expressed as percentages of the average value, calculated for one of the other time frames of the same experiment – usually the one with the highest activity during a stimulus. Finally, it was averaged for the different time frames

across experiments. Therefore, to distinguish this method of evaluation from the preceding one, the bars in the respective bar graphs are dark gray and the y-axis was labelled with "% *overall* average amplitude change". In this case the "Ca^{2+} signaling activity" served as a general measure for activity to obtain an "average activity profile" of the recordings, including all cells, and not only to compare the activity of active cells between the control and the pharmacological condition.

2.6.3 Statistical tests

All values are expressed as mean ± standard deviation. Means and standard deviations were calculated with Excel (Microsoft). Further statistical analysis of the data was performed with the SPSS software (SPSS Inc.) and Excel. For revealing significant differences between reacting cell numbers and average amplitude changes, the multiple Kruskal-Wallis Test was used to test for significant differences between all groups (corresponding to one bar within a bar diagram) within a set of groups (corresponding to a diagram). If it was positive, each group was separately compared with its respective control (the bar in the diagram set 100%) by the paired Mann-Whitney Test. These tests are only suitable for independent variables. Since the values of the overall average amplitude changes in the activity profile diagrams were dependent, significance between the different time frames was tested by the two-tailed Student's T-test for unequal variance or by the Willcoxon Test. Differences were considered as significant when $p < 0.05$, as highly significance when $p < 0.005$.

2.7 Manufacturers

Agar Scientific	Stansted, Essex, UK
BD Bioscience	Heidelberg, Germany
Boehringer Ingelheim Pharma GmbH & Co. KG	Mannheim, Germany
Cedarlane Labs	Ontario, Canada
Charles River Laboratories	Sulzfeld, Germany
Dako Cytomation (Dako)	Glostrup, Denmark
Heka	Lambrecht/Pfalz, Germany
Jackson/Dianova	Hamburg, Germany

Leica	Bensheim, Germany
Life Technologies/Gibco BRL (Gibco)	Eggenstein, Germany
Media Cybernetics	Georgia, USA
Merck	Darmstadt, Germany
Microm International GmbH	Walldorf, Germany
Microsoft	Silicon Valley, CA, USA
Molecular Dynamics	Sunnyvale, CA, USA
Molecular Probes/Invitrogen GmbH	Karlsruhe, Germany
Nunc GmbH & Co. KG	Wiesbaden, Germany
Olympus	Hamburg, Germany
Omega Optical inc.	Brattleboro, VT, USA
Petro Gas Ausrüstung	Berlin, Germany
PCO	Kelheim, Germany
C. Roth GmbH & Co.	Karlsruhe, Germany
Sanofi	Paris, France
Sakura Finetek	Torrance, USA
Seromed/Biochrome	Berlin, Germany
Serva Feinbiochemika GmbH & Co. KG	Heidelberg, Germany
Sigma-Aldrich Chemie GmbH	Deisenhofen, Germany
SPSS Inc.	Chicago, IL, USA
SuperAttack	Loctite, England
Swant	Bellinzona, Switzerland
Tierzucht Schönwalde GmbH	Schönwalde, Germany
Till Photonics	München, Germany
Tocris Bioscience	Cologne, Germany
TPP	Trasadingen, Switzerland
Wilkinson Sword GmbH	Solingen, Germany
Worthington Biochem. Corp.	Freehold, NY, USA