GABA uptake-dependent Ca^{2+} signaling in developing olfactory bulb astrocytes

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Edited by Tullio Pozzan, University of Padua, Padua, Italy, and approved August 21, 2009 (received for review November 25, 2008)

We studied GABAergic signaling in astrocytes of olfactory bulb slices using confocal Ca^{2+} imaging and two-photon Na^+ imaging. GABA evoked Ca^{2+} transients in astrocytes that persisted in the presence of $GABA_A$ and $GABA_B$ receptor antagonists, but were suppressed by inhibition of GABA uptake by SNAP 5114. Withdrawal of external Ca^{2+} blocked GABA-induced Ca^{2+} transients, and depletion of Ca^{2+} stores with cyclopiazonic acid reduced Ca^{2+} transients by approximately 90%. This indicates that the Ca^{2+} transients depend on external Ca^{2+}, but are mainly mediated by intracellular Ca^{2+} release, conforming with Ca^{2+}-induced Ca^{2+} release. Inhibition of ryanodine receptors did not affect GABA-induced Ca^{2+} transients, whereas the $InsP_3$ receptor blocker 2-APB inhibited the Ca^{2+} transients. GABA also induced Na^+ increases in astrocytes, potentially reducing Na^+/Ca^{2+} exchange. To test whether reduction of Na^+/Ca^{2+} exchange induces Ca^{2+} signaling, we inhibited Na^+/Ca^{2+} exchange with KB-R7943, which mimicked GABA-induced Ca^{2+} transients. Endogenous GABA release from neurons, activated by stimulation of afferent axons or NMDA application, also triggered Ca^{2+} transients in astrocytes. The significance of GABAergic Ca^{2+} signaling in astrocytes for control of blood flow is demonstrated by SNAP 5114-sensitive constriction of blood vessels accompanying GABA uptake. The results suggest that GABAergic signaling is composed of GABA uptake-mediated Na^+ rises that reduce Na^+/Ca^{2+} exchange, thereby leading to a Ca^{2+} increase sufficient to trigger Ca^{2+}-induced Ca^{2+} release via $InsP_3$ receptors. Hence, GABA transporters not only remove GABA from the extracellular space, but may also contribute to intracellular signaling and astrocyte function, such as control of blood flow.

calcium-induced calcium release | GABA transporter | Neuron-glia interaction | sodium imaging | vasoconstriction

Glial cells are electrically inexcitable cells in the nervous system and have long been considered to be supporting cells with little direct impact on neuronal performance. It was only in the last decade when it was recognized that astrocytes, the major class of glial cells in the mammalian brain, participate in synaptic transmission and contribute to information processing. This led to the model of the "tripartite synapse" consisting of pre- and postsynaptic neuronal elements and glial processes (1). Astrocytes detect neuronal release of excitatory neurotransmitters such as glutamate and acetylcholine via G protein-coupled receptors and respond with cytosolic Ca^{2+} signaling (2–4). Astrocytes can then release "gliotransmitters" such as glutamate, D-serine and arachidonic acid in a Ca^{2+}-dependent manner, thus modulating neuronal performance and local cerebrovascular blood flow (5–8). While most studies describe the integration of astrocytes in excitatory neuronal networks in great detail (reviewed in ref. 1), only little information exists about how astrocytes are affected by inhibitory neurotransmitters such as GABA and glycine. $GABA_A$ and $GABA_B$ receptor-mediated Ca^{2+} signaling has been reported in hippocampal astrocytes (9–11), where $GABA_B$ receptors mediate heterosynaptic depression (10). Only a minority of hippocampal astrocytes, however, respond to $GABA_B$ receptor activation with Ca^{2+} increases (11), raising the question how astrocytes are integrated in inhibitory neuronal networks.

Besides GABA receptors, astrocytes express GABA transporters (GATs), which significantly contribute to the clearance of GABA molecules from the synaptic cleft (12), but have not yet been considered to directly participate in neuronal signal processing. Glial GATs are also involved in pathological processes in the brain, for example, in patients with temporal lobe epilepsy, where astrocytic expression of GATs is increased (13), and inhibitors of glial GATs have anticonvulsant effects (14), indicating the involvement of glial GATs in the generation of epileptic seizures. It is not known, however, how glial GATs contribute to epileptic seizures.

In this study, we have investigated the role of GATs for neuron-glia signaling. We have chosen the olfactory bulb for this study, because astrocytic processes ensheath dendrodendritic, mostly GABAergic synapses (15, 16). Our results indicate a principle of GABAergic signaling between neurons and glial cells mediated by GATs. Activation of GABA uptake elicited an intracellular Na^+ rise in olfactory bulb astrocytes. Our results suggest that this Na^+ rise reduced Na^+/Ca^{2+} exchange, thereby leading to a Ca^{2+} increase sufficient to trigger Ca^{2+}-induced Ca^{2+} release via $InsP_3$ receptors. Thus, in addition to clearance of GABA from the synaptic cleft, GABA uptake might serve as a mediator for neuron-glia signaling.

Results

$GABA_A$ Receptors Mediate Ca^{2+} Signaling in Neurons But Not in Astrocytes. In brain slices of 2- to 7-day-old mice (P2–7), application of GABA (300 μM) evoked Ca^{2+} transients in 69% of olfactory bulb astrocytes (n = 899 cells/39 slices/29 animals) and 75% of periglomerular neurons (n = 261/10/6), with mean amplitudes of 128.7 ± 2.5% ΔF (n = 621/39/29) and 76.1 ± 3.2% ΔF (n = 195/10/6), respectively (Fig. 1*A*). In 3-week-old animals (P18–22), the fraction of responsive cells decreased to 34% of astrocytes (n = 47/8/4) and 29% of neurons (n = 306/8/3), with amplitudes of 76.8 ± 14.5% ΔF (n = 16/8/4) and 81.7 ± 5.4% ΔF (n = 88/8/3) (Fig. S1), indicating that GABAergic Ca^{2+} signaling in astrocytes and neurons is developmentally regulated.

We further studied the mechanism underlying GABA-induced Ca^{2+} signaling using mice at an age of P2–7. In addition to GABA-evoked Ca^{2+} signaling, spontaneous Ca^{2+} transients or Ca^{2+} oscillations could also be measured in 68% of the astrocytes (n = 115/2/2; Fig. 1*A*, asterisks) and 25% of the periglomerular neurons (n = 56/2/2). If spontaneous Ca^{2+} signaling occurred shortly before the application of GABA, cells were not used for analysis.

Author contributions: M.D., D.H., J.W.D., and C.L. designed research; M.D., D.H., and P.C. performed research; M.D., D.H., and C.L. analyzed data; and H.-C.P., J.W.D., and C.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0809513106/DCSupplemental.

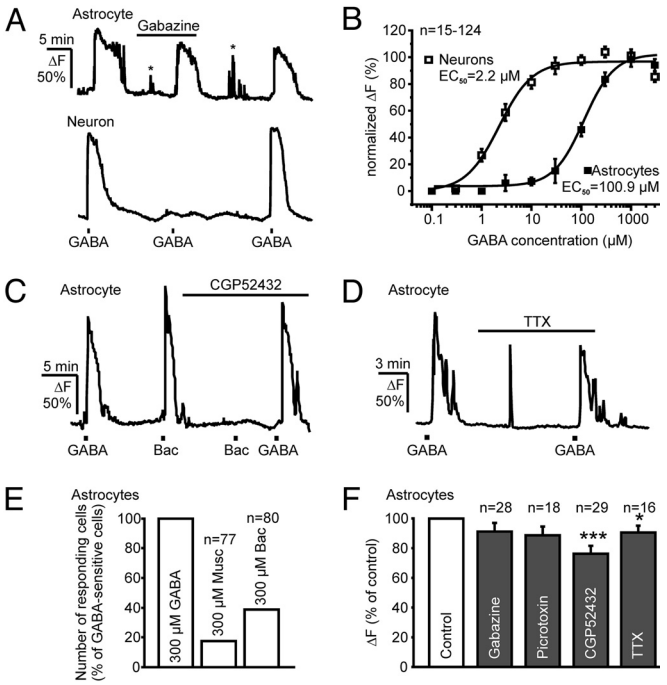

Fig. 1. GABA-mediated Ca^{2+} signaling in olfactory bulb astrocytes. (*A*) The $GABA_A$ receptor antagonist gabazine (60 μM) blocked Ca^{2+} transients induced by GABA (300 μM) in neurons (lower trace), but not in astrocytes (upper trace) at P2–7. Asterisks indicate spontaneous Ca^{2+} signaling. (*B*) Dose-response curve of GABA-induced Ca^{2+} transients in neurons (open squares) and astrocytes (closed squares). (*C*) The $GABA_B$ receptor antagonist CGP52432 (10 μM) blocked Ca^{2+} transients elicited by baclofen (Bac, 300 μM), but not Ca^{2+} transients elicited by GABA (300 μM). (*D*) TTX (2 μM) only weakly reduced GABA-induced Ca^{2+} transients in astrocytes. (*E*) Agonist profile of Ca^{2+} signaling in astrocytes. (*F*) Antagonist profile of GABA-induced Ca^{2+} signaling. CGP52432 (10 μM) and TTX (2 μM) significantly reduced GABA-induced Ca^{2+} transients in astrocytes. *, $P < 0.05$; ***, $P < 0.005$. Bars, means + error of the mean; *n*, the number of cells investigated.

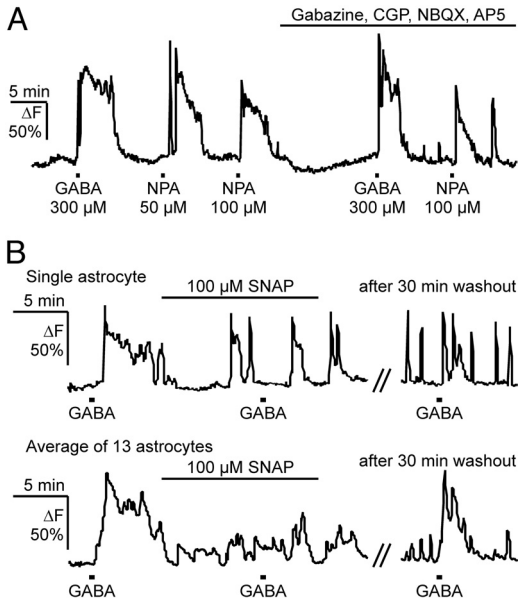

Fig. 2. GABA uptake mediates Ca^{2+} signaling in olfactory bulb astrocytes. (*A*) The GABA transporter substrate nipecotic acid (NPA) induced Ca^{2+} transients similar to GABA-induced Ca^{2+} transients. The Ca^{2+} transients were not blocked by GABA receptor antagonists (60 μM gabazine, 10 μM CGP52432). NBQX (30 μM) and D-AP5 (100 μM) were applied to suppress epileptiform activity induced by gabazine. (*B*) Upper trace, the GAT inhibitor SNAP 5114 evoked Ca^{2+} oscillations in astrocytes, rendering analysis of putative GABA-induced Ca^{2+} transients in single astrocytes difficult. Lower trace, averaged Ca^{2+} signal from 13 individual astrocytes of one experiment to reduce unsynchronized Ca^{2+} oscillations and unmask GABA-evoked Ca^{2+} transients. Inhibiting GABA uptake with SNAP 5114 (100 μM) suppressed GABA-induced Ca^{2+} signaling.

Blocking $GABA_A$ receptors with gabazine suppressed GABA-induced Ca^{2+} transients in periglomerular neurons ($n = 132/7/6$; $P < 0.005$), but had no effect on the amplitude of Ca^{2+} transients in astrocytes ($n = 28/5/4$; Fig. 1 *A* and *F*). The $GABA_A$ receptor channel blocker picrotoxin (100 μM) reduced neuronal GABA-evoked Ca^{2+} transient amplitudes to 34.2 ± 4.1% ($n = 49/3/2$; $P < 0.005$), whereas astrocytic Ca^{2+} transients remained unaffected ($n = 18/3/2$; Fig. 1*F*). The $GABA_A$ receptor agonist muscimol (300 μM) evoked Ca^{2+} transients in all periglomerular neurons that responded to GABA ($n = 77/4/3$), but only in 18% of the GABA-sensitive astrocytes ($n = 51/4/3$; Fig. 1*E*), indicating that olfactory bulb astrocytes employ an additional pathway for GABA-mediated Ca^{2+} rises that neurons lack. This is supported by the delay of the Ca^{2+} response of 79.7 ± 3.4 s in astrocytes ($n = 256/14/8$) with respect to the Ca^{2+} response in periglomerular neurons (Fig. S2). In addition, the GABA sensitivity of astrocytes and neurons is strikingly different. The apparent EC_{50} value of the GABA-induced Ca^{2+} response, as calculated from measurements from at least three individual animals per data point, was 2.2 μM ($n = 52$–172 cells) in neurons and 100.9 μM ($n = 15$–124 cells) in astrocytes (Fig. 1*B*).

Application of baclofen (300 μM), a specific agonist of $GABA_B$ receptors, evoked Ca^{2+} transients in 39% of the astrocytes that responded to GABA ($n = 80/7/6$; Fig. 1 *C* and *E*). The $GABA_B$ antagonist CGP52432 (10 μM) entirely blocked the baclofen-induced Ca^{2+} transients ($P < 0.005$), but reduced the amplitude of GABA-induced Ca^{2+} transients in astrocytes by only 24 ± 4% ($n = 29/3/3$; $P < 0.005$; Fig. 1*F*). Hence, in the majority of astrocytes, GABA-induced Ca^{2+} signaling cannot be explained by $GABA_B$ receptor activation.

The results show that GABA receptors mediate Ca^{2+} signaling only in a minority of olfactory bulb astrocytes. They also argue against the possibility that Ca^{2+} transients in astrocytes depend on GABA-driven activity of neurons or any other neuronal activity, since inhibiting action potential firing with TTX (2 μM) only marginally reduced GABA-induced Ca^{2+} transient amplitudes in astrocytes by 9.5 ± 4.5% ($n = 16/3/3$; $P = 0.011$; Fig. 1*D*), and gabazine blocked Ca^{2+} responses in periglomerular neurons, but not in astrocytes. In neurons, GABA-induced Ca^{2+} transients were also suppressed in Ca^{2+}-free solution ($n = 86/4/3$; $P < 0.005$) and in the presence of the voltage-gated Ca^{2+} channel blocker diltiazem (400 μM; $n = 55/2/2$; $P < 0.005$), suggesting Ca^{2+} influx via voltage-gated Ca^{2+} channels after GABA-evoked depolarization (Fig. S3).

Ca^{2+} Signaling Mediated by GABA Uptake. Since GABA receptor antagonists only weakly reduced GABA-induced Ca^{2+} transients in olfactory bulb astrocytes, we tested the contribution of GATs to GABAergic signaling in astrocytes. Application of the GABA transporter substrate nipecotic acid at concentrations of 50 and 100 μM mimicked the effect of GABA and evoked Ca^{2+} transients in 54% and 68% of the GABA-sensitive astrocytes with amplitudes of 165.3 ± 5.8% ΔF and 167.1 ± 5.9% ΔF ($n = 100/4/3$), respectively (Fig. 2*A*). Next, we tested the effect of gabazine (60 μM) and CGP52432 (10 μM) on Ca^{2+} transients evoked by nipecotic acid. To prevent epileptiform activity of olfactory bulb neurons upon gabazine treatment, we added the glutamate receptor antagonists NBQX (30 μM) and D-AP5 (100 μM; Fig. 2*A*). In the presence of inhibitors of GABA receptors and ionotropic glutamate receptors, the Ca^{2+} transients evoked by 300 μM GABA and 100 μM nipecotic acid were reduced by 34.3 ± 3.8% ($n = 116/7/6$; $P <$

0.005) and 11.1 ± 7.2% (n = 23/2/2; P = 0.018), respectively (Fig. 2*A*). All of the following experiments were performed in the presence of the GABA receptor blockers and, unless otherwise noted, glutamate receptor blockers, to investigate GABA-induced Ca^{2+} signaling independent of GABA receptor activation.

GATs are localized in the rat olfactory bulb, where neurons express GAT1 and astrocytes express GAT3 (17), which corresponds to the murine transporter mGAT4. The involvement of astrocytic GATs in GABA-induced Ca^{2+} signaling was tested by using the non-competitive mGAT4 inhibitor, SNAP 5114 (18). Incubation of the slice in 100 μM SNAP 5114 resulted in Ca^{2+} oscillations in most astrocytes, which made it difficult to distinguish between Ca^{2+} oscillations and putative GABA-induced Ca^{2+} transients in single cells (Fig. 2*B*, upper trace). Therefore, we analyzed the averaged signal derived from all astrocytes that responded to GABA under control conditions within a given slice preparation, thus reducing the randomly occurring Ca^{2+} oscillations, but conserving the Ca^{2+} transients evoked by GABA (Fig. 2*B*, lower trace). GABA-evoked Ca^{2+} responses were strongly reduced by 70.8 ± 1.5% (n = 60/5/5; $P < 0.005$) in the presence of SNAP 5114, indicating that GABA transporters are involved in mediating Ca^{2+} signaling in olfactory bulb astrocytes. The effect of SNAP 5114 on GABA-induced Ca^{2+} signaling was only partly reversible after washout for 30–40 min. The GAT1 inhibitor NNC 711 did not significantly reduce GABA-evoked Ca^{2+} transients (n = 24/3/3; Fig. S4). Hence, GABA-induced Ca^{2+} transients in olfactory bulb astrocytes are mediated by mGAT4, but not by GAT1.

Mechanism of GABA Uptake-Mediated Ca^{2+} Signaling. GABA transporters use the inwardly directed Na^+ gradient as the driving force (19), but do not cotransport Ca^{2+}. This raises the question how GABA uptake is linked to cytosolic Ca^{2+} rises. We first investigated the source of Ca^{2+} to reveal the mechanism underlying GABA uptake-induced Ca^{2+} signaling in olfactory bulb astrocytes. Ca^{2+} transients evoked by GABA were entirely and reversibly suppressed in the absence of external Ca^{2+}, indicating that Ca^{2+} influx is necessary for GABA-induced Ca^{2+} signaling (n = 33/4/3; $P < 0.005$; Fig. 3*A*). Upon re-addition of external Ca^{2+}, a slow increase in intracellular Ca^{2+} was measured (Fig. 3*A*, arrowhead). On top of this slow Ca^{2+} increase, Ca^{2+} transients with a time course similar to GABA-induced Ca^{2+} transients were recorded. When intracellular Ca^{2+} stores were depleted by the endoplasmic Ca^{2+}-ATPase inhibitor cyclopiazonic acid (CPA; 25 μM), GABA-induced Ca^{2+} transients were also largely reduced, on average by 87.2 ± 1.5% (n = 87/4/3; $P < 0.005$; Fig. 3*B*). Ca^{2+} store depletion by CPA resulted in an elevated baseline Ca^{2+} concentration, presumably due to store-operated Ca^{2+} entry (20). The results demonstrate that GABA-induced Ca^{2+} transients in olfactory bulb astrocytes require Ca^{2+} release from intracellular stores, but also depend on Ca^{2+} influx from the extracellular space.

GABA-induced Ca^{2+} increases might result from reduced Na^+/Ca^{2+} exchanger (NCX) efficacy due to elevated intracellular Na^+ concentrations upon GABA uptake, which would lead to a reduced driving force for the NCX. To test whether GABA uptake indeed evoked significant Na^+ rises, we measured changes in the Na^+ concentration by two-photon imaging using the Na^+ indicator SBFI (21). Application of 300 μM and 600 μM GABA evoked Na^+ rises of 2.2 ± 0.1 mM (n = 74/3/2) and 3.2 ± 0.1 mM (n = 188/6/3), respectively, in the cell bodies (Fig. 3*C*), which corresponds to transporter-mediated Na^+ transients in other glial cells (22). Larger Na^+ transients are expected in the fine astrocyte processes due to the larger surface-to-volume ratio (22), but the detector noise rendered SBFI fluorescence recordings impossible in astrocytic processes. Concentrations of 100 μM and 600 μM nipecotic acid evoked Na^+ rises with an amplitude of 1.8+/−0.1 mM (n = 47/3/2) and 2.3+/−0.1 mM (n = 84/3/2), respectively. SNAP 5114 (100 μM) entirely blocked GABA-induced Na^+ transients (n = 240/6/4), indicating that they were mediated by GABA uptake via mGAT4

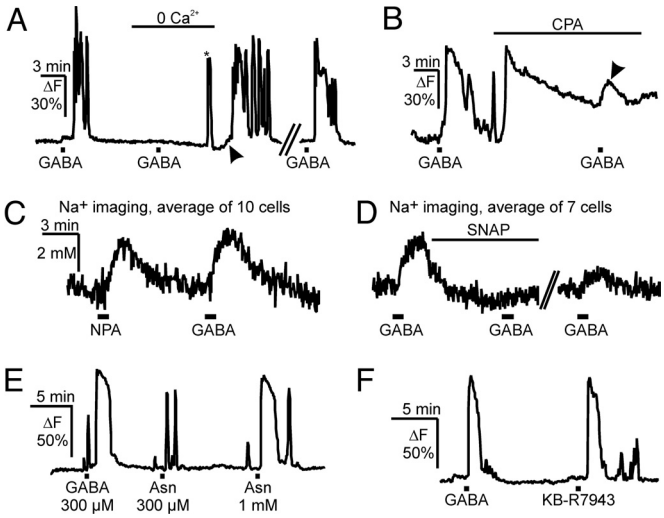

Fig. 3. GABA-induced Ca^{2+} signaling depends on external and internal sources and can be mimicked by NCX inhibition. All experiments were performed in the presence of GABA and glutamate receptor antagonists (gabazine, CGP52432, NBQX, D-AP5). (*A*) Withdrawal of external Ca^{2+} reversibly abolished GABA-induced Ca^{2+} transients. Re-addition of Ca^{2+} evoked a slow Ca^{2+} increase (arrowhead), on top of which Ca^{2+} transients or Ca^{2+} oscillations were recorded. A spontaneous Ca^{2+} transient is indicated by an asterisk. (*B*) Cyclopiazonic acid (CPA, 25 μM) reduced GABA-induced Ca^{2+} transients, unmasking a Ca^{2+} signaling component independent of internal Ca^{2+} release (arrowhead). (*C*) Changes in intracellular Na^+ measured by SBFI. GABA (600 μM) and nipecotic acid (NPA, 600 μM) evoked Na^+ transients in astrocytes. (*D*) SNAP 5114 (100 μM) abolished GABA-induced Na^+ transients. (*E*) GABA- and asparagine-induced Ca^{2+} transients. (*F*) Inhibition of Na^+/Ca^{2+} exchange by KB-R7943 mimics GABA-induced Ca^{2+} transients.

(Fig. 3*D*). To test whether activation of Na^+-driven transporters were sufficient to evoke Ca^{2+} signaling in astrocytes, we applied L-asparagine, which is a substrate for the glial Na^+-dependent neutral amino acid transporter SNAT3 (system N; 23). Asparagine has no significant effect on neurotransmitter receptors or Ca^{2+} release mechanisms, but increases intracellular Na^+ when cotransported with Na^+ by SNATs (23). Application of asparagine evoked Ca^{2+} transients comparable to GABA-induced Ca^{2+} transients in a dose-dependent manner (Fig. 3*E*). One hundred micromolar asparagine evoked Ca^{2+} transients in 25% (n = 16/2/2) of the astrocytes, whereas 300 μM and 1 mM asparagine evoked Ca^{2+} transients in 28% (n = 53/4/4) and 67% (n = 58/5/5) of GABA-sensitive astrocytes, respectively (Fig. 3*D*). The mean amplitudes of the asparagine-induced Ca^{2+} transients were 11.8 ± 5.2% ΔF (100 μM), 29.4 ± 5.8% ΔF (300 μM), and 71.8 ± 6.4% ΔF (1 mM). The mean amplitudes were significantly different from each other ($P < 0.005$, unpaired t test). The results are in line with the assumption that Na^+ rises due to transporter activity in astrocytes are sufficient to induce Ca^{2+} signaling.

If Na^+ rises induce Ca^{2+} transients in astrocytes by reducing NCX activity, pharmacological inhibition of NCX should mimic GABA-induced Ca^{2+} transients. Therefore, we checked whether reduced NCX activity can evoke Ca^{2+} transients by using the NCX inhibitor KB-R7943. Application of 50 μM KB-R7943 for 30 s and 60 s resulted in Ca^{2+} transients similar to GABA-induced Ca^{2+} transients in 58% and 82% of all astrocytes, with a mean amplitude of 105.3 ± 4.8% ΔF (n = 93/6/4) and 106.9 ± 6.5% ΔF (n = 45/4/4), respectively (Fig. 3*F*). Thus, inhibition of NCX activity can mimic GABA-induced Ca^{2+} transients, suggesting that reduced NCX activity mediates Ca^{2+} signaling evoked by GABA uptake into olfactory bulb astrocytes.

$InsP_3$ Receptors Mediate Ca^{2+}-Induced Ca^{2+} Release. GABA-induced Ca^{2+} transients in olfactory bulb astrocytes appear to require a

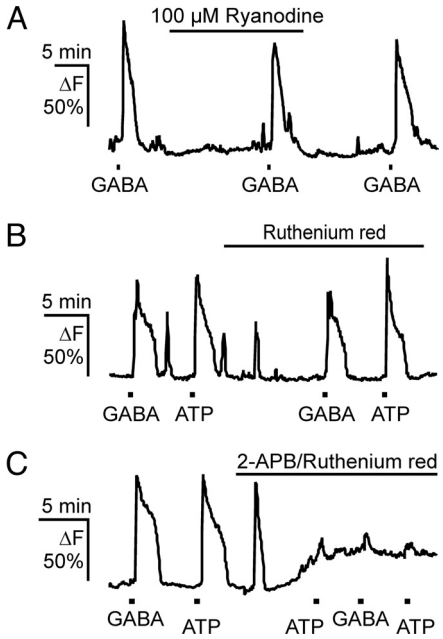

Fig. 4. $InsP_3$ receptors, but not ryanodine receptors, mediate GABA-induced Ca^{2+} signaling in olfactory bulb astrocytes. (*A*) Ryanodine (100 μM) had no effect on GABA-induced Ca^{2+} transients. (*B*) Ruthenium red (10 μM) had no effect on Ca^{2+} transients evoked by GABA and ATP. (*C*) Inhibition of $InsP_3$ receptors with 2-APB (600 μM) blocked GABA- and ATP-induced Ca^{2+} transients almost completely. Ruthenium red (10 μM) was added to suppress 2-APB-mediated Ca^{2+} oscillations.

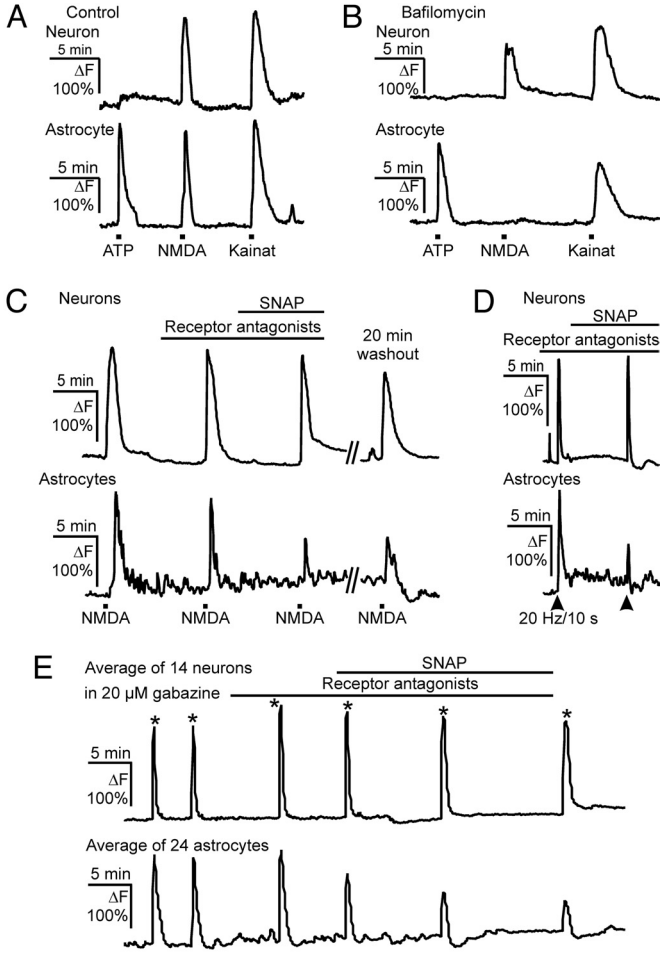

Fig. 5. Endogenous GABA release triggers Ca^{2+} signaling in astrocytes. (*A*) Ca^{2+} transients evoked by ATP (30 μM), NMDA (100 μM) and kainate (100 μM) in neurons (upper trace) and astrocytes (lower trace). (*B*) Suppressing vesicular neurotransmitter release by 10 μM bafilomycin A1 abolished NMDA-mediated Ca^{2+} signaling in astrocytes (lower trace), but not in neurons (upper trace). (*C*) Inhibiting astrocytic neurotransmitter receptors by a mixture of antagonist [30 μM MRS2179 ($P2Y_1$), 1 μM SCH58261 (A_{2A}), 10 μM CGP52432 ($GABA_B$), 60 μM gabazine ($GABA_A$), 10 μM MPEP (mGluR5), and 30 μM NBQX (AMPAR)] did not affect NMDA-induced Ca^{2+} transients in astrocytes. Addition of SNAP 5114 (100 μM) reduced NMDA-induced Ca^{2+} transients in astrocytes (average of 13 cells), but not in neurons (average of 41 cells). (*D*) Ca^{2+} transients evoked by electrical stimulation of receptor axons (50 Hz, 10 s) in the presence of the antagonist mixture were reduced by SNAP 5114 in astrocytes (lower trace, average of 42 cells), but not in neurons (upper trace, average of 35 cells). (*E*) Synchronous neuronal discharges (*, upper trace) induced by 20 μM gabazine. Using a receptor antagonist mixture as above, but with 50 μM IEM 1460 instead of NBQX to block only GluR2-containing, Ca^{2+}-permeable AMPA receptors while allowing for fast glutamatergic synaptic transmission maintained neuronal discharges, which were accompanied by SNAP 5114-sensitive Ca^{2+} transients in astrocytes (lower trace).

small Ca^{2+} increase dependent on external Ca^{2+}, but are mainly attributable to Ca^{2+} release from intracellular stores, in line with Ca^{2+}-induced Ca^{2+} release (CICR). Often, CICR is mediated by ryanodine receptors of the endoplasmic reticulum, but it has also been reported that inositol (1, 4, 5)-trisphosphate ($InsP_3$) receptors can be stimulated by increases in cytosolic Ca^{2+} (24, 25). Ryanodine receptors can be blocked by ryanodine at concentrations >10 μM and by ruthenium red (26). In the present study, neither 100 μM ryanodine (n = 39/3/3) nor 10 μM ruthenium red (n = 21/3/2) reduced GABA-induced Ca^{2+} signaling (Fig. 4 *A* and *B*). These results suggest that ryanodine receptors were not involved in Ca^{2+} increases evoked by GABA.

$InsP_3$ receptors can be blocked by 2-APB at concentrations of several hundred micromolar (27, 28). However, 2-APB can also act on store-operated Ca^{2+} channels and transient receptor potential (TRP) channels (20, 29), thereby either blocking the channels (some TRPCs and TRPMs) or activating the channels (some TRPVs). Application of 600 μM 2-APB resulted in Ca^{2+} oscillations in olfactory bulb astrocytes, suggesting activation of TRP channels. Addition of 10 μM ruthenium red, which also acts as a TRPV inhibitor (30), suppressed the generation of Ca^{2+} oscillations, although an initial Ca^{2+} transient was elicited frequently upon incubation with 2-APB/ruthenium red (Fig. 4*C*). Under these conditions, GABA as well as ATP, which was used as control for $InsP_3$-mediated Ca^{2+} release, failed to induce Ca^{2+} transients in astrocytes (n = 64/4/3; $P < 0.005$). Baseline Ca^{2+} slightly increased upon application of 2-APB, presumably due to Ca^{2+} leakage from internal stores (28). These results suggest that GABA uptake induces Ca^{2+} signaling that is mediated by 2-APB-sensitive $InsP_3$ receptors.

Endogenous GABA Release Triggers Ca^{2+} Signaling in Astrocytes. We asked whether endogenous GABA release after stimulation of GABAergic neurons by either NMDA/kainate application or electrical stimulation of receptor axons would activate astrocytic Ca^{2+} signaling. NMDA (100 μM) and kainate (100 μM) evoked Ca^{2+} transients in periglomerular neurons (n = 88/5/4) and astrocytes (n = 201/5/4; Fig. 5*A*). The V-ATPase inhibitor bafilomycin A1 suppressed NMDA-induced astrocytic Ca^{2+} transients ($P < 0.005$, unpaired t test), while ATP- and kainate-induced Ca^{2+} transients were not blocked (n = 47/3/2; Fig. 5*B*). Hence, kainate appears to induce Ca^{2+} transients in astrocytes directly, presumably via Ca^{2+}-permeable AMPA/kainate receptors, as shown for other glial cells (31, 32). NMDA, in contrast, triggered vesicular neurotransmitter release from neurons and subsequent activation of astrocytes. To suppress Ca^{2+} signaling in astrocytes that was mediated by neuro-

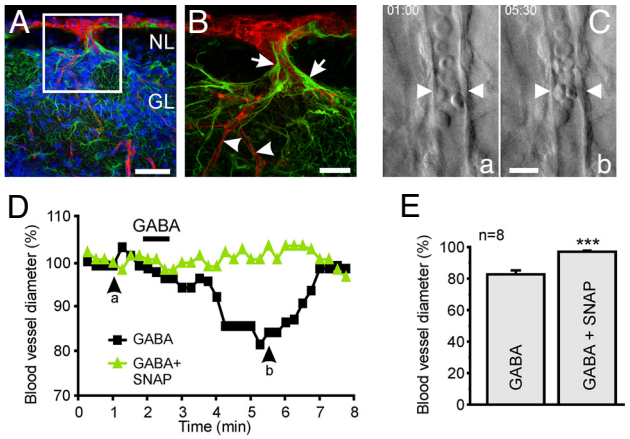

Fig. 6. Glial GABA uptake triggers vasoconstriction. (*A*) Immunostaining against GFAP (green), mouse IgG (blood vessels, red), and nuclear staining (Hoechst 33342, blue) in the nerve layer (NL) and glomerular layer (GL). (Scale bar, 40 μm.) (*B*) Area indicated in *A* at higher magnification. Blood vessels penetrating the nerve layer are densely covered by astrocytic processes (arrows), while blood capillaries surrounding glomeruli are only weakly contacted by GFAP-positive astrocyte endfeet (arrowheads). (Scale bar, 15 μm.) (*C*) Blood vessel in the nerve layer in an intact olfactory bulb before (a) and after (b) application of 3 mM GABA. Arrowheads indicate the center of vasoconstriction. (Scale bar, 10 μm.) (*D*) Changes in blood vessel diameter after GABA application in the absence (black) and presence (green) of SNAP 5114. Arrowheads a and b indicate time points when the images in *C* were taken. (*E*) SNAP 5114 significantly blocked the GABA-induced reduction in blood vessel diameter (***, $P < 0.005$).

transmitter receptors during NMDA application and receptor axon stimulation, we blocked GABA receptors, metabotropic glutamate receptors, AMPA/kainate receptors, $P2Y_1$ receptors and A_{2A} receptors (Fig. 5 *C* and *D*). Under these conditions, NMDA and receptor axon stimulation still evoked Ca^{2+} transients in astrocytes, which were significantly reduced by 56 ± 3% (n = 119/3/2; $P <$ 0.005) and 51.8 ± 2.7% (n = 63/3/3; $P < 0.005$), respectively, by the mGAT4 inhibitor SNAP 5114, suggesting that GABA released by periglomerular neurons induced Ca^{2+} transients in astrocytes via mGAT4. In the presence of SNAP 5114, NMDA-evoked Ca^{2+} transients in neurons were not significantly reduced (n = 74/4/3), while stimulation-evoked Ca^{2+} transients in neurons were reduced by 13.4 ± 2.1% (n = 78/3/3; P = 0.005).

We also measured Ca^{2+} changes in neurons and astrocytes during synchronous (epileptiform) discharges of neurons induced by disinhibition using gabazine (20 μM) in the presence of glial receptor blockers (Fig. 5*E*). The Ca^{2+} transients had an average amplitude of 202 ± 13.8% ΔF (n = 66/4/4) in neurons and 141.9 ± 8.4% ΔF (n = 91/4/4) in astrocytes. SNAP 5114 reduced the amplitude of the Ca^{2+} transients by 16.9 ± 2.7% in neurons (n = 66/4/4); $P < 0.005$) and by 53.4 ± 2.0% in astrocytes (n = 91/4/4; $P < 0.005$).

Astrocytic GABA Uptake Triggers Vasoconstriction. Ca^{2+} transients in astrocytes have been shown to evoke both vasoconstriction and vasodilation, depending on the metabolic state of the tissue (8, 33, 34). In the olfactory bulb, blood vessels that penetrate the nerve layer and enter the glomerular layer are densely covered by astrocytic processes as assessed by GFAP staining (Fig. 6 *A* and *B*). In contrast, only weak GFAP staining was detected around capillaries surrounding glomeruli. In a preparation of the intact olfactory bulb, we measured changes in the diameter of vessels in the nerve layer upon application of GABA in the presence of GABA receptor blockers and TTX. Because the intact pia mater and the nerve layer provide a major diffusion barrier (Fig. S5), we applied 3 mM GABA (1 min) to stimulate GABA uptake into astrocytes in deeper layers. After application of GABA, a constriction of blood vessels in the nerve layer was recorded (Fig. 6 *C* and *D* and Movie S1). On average, the diameter of blood vessels significantly decreased by 17.3 ± 2.3% (n = 8 preparations; $P < 0.005$; Fig. 6*E*). SNAP 5114 significantly reduced the GABA-induced vasoconstriction to 3.2 ± 0.7% (n = 8; $P < 0.005$).

Discussion

In the present study, we have investigated GABA-induced Ca^{2+} signaling in olfactory bulb astrocytes and found a GAT-mediated Ca^{2+}-signaling mechanism. GABA-induced Ca^{2+} transients were independent of GABA receptors, but were a consequence of Na^+-driven GABA uptake. They could be mimicked by inhibition of Na^+/Ca^{2+} exchange and depended on both extracellular Ca^{2+} and $InsP_3$ receptor-mediated intracellular Ca^{2+} release. This suggests that GABA uptake-evoked cytosolic Na^+ transients reduce Na^+/Ca^{2+} exchange, leading to Ca^{2+} rises in astrocytes, which trigger Ca^{2+}-induced Ca^{2+} release via $InsP_3$ receptors (Fig. S6). This study demonstrates that GATs not only take up GABA, but also mediate intracellular signaling in astrocytes.

In immature neurons, which maintain a high intracellular Cl^- concentration, $GABA_A$ receptor activation can lead to depolarization and thereby Ca^{2+} influx via voltage-gated Ca^{2+} channels (35, 36). We studied olfactory bulbs of mice of postnatal days 2–7. At this age, mitral cells already maintain a low intracellular chloride concentration and are inhibited by GABA, whereas olfactory bulb granule cells are depolarized upon $GABA_A$ receptor activation and display Ca^{2+} influx via voltage-gated Ca^{2+} channels (37). Our results show that in the majority of periglomerular neurons, activation of $GABA_A$ receptors triggers Ca^{2+} influx via voltage-gated Ca^{2+} channels. In olfactory bulb astrocytes, $GABA_A$ receptors appear not to play a predominant role in Ca^{2+} signaling, which is in contrast to hippocampal astrocytes (11). The presence of voltage-gated Ca^{2+} channels in astrocytes is controversial, since Carmignoto et al. (38) failed to detect voltage-dependent Ca^{2+} signaling in hippocampal astrocytes. In olfactory bulb astrocytes, voltage-gated Ca^{2+} channels appear not to play a major role in Ca^{2+} signaling, since high K^+ evoked much smaller Ca^{2+} transients in astrocytes as compared to neurons (Fig. S7). The GABA transporter substrate nipecotic acid was able to mimic the effect of GABA, and the mGAT4 inhibitor SNAP 5114 reduced GABA-induced Ca^{2+} transients, suggesting GABA uptake to be the mechanism underlying GABA-induced Ca^{2+} signaling.

Our results suggest that GABA uptake-mediated Na^+ rises trigger Ca^{2+} signaling in astrocytes by reducing NCX efficacy. It has been shown that inhibition of NCX can result in increased Ca^{2+} concentrations in other cells, including glial cells (39). In olfactory bulb astrocytes, this increase in the cytosolic Ca^{2+} concentration depended on extracellular Ca^{2+}. However, the major fraction of the GABA-induced Ca^{2+} transient was dependent on Ca^{2+} release from intracellular Ca^{2+} stores, and GABA evoked only small Ca^{2+} increases after Ca^{2+} stores had been depleted with CPA. Small Ca^{2+} increases as evoked by GABA in the presence of CPA can trigger Ca^{2+}-induced Ca^{2+} release (CICR) from intracellular stores. This mechanism is different from the mechanism in NG2 cells, in which $GABA_A$ receptor activation leads to depolarization, Na^+ influx through persistent Na^+ channels and subsequent reversal of NCX, while CICR has not been reported in NG2 cells (40).

The mechanism presented in this study requires high concentrations of GABA, since the Ca^{2+} response saturated only at GABA concentrations in the millimolar range. Millimolar concentrations of GABA are expected in the vicinity of the synaptic cleft (36), where astrocytic processes, together with pre- and postsynaptic neuronal elements, contribute to the "tripartite synapse" (1). In the olfactory bulb, the neuropil of the glomeruli is strongly pervaded by processes of astrocytes (41), which ensheath dendrodendritic, putative GABAergic, synapses (15, 16). Thus, olfactory bulb astrocytes may detect GABA directly at the synaptic cleft and, as shown

in the present study, respond to GABA released from periglomerular neurons and possibly granule cells with Ca^{2+} signaling. Strong stimulation of neurons triggered a robust global Ca^{2+} signal in the entire astrocyte. Under more physiological conditions such as odorant stimulation, local Ca^{2+} signals might be smaller and may be restricted to astrocyte processes ensheathing GABAergic synapses. A global Ca^{2+} signal could be evoked under pathophysiological conditions such as synchronized neuronal discharges occurring during epileptiform activity. Due to the GABA uptake-dependent mechanism, astrocytes would be able to respond not only to glutamatergic, but also to GABAergic neurotransmission with Ca^{2+} signaling, which might contribute to control of blood flow by vasodilation (34) and vasoconstriction (this study) and, hence, affect neuronal performance.

Materials and Methods

Solutions and Slice Preparation. The standard artificial cerebrospinal fluid (aCSF) for acute brain slices contained (in mM): NaCl 125, KCl 2.5, $CaCl_2$ 2, $MgCl_2$ 1, D-glucose 25, $NaHCO_3$ 26, NaH_2PO_4 1.25, and L-lactate 0.5, gassed during the entire experiment by carbogen to adjust the pH to 7.4. In Ca^{2+}-reduced saline (0.5 mM), 1.5 mM $CaCl_2$ was replaced by 1.5 mM $MgCl_2$. In Ca^{2+}-free saline, Ca^{2+} was replaced by 2 mM Mg^{2+}, and 0.5 mM EGTA was added. In saline with altered sodium concentrations used for SBFI calibration (Fig. S8), K^+-free saline ($0K^+$) or high-K^+ solution (50 mM K^+, $50K^+$), KCl was exchanged by/for NaCl. Olfactory bulb slices were prepared from NMRI mice (P2–P22) as described before (42).

Confocal Ca^{2+} Imaging. Acute brain slices were incubated in the dark at room temperature (21–24 °C) for 60 min in Ca^{2+}-reduced aCSF containing 2 μM Fluo-4-AM, which labels glial cells and periglomerular neurons (41). Ca^{2+} changes in cells of an acute brain slice were measured with confocal laser scanning microscopes (Zeiss LSM 510 and Nikon eC1 plus). To measure Ca^{2+} dynamics, images were acquired in one focal plane at 0.3 Hz. Ca^{2+} signaling was evoked by either drug application or electrical stimulation of olfactory receptor axons using a bipolar tungsten electrode (MPI) connected to a stimulator (SD9K, Grass) and inserted into the olfactory nerve layer.

Data Analysis and Statistics. For analysis of time series, Fluo-4-stained cell bodies were defined as regions of interest (ROIs), and the fluorescence intensity was measured in each ROI. Only cells of the glomerular layer and the external plexiform layer were chosen. Ca^{2+} changes are given as relative fluorescence changes (ΔF) with respect to the resting fluorescence that was normalized to 100%. Only cells that responded with an amplitude of at least 3% ΔF were considered as responsive. Measurements are given as mean values ± SEM, with n giving the number of cells/number of slices/number of animals tested. If not stated otherwise, significance of statistical difference was calculated using Student's paired *t* test, with $P < 0.05$.

Further information is given in the *SI Text*.

ACKNOWLEDGMENTS. We thank P. Meuth (Münster) for software programming, F. Barros (Valdivia, Chile) for helpful discussions, and K. Kaila (Helsinki, Finland) for critical comments on an earlier version of the manuscript. This work was funded by the Deutsche Forschungsgemeinschaft (LO 779/3 and SFB 530, TP B1) and Interdisziplinäres Zentrum für Klinische Forschung Münster (IZKF-FG6).

1. Haydon PG, Carmignoto G (2006) Astrocyte control of synaptic transmission and neurovascular coupling. *Physiol Rev* 86:1009–1031.
2. Porter JT, McCarthy KD (1996) Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals. *J Neurosci* 16:5073–5081.
3. Grosche J, et al. (1999) Microdomains for neuron-glia interaction: Parallel fiber signaling to Bergmann glial cells. *Nat Neurosci* 2:139–143.
4. Perea G, Araque A (2005) Properties of synaptically evoked astrocyte calcium signal reveal synaptic information processing by astrocytes. *J Neurosci* 25:2192–2203.
5. Bezzi P, et al. (1998) Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature* 391:281–285.
6. Araque A, Parpura V, Sanzgiri RP, Haydon PG (1998) Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. *Eur J Neurosci* 10:2129–2142.
7. Newman EA, Zahs KR (1998) Modulation of neuronal activity by glial cells in the retina. *J Neurosci* 18:4022–4028.
8. Zonta M, et al. (2003) Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. *Nat Neurosci* 6:43–50.
9. Kang J, Jiang L, Goldman SA, Nedergaard M (1998) Astrocyte-mediated potentiation of inhibitory synaptic transmission. *Nat Neurosci* 1:683–692.
10. Serrano A, Haddjeri N, Lacaille JC, Robitaille R (2006) GABAergic network activation of glial cells underlies hippocampal heterosynaptic depression. *J Neurosci* 26:5370–5382.
11. Meier SD, Kafitz KW, Rose CR (2008) Developmental profile and mechanisms of GABA-induced calcium signaling in hippocampal astrocytes. *Glia* 56:1127–1137.
12. Conti F, Minelli A, Melone M (2004) GABA transporters in the mammalian cerebral cortex: Localization, development and pathological implications. *Brain Res Rev* 45:196–212.
13. Lee TS, et al. (2006) GAT1 and GAT3 expression are differently localized in the human epileptogenic hippocampus. *Acta Neuropathol* 111:351–363.
14. Schousboe A, Sarup A, Bak LK, Waagepetersen HS, Larsson OM (2004) Role of astrocytic transport processes in glutamatergic and GABAergic neurotransmission. *Neurochem Int* 45:521–527.
15. Higashi K, et al. (2001) An inwardly rectifying K^+ channel, Kir4.1, expressed in astrocytes surrounds synapses and blood vessels in brain. *Am J Physiol Cell Physiol* 281:C922–931.
16. Chao TI, Kasa P, Wolff JR (1997) Distribution of astroglia in glomeruli of the rat main olfactory bulb: Exclusion from the sensory subcompartment of neuropil. *J Comp Neurol* 388:191–210.
17. Nishimura M, et al. (1997) Differential expression patterns of GABA transporters (GAT1–3) in the rat olfactory bulb. *Brain Res Mol Brain Res* 45:268–274.
18. Borden LA, et al. (1994) Cloning of the human homologue of the GABA transporter GAT-3 and identification of a novel inhibitor with selectivity for this site. *Receptors Channels* 2:207–213.
19. Hog S, et al. (2006) Structure-activity relationships of selective GABA uptake inhibitors. *Curr Top Med Chem* 6:1861–1882.
20. Singaravelu K, Lohr C, Deitmer JW (2006) Regulation of store-operated calcium entry by calcium-independent phospholipase A2 in rat cerebellar astrocytes. *J Neurosci* 26:9579–9592.
21. Rose CR, Kovalchuk Y, Eilers J, Konnerth A (1999) Two-photon Na^+ imaging in spines and fine dendrites of central neurons. *Pflugers Arch* 439:201–207.
22. Bennay M, Langer J, Meier SD, Kafitz KW, Rose CR (2008) Sodium signals in cerebellar Purkinje neurons and Bergmann glial cells evoked by glutamatergic synaptic transmission. *Glia* 56:1138–1149.
23. Chaudhry FA, et al. (1999) Molecular analysis of system N suggests novel physiological roles in nitrogen metabolism and synaptic transmission. *Cell* 99:769–780.
24. Bootman MD, Missiaen L, Parys JB, De Smedt H, Casteels R (1995) Control of inositol 1,4,5-trisphosphate-induced Ca^{2+} release by cytosolic Ca^{2+}. *Biochem J* 306:445–451.
25. Taylor CW, Laude AJ (2002) IP_3 receptors and their regulation by calmodulin and cytosolic Ca^{2+}. *Cell Calcium* 32:321–334.
26. Fill M, Copello JA (2002) Ryanodine receptor calcium release channels. *Physiol Rev* 82:893–922.
27. Maruyama T, Kanaji T, Nakade S, Kanno T, Mikoshiba K (1997) 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P_3-induced Ca^{2+} release. *J Biochem* 122:498–505.
28. Bootman MD, et al. (2002) 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca^{2+} entry but an inconsistent inhibitor of $InsP_3$-induced Ca^{2+} release. *FASEB J* 16:1145–1150.
29. Nilius B, Owsianik G, Voets T, Peters JA (2007) Transient receptor potential cation channels in disease. *Physiol Rev* 87:165–217.
30. Hoenderop JG, et al. (2001) Function and expression of the epithelial Ca^{2+} channel family: Comparison of mammalian ECaC1 and 2. *J Physiol* 537:747–761.
31. Müller T, Möller T, Berger T, Schnitzer J, Kettenmann H (1992) Calcium entry through kainate receptors and resulting potassium-channel blockade in Bergmann glial cells. *Science* 256:1563–1566.
32. Seifert G, Steinhäuser C (1995) Glial cells in the mouse hippocampus express AMPA receptors with an intermediate Ca^{2+} permeability. *Eur J Neurosci* 1872–1881.
33. Gordon GR, Choi HB, Rungta RL, Ellis-Davies GC, MacVicar BA (2008) Brain metabolism dictates the polarity of astrocyte control over arterioles. *Nature* 456:745–749.
34. Petzold GC, Albeanu DF, Sato TF, Murthy VN (2008) Coupling of neural activity to blood flow in olfactory glomeruli is mediated by astrocytic pathways. *Neuron* 58:897–910.
35. Ben-Ari Y, Gaiarsa JL, Tyzio R, Khazipov R (2007) GABA: A pioneer transmitter that excites immature neurons and generates primitive oscillations. *Physiol Rev* 87:1215–1284.
36. Farrant M, Kaila K (2007) The cellular, molecular and ionic basis of $GABA_A$ receptor signalling. *Prog Brain Res* 160:59–87.
37. Wang C, et al. (2005) Differential expression of KCC2 accounts for the differential GABA responses between relay and intrinsic neurons in the early postnatal rat olfactory bulb. *Eur J Neurosci* 21:1449–1455.
38. Carmignoto G, Pasti L, Pozzan T (1998) On the role of voltage-dependent calcium channels in calcium signaling of astrocytes in situ. *J Neurosci* 18:4637–4645.
39. Goldman WF, Yarowsky PJ, Juhaszova M, Krueger BK, Blaustein MP (1994) Sodium/calcium exchange in rat cortical astrocytes. *J Neurosci* 14:5834–5843.
40. Tong XP, et al. (2009) Ca^{2+} signaling evoked by activation of Na^+ channels and Na^+/Ca^{2+} exchangers is required for GABA-induced NG2 cell migration. *J Cell Biol* 186:113–128.
41. Doengi M, Deitmer JW, Lohr C (2008) New evidence for purinergic signaling in the olfactory bulb: A_{2A} and $P2Y_1$ receptors mediate intracellular calcium release in astrocytes. *FASEB J* 22:2368–2378.
42. Rieger A, Deitmer JW, Lohr C (2007) Axon-glia communication evokes calcium signaling in olfactory ensheathing cells of the developing olfactory bulb. *Glia* 55:352–359.