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Nonsynaptic GABA signaling in postnatal subventricular zone controls GFAP-expressing progenitor proliferation

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Abstract

In the postnatal subventricular zone (SVZ) local cues or signaling molecules released from neuroblasts limit the proliferation of glial fibrillary acidic protein (GFAP)-expressing progenitors thought to be stem cells. However, signals between SVZ cells have not been identified. We show that depolarizations of neuroblasts induce nonsynaptic SNARE-independent GABA_A receptor currents in GFAP-expressing cells whose time-course depends on GABA uptake in acute mouse slices. We found that GABA_A receptors are tonically activated in GFAP-expressing cells consistent with the presence of spontaneous depolarizations in neuroblasts that are sufficient to induce GABA release. These data demonstrate the existence of nonsynaptic GABAergic signaling between neuroblasts and GFAP-expressing cells. Furthermore, we show that GABA_A receptor activation in GFAP-expressing cells limits their progression through the cell cycle. Thus, as GFAP-expressing cells generate neuroblasts, GABA released from neuroblasts provides a feedback mechanism to control the proliferation of GFAP-expressing progenitors by activating GABA_A receptors.

INTRODUCTION

The postnatal SVZ contains the largest pool of dividing neural progenitors in the adult brain. The SVZ consists of interconnected channels comprised of three major cell types¹. The channel lumen is packed with neuroblasts. Many of these proliferate and migrate towards the olfactory bulb where they become interneurons²⁻⁵. GFAP-immunopositive progenitors, also called astrocytes^{1,6}, line the channel walls and thus closely encapsulate neuroblasts. These GFAP-expressing cells constitute a pool of stem cells⁶⁻⁸. A small number of highly proliferative progenitors are present in clusters along the channels. Overall, the anterior SVZ is mostly composed of neuroblasts (~60-70%) ensheathed by GFAP-expressing cells (~20%), which are thus in a prime location to send and receive signals from neuroblasts. Furthermore, elimination of neuroblasts stimulates the proliferation of GFAP-expressing cells that regenerate the entire SVZ⁹, indicating that local cues or signaling molecules released from neuroblasts regulate the proliferation of GFAP-expressing cells. However, the signals between neuroblasts and GFAP-expressing cells have not been identified. Identifying these signals and their receptors is of considerable interest because they provide therapeutic targets for controlling the proliferation of GFAP-expressing progenitors and ultimately neurogenesis.

Among the possible signaling molecules, the neurotransmitter GABA is a prominent candidate for providing intercellular signals between neuroblasts and GFAP-expressing progenitors because SVZ progenitors synthesize and release GABA and GFAP-expressing cells express

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GABA transporters (GATs)^{10,11}. GABA plays an important signaling role in developmental processes, such as embryonic cell proliferation and migration¹²⁻¹⁷ as well as regulating the migratory speed and production of neuroblasts in postnatal SVZ^{11,18}. To support GABAergic signaling from neuroblasts to GFAP-expressing cells, the expression of GABA receptors in GFAP-expressing cells has yet to be demonstrated. Furthermore, it is unknown whether neuroblasts release GABA that activates GABA receptors on GFAP-expressing cells in a synaptic or nonsynaptic fashion, and whether this release depends on the classical SNARE complex. The SNARE complex, formed by the vesicle protein synaptobrevin/VAMP (R-SNARE) and by the plasmamembrane proteins SNAP25 and syntaxin (Q-SNARE), is the minimal machinery required for vesicle exocytosis. GABA release onto synaptically silent hippocampal neurons¹⁹ and from CA1 neurons²⁰ is Q-SNARE-independent.

To determine whether neuroblasts communicate with GFAP-expressing cells via GABA signaling, we recorded from GFAP-expressing cells in the postnatal SVZ using transgenic mice expressing GFP driven by the human GFAP promoter (GFAP^P-GFP). Our data demonstrate the existence of nonsynaptic, SNARE-independent GABAergic communication between neuroblasts and presumed stem cells. Furthermore, we found that GABA_A receptor (GABA_AR) inhibition increases the number of proliferative GFAP-expressing cells. Thus, GABA released from neuroblasts acts as a stop signal to limit GFAP-expressing cell proliferation, and this GABAergic communication between SVZ progenitors may contribute to maintaining a balance between amplification and mobilization of progenitors.

RESULTS

GFAP-expressing cells of the SVZ express functional GABA_ARs

Whole-cell patch-clamp recordings were performed in acute slices from postnatal transgenic GFAP^P-GFP mice²¹. Fluorescent (GFP⁺) cells could be visualized before recording and then filled with lucifer yellow during recording to better examine their morphology. GFP⁺ cells recorded in the SVZ had cell bodies of irregular shape (~10 μm-long) with one or two main processes (~30-60 μm-long, Fig. 1a). Although striatal astrocytes adjacent to the SVZ are also GFP⁺, they were easily distinguished due to their more complex morphology (Supplemental Fig. 1) compared to GFP⁺ cells in the SVZ. Striatal astrocytes near the SVZ were characterized by several main processes confined to a 80-150 μm-diameter sphere or ellipse. GFP⁺ cells in the SVZ had a low mean input resistance (R_{IN} , 50.9 ± 4.7 MΩ mean $\pm$ SEM, $n = 48$) and a hyperpolarized mean resting potential (V_R , -80.7 ± 0.6 mV, $n = 48$) similar to those of astrocytes²² and radial glia²³ but different from those of GFP-negative progenitors (mean R_{IN} of ~3 GΩ and zero-current V_R of -28 mV), which are presumably neuroblasts^{11,24}. We also performed immunostaining for nestin, a class VI intermediate filament protein, expressed in SVZ cells but not in astrocytes^{1,25}. Lucifer yellow-filled GFP⁺ cells recovered after fixation of slices stained positive for nestin ($n = 5/5$, Fig. 1b), confirming that GFP⁺ cells with an immature morphology and recorded in the SVZ are the GFAP-expressing progenitors thought to be stem cells⁶⁻⁸.

All GFAP-expressing cells examined responded to focal applications of GABA (100 μM). GABA induced inward currents that ranged from -20 to -300 pA in amplitude at a holding potential of -80 mV ($n = 42$, Fig. 1c). The variability in current amplitude is likely due to variability in the GABA concentration that reaches cells recorded at variable depths (50-150 μm) in the slice. GABA-induced currents slowly desensitized and were accompanied by an increase in noise, which was mostly visible in the presence of 100 μM meclofenamic acid (MFA), a gap junction blocker²⁶. This increase in noise with MFA was accompanied by a decrease in GABA current amplitudes and a significant increase in R_{IN} (to 305.4 ± 14.8 MΩ, $n = 178$, $P < 0.001$) without affecting the series (access) resistance (Fig. 1d). These effects were likely due to gap junction blockade by MFA²⁶. Indeed, no dye coupling was observed

in GFP⁺ cells recorded in the presence of MFA (Fig 1a, b) while GFP⁺ cells recorded without MFA displayed dye coupling to 4 or 8 surrounding GFP⁺ cells (data not shown). Although MFA affects responses at certain GABA_ARs²⁷, MFA did not alter the amplitude of gabazine-sensitive currents in outside-out patches of GFAP-expressing cells (data not shown). MFA (100 μ M) was then routinely bath applied after obtaining whole cell recordings of GFP⁺ cells to improve the space clamp of the recording. GABA responses in GFAP-expressing cells were reversibly blocked by the GABA_AR antagonists, 20–50 μ M gabazine ($n = 5$, Fig. 1e), 100 μ M bicuculline (BIC) or 30–50 μ M picrotoxin (PTX, $n = 6$ and 5, respectively, data not shown), and were mimicked by pressure application of a GABA_AR agonist isoguvacine (10 μ M, $n = 3/3$ and 100 μ M, $n = 3/3$, data not shown). As expected for GABA_AR-mediated (GABA_A) currents, the mean reversal potential for GABA-induced currents (mean of -52.8 ± 6.8 mV, $n = 7$) was near the Nernst equilibrium potential for chloride ($E_{Cl} = -54$ mV with an intracellular $[Cl^-]$ of 18.5 mM, Fig. 1f). The mean EC₅₀ of GABA was 15.1 μ M ($n = 5$, Fig. 2a) obtained in cells pulled out and raised above the slice. Furthermore, single channel activity could be induced with 3 and 10 μ M GABA in outside-out patches of GFAP-expressing progenitors (data not shown). The dose-dependent inhibition for PTX on 100 μ M GABA responses gave an IC₅₀ of 11.9 μ M ($n = 4$, Fig. 2b).

Stimulus-evoked nonsynaptic GABA_A currents in GFAP cells

SVZ neuroblasts synthesize and contain GABA^{10,11}. We thus set out to determine whether GABA_ARs could be activated by GABA released from SVZ progenitors following focal electrical stimulation in the SVZ. Single-pulse electrical stimulation in the SVZ failed to induce currents in GFAP-expressing cells (Fig. 3a). However, increasing the number of pulses (2–5 pulses at 50 Hz) induced slow, long-lasting inward currents of increasing amplitudes (Fig. 3a). These evoked inward currents were usually accompanied with an increase in noise and were reversibly reduced by BIC (100 μ M, $n = 3$, Fig. 3b) and PTX (50 μ M, $n = 3$, data not shown), suggesting that they were mediated by GABA_AR activation in GFAP-expressing cells. In addition, evoked currents persisted in the presence of AMPA/kainate and NMDA receptor antagonists (20 μ M DNQX and 50 μ M D-APV, respectively, $n = 5$). Evoked GABA_A currents ranged from -20 to -60 pA, suggesting that GABA release was small. Evoked currents displayed slower kinetics than those of synaptic currents recorded in other brain regions. Evoked currents developed slowly with a rise-time between 200 and 700 ms, and were long-lasting with a mean mono-exponential decay time constant (τ) of 1.16 ± 0.10 s ($n = 24$, Fig. 3c). Fast inward currents resembling synaptic currents were never observed following either a single or tetanic stimulation (Fig. 3c inset), or during bath application of 1 nM α -latrotoxin (Fig. 3d, $n = 4$). Although α -latrotoxin increased the frequency of spontaneous synaptic currents in striatal neurons (Fig. 3d, $n = 6$), no effect of α -latrotoxin in GFAP-expressing cells could be due to the lack of receptors necessary for the toxin internalization to the cytoplasm²⁸.

To further ensure that GABA released by SVZ progenitors diffuses and activates receptors in GFAP-expressing cells in a nonsynaptic manner, we tested whether the amplitude and kinetics of evoked currents changed with the distance between the stimulating electrodes and recorded cells. The rise-time of evoked GABA_A currents decreased and amplitude increased when the stimulating electrode was moved closer to the recorded cell (Fig. 3e,f). Stimulating electrodes were usually placed 40 to 60 μ m from the recorded cell (between positions b and c in Fig. 3e) close to the lateral ventricle and GFP⁺ cells were recorded midway in the SVZ except for experiments displayed in Figure 3e. Variability in the distance between the stimulating electrode and recorded cell, and the presence of GATs (see Fig. 4) likely contribute to the variability in the evoked current amplitude and kinetics. We next examined whether evoked currents were independent of action potentials and extracellular or intracellular Ca²⁺. Evoked GABA_A currents in GFAP-expressing cells persisted in the presence of the voltage-dependent sodium channel blocker, tetrodotoxin (TTX, 1 μ M, $n = 4$), in a Ca²⁺-free extracellular solution

applied for 30 min (0 mM Ca^{2+} plus 1 or 2 mM EGTA, $n = 4$), or in the presence of the voltage-gated Ca^{2+} channel blockers, Ni^{2+} and Cd^{2+} (each at 100 μM , $n = 4$, Fig. 3g). These data suggested a nonsynaptic release of GABA. TTX, Ni^{2+} and Cd^{2+} or a Ca^{2+} -free solution abolished or significantly reduced GABA_A synaptic currents in Purkinje neurons induced by one pulse stimulation in the molecular layer (data not shown). When slices were incubated for 30 min in 30 μM BAPTA-AM in a Ca^{2+} -free solution to buffer intracellular Ca^{2+} , no evoked currents could be induced in GFAP-expressing progenitors ($n = 9$ cells, 72 stimulations, 5 slices, Fig. 3h). Nevertheless, GABA responses could still be induced in SVZ progenitors by exogenous GABA applications ($n = 4$, data not shown). These data suggest that increases in intracellular Ca^{2+} levels are necessary for inducing GABA release. Consistent with a nonsynaptic release, we found no immunostaining for synapsin I near GFAP-immunopositive cells in the SVZ (Supplemental Fig. 2). Synapsin I is a specific marker of synapses²⁹. Because the striatum contains GABAergic terminals, we next examined whether tetanic stimulation in the striatum adjacent to the SVZ induced GABA responses in GFAP-expressing cells. While tetanic stimulation in the SVZ induced inward currents in GFAP-expressing cells recorded midway in the SVZ, the same stimulation applied in the striatum within the same distance from the recorded cells failed to induce such currents ($n = 4$, Fig. 3i). Although GABA is released from synaptic terminals in the striatum, dilution by diffusion and uptake into both astrocytes and GFAP-expressing cells likely prevent significant levels of synaptically released GABA to reach recorded cells in the SVZ. These data indicate that GABA or a GABA_A agonist is nonsynaptically released from SVZ progenitors and activates GABA_ARs in GFAP-expressing cells.

SNARE-independent GABA release from SVZ progenitors

Because evoked GABA currents were dependent on intracellular Ca^{2+} , we examined whether GABA release was abolished after inhibition of the SNARE complex. We incubated slices with botulinum neurotoxin A (BoNT/A) or B (BoNT/B) to block the Q- or R-SNARE complex, respectively. Bath applications of BoNT/A or B (each at 200 ng/ml) for 30 min significantly ($P < 0.001$) reduced the frequency of spontaneous gabazine (20 μM)-sensitive synaptic currents by 80% in striatal neurons without affecting cell capacitance and R_{IN} ($n = 4$, Fig. 4a). Synaptic activity was studied in the presence of AMPA/kainate and NMDA receptor antagonists (20 μM DNQX and 50 μM D-APV). However, similar treatment with BoNT/A or B for 30 min did not affect evoked GABA_A currents in GFAP-expressing cells ($n = 4$ for each toxin, Fig. 4b). Treated and control slices were then incubated for 18 hrs in a solution containing 100 ng/ml of BoNT/A or BoNT/B (without MFA) and no toxin, respectively. Spontaneous GABAergic synaptic currents were absent in neurons recorded in treated slices but were present in neurons from control slices (Fig. 4c for BoNT/B, data not shown for BoNT/A). Furthermore, α -latrotoxin (1 nM) did not induce spontaneous synaptic currents in neurons from treated slices ($n = 5$, data not shown). Evoked slow GABA_A currents in GFAP-expressing cells persisted in slices treated with either toxin ($n = 5$ and 6 with BoNT/A and B, respectively, Fig. 4d for BoNT/B). These data suggest that GABA release from SVZ progenitors is SNARE-independent.

GABA uptake controls GABA_AR activation in GFAP cells

Four subtypes of GATs, called mouse GAT1 to GAT4 have been reported³⁰. GAT2 is less widespread in the brain and has the lowest affinity for GABA³¹. Bath application of non-transportable antagonists of the three major GATs, 50 μM NO-711 for GAT1 and 50 μM SNAP5114 for GAT3/4, significantly enhanced the amplitude and prolonged the decay of PTX- and BIC-sensitive inward currents evoked by tetanic stimulation ($n = 5$, $P < 0.05$, Fig. 5a,b). Furthermore, NO-711 and SNAP5114 induced inward shifts of the holding current of about -5 to -20 pA ($n = 6/10$, Fig. 5c). These results suggest that GATs decrease ambient GABA levels and limit GABA_AR activation in GFAP-expressing cells. To determine whether both GAT1 and GAT3/4 contributed to the clearance of GABA, we tested the effects of each

blocker on GABA responses induced by pressure applications of GABA (100 μ M, 100 ms, Fig. 5e). SNAP5114 significantly ($P < 0.05$) increased the amplitude of GABA-induced responses by $61 \pm 39\%$ in 3/5 cells while NO711 had no effect on the amplitude ($n = 5$, Fig. 5e). Both SNAP5114 and NO-711 significantly increased τ of GABA responses by $91 \pm 26\%$ ($n = 7$) and $33 \pm 15\%$ ($n = 5$), respectively ($P < 0.05$, Fig. 5f,g). These data suggest that GAT3/4 transporters are more efficient than GAT1 transporter at regulating ambient and nonsynaptically released GABA levels in the SVZ.

Depolarization-induced GABA_A currents in GFAP cells

We examined whether a subtle and near-physiological depolarizations of SVZ progenitors induces GABA_AR activation in GFAP-expressing cells. Bath applications of 7.5 and 12.5 mM K⁺ or focal (pressure) applications of 12.5 mM K⁺ depolarized neuroblasts by 10 and 15 mV, respectively ($n = 9$, without MFA in the bath, data not shown). Bath and pressure applications of K⁺, which depolarizes every progenitor or a local group of SVZ progenitors, respectively, induced inward currents in GFAP-expressing cells that were reversibly reduced by BIC ($n = 8$, Fig. 6a) or PTX ($n = 6$, Fig. 6b). Neither PTX nor BIC affected the series (access) resistance or capacitance calculated in response to a 10 mV-depolarizing pulse (Fig. 6b for PTX). As a control for high K⁺ application, application of a solution into which 5 mM sucrose was added induced no currents in GFAP-expressing cells ($n = 3$). As expected for GABA_A currents, the mean reversal potentials for high K⁺-induced GABA_A currents were -58.7 ± 2.3 mV and $+1.9 \pm 1.4$ mV ($n = 6$, data not shown) when using a low ($E_{Cl} = -54$ mV) and high internal Cl⁻ solution ($E_{Cl} = 1.3$ mV), respectively. High K⁺-induced GABA_A currents persisted in the presence of TTX (data not shown) or in a Ca²⁺-free solution ($n = 4$, Fig. 6a). Furthermore, no fast, transient currents resembling synaptic currents were observed during high K⁺ applications. PTX-sensitive currents induced by 12.5 mM K⁺ (bath) had significantly larger amplitudes (-34.0 ± 5.8 pA) than those induced by 7.5 mM K⁺ (-22.0 ± 4.0 pA, $n = 6$, $P < 0.05$). Focal (pressure) application of 12.5 mM K⁺ induced GABA_A currents of -10 to -20 pA ($n = 5$). High K⁺ applications also induced BIC- and PTX-insensitive currents (Fig. 6a,b). Because GFAP-expressing cells have a high resting K⁺ conductance like astrocytes (data not shown), BIC- and PTX-insensitive inward currents induced by K⁺ likely represent a change in the resting K⁺ current due to a shift in the Nernst equilibrium potential for K⁺.

We next examined whether 10 to 15 mV-depolarizations spontaneously occurred in neuroblasts. Neuroblasts were recorded in current clamp from -60 mV, which is near their estimated resting potential¹⁰, and without MFA. Nevertheless, MFA had no effect on the resting potential, R_{IN} , and the spontaneous depolarizations observed in neuroblasts (data not shown). 71% of the neuroblasts displayed spontaneous depolarizations of 15 to 20 mV (Fig. 6c), which occurred at a frequency of 0.3 to 12/min and were insensitive to GABA_AR blockers. These large spontaneous depolarizations were observed during the whole length (45-75 min) of stable whole-cell and perforated recordings, suggesting that they were not due to cell damage. Spontaneous single channel currents of -5 pA-amplitude that correspond to a 15 to 20 mV depolarization due to the large R_{IN} of neuroblasts (3-4 G Ω) were also observed in voltage clamped neuroblasts. 40% of the neuroblasts also displayed small gabazine-sensitive depolarizations of ~ 5 -8 mV (data not shown), consistent with tonic GABA_AR activation in neuroblasts^{11,18}. These results show that K⁺-induced depolarizations of SVZ progenitors similar in amplitude to the spontaneous depolarizations found in SVZ neuroblasts can induce nonsynaptic GABA_AR activation in surrounding GFAP-expressing cells.

Neuroblasts mediate tonic GABA_A current in GFAP cells

The presence of spontaneous depolarizations in neuroblasts suggests that GABA_ARs are tonically activated in some GFAP-expressing cells due to spontaneous GABA release. Bath application of BIC (100 μ M, Fig. 6d) or PTX (50 μ M, Fig. 6e) induced reversible $+5$ to $+30$

pA outward shifts of the holding currents when recorded in a 2.5 or 5 mM K⁺ extracellular solution with and without GABA transporter inhibitors, 50 μ M NO-711 and 50 μ M SNAP5114 ($n = 12/17$ and $n = 7/12$, respectively). This outward shift was not accompanied by a change in series resistance or cell capacitance (Fig. 6d). These results indicate that there is sufficient ambient GABA in the SVZ to tonically activate GABA_ARs in GFAP-expressing cells.

Although electrical and high K⁺ stimulations indiscriminately depolarize all SVZ cells, ~70% of the SVZ cells that stain positive for GABA are neuroblasts¹¹, suggesting that neuroblasts are the major source of GABA in the SVZ. Furthermore, GFP⁺ cells did not stain positive for GABA (Fig. 7a). To conclusively show that neuroblasts release GABA spontaneously and upon depolarization, we used outside-out patches of striatal neurons or GFAP-expressing cells held at -80 mV to monitor GABA_A single channels (without MFA) and detect GABA release from simultaneously recorded neuroblasts (Fig. 7b-e). Neuroblasts were recorded in the cell-attached mode to keep the intracellular milieu intact. Cell-attached patches were held at a pipette potential (V_{pip}) of 0 mV (patch potential $V_{\text{patch}} = V_R$, using $V_{\text{patch}} = V_R - V_{\text{pip}}$). Experiments were performed with AMPA/kainate and NMDA receptor blockers (DNQX and D-APV). Because gabazine (20 μ M)-sensitive single channel activity was detected when outside-out patches were ~5 μ m above the slices, outside-out patches were raised at ~50 μ m above the slice where no single channel activity was observed (Fig. 7c). The cell-attached neuroblasts were at first positioned at 50 μ m from the patches and from the slice. A total of 21 paired recordings (4 neuron- and 17 GFAP-expressing cell-neuroblast pairs) were obtained. 52% of the outside-out patches (7 from GFAP-expressing cells and 4 from striatal neurons) displayed single channel activity when placed < 5 μ m from the recorded neuroblast (Fig. 7b,c,d). This activity was abolished by 20 μ M gabazine in outside-out patches from neurons ($n = 3$, Fig. 7c) and GFAP-expressing cells ($n = 3$, Fig. 7d). Furthermore, single channels had a mean amplitude of -2.0 ± 0.1 pA in GFAP-expressing cells ($n = 13$) and -2.2 ± 0.1 pA in neurons ($n = 4$) at -80 mV corresponding to a typical GABA_AR conductance of 24.5 ± 0.7 pS and 27.7 ± 0.9 pS, respectively (assuming a reversal potential of ~0 mV in our recording condition). 6/10 of the remaining silent patches from GFAP-expressing cells placed at < 5 μ m from a recorded neuroblast displayed single channel activity upon a 5 s-long depolarization of the progenitor from a V_{patch} of -60 to +60 mV (using V_R of -60 mV^{10,24}). The single channel activity lasted 30 to 60 s and were blocked by gabazine ($n = 3$). Insets in Fig. 7c-e display single channels at a higher time scale and corresponding amplitude histograms. These data demonstrate that neuroblasts release GABA both spontaneously and upon depolarization, and that released GABA can directly activate GABA_ARs on neighboring GFAP-expressing cells.

GABA_AR activation reduces the proliferation of GFAP cells

We examined whether activation of GABA_ARs in GFAP-expressing cells influence the proliferation of these cells, as previously shown for embryonic cells¹⁴, including SVZ cells¹³. Pairs of hemisected slices were incubated in the presence of the mitotic marker 5-bromo-2'-deoxyuridine (0.05% BrdU) for 18 hrs either in control conditions (without MFA) or in the presence of BIC (50 μ M). After 18 hrs, hemisected slices were fixed and processed for GFP and BrdU immunostaining and confocal z-stacks (10 sections spaced by 1 μ m) of 2 to 3 fields of view were taken in control and treated (opposite) hemispheres. Fig. 8a represents a reconstruction of a 10 micron z-stack in control and in bicuculline-treated sections. Inhibition of GABA_ARs with BIC alone significantly ($P < 0.001$, unpaired t-test with unequal variance) increased the percentage of GFP-immunopositive cells that stained positive for BrdU from $8.8 \pm 1.5\%$ (665 GFP⁺ cells counted, 95 ± 41 cells, mean $\pm$ SEM) to $17.9 \pm 1.5\%$ (1064 cells, mean of 142 ± 47 cells, Fig. 8b, 6 pairs of hemisected slices from 5 mice). To prevent an increase in the slice excitability and release of various factors from the slice, these experiments were repeated in the presence of the glutamate receptor blockers DNQX (20 μ M) to block AMPA/kainate receptors and D-APV (50 μ M) to block NMDA receptors. In this condition, inhibition

of GABA_ARs also significantly ($P < 0.001$) increased the percentage of GFP-immunopositive cells that stained positive for BrdU from $8.8 \pm 1.1\%$ (1643 GFP⁺ cells, mean of 182 ± 7 cells) to $19.7 \pm 1.7\%$ (1238 cells, mean of 137 ± 15 cells, Fig. 8c, 4 pairs of slices, 9 resected sections, 3 mice). We next tested whether the blocker of GAT4 transporters SNAP5114 (100 μ M) would limit the number of proliferative GFAP-expressing cells since we previously showed that SNAP5114 increased ambient GABA levels in the SVZ¹¹ (see also Fig. 5). Inhibition of GAT4 transporters with SNAP5114 significantly ($P < 0.05$) reduced the percentage of GFP-immunopositive cells that stained positive for BrdU from $10.9 \pm 1.9\%$ (1909 GFP⁺ cells, mean of 127 ± 15) to $6.7 \pm 1.2\%$ (1979 cells, mean of 116 ± 13 , Fig. 8c, 6 pairs of slices, 15 resected sections, 4 mice). These data suggest that GABA_AR activation in GFAP-expressing cells by ambient GABA reduces the proliferation of these cells.

DISCUSSION

We demonstrate here that nonsynaptically, SNARE-independent released GABA from neuroblasts activates GABA_ARs in GFAP-expressing cells of the SVZ, thought to be stem cells⁶⁻⁸, and that tonic GABA_AR activation in GFAP-expressing cells reduces their proliferation.

Nonsynaptic GABAergic signaling between SVZ progenitors

Recorded cells were identified as GFAP-expressing cells prior to recording by direct visualization of fluorescence in GFAP^P-GFP mice²¹. Recorded GFP⁺ cells had passive membrane properties similar to those of astrocytes but displayed a less elaborated morphology²². In addition, recorded GFP⁺ cells stained positive for nestin, a marker of immature cells but not astrocytes^{1,25}. GABA currents in GFAP-expressing cells were blocked by several GABA_AR antagonists and were carried by Cl⁻ ions, identifying these currents as GABA_AR-mediated responses. Electrical or high K⁺ stimulations of SVZ progenitors induced nonsynaptic GABA_A currents in GFAP-expressing cells, suggesting that SVZ progenitors released GABA. Evoked currents developed slowly and decayed over several seconds. This time course is significantly slower than that of synaptic currents. The four following results support nonsynaptic GABA release. (1) Synapsin I staining was absent in the SVZ, (2) The kinetics course and amplitude of evoked GABA_A currents were dependent on the distance between stimulating electrodes and recorded cells. (3) Blockade of action potentials or voltage-gated Ca²⁺ channels, or removal of extracellular Ca²⁺ did not prevent evoked GABA_A currents. (4) α -latrotoxin, which stimulates vesicular exocytosis from presynaptic terminals²⁸, did not induce any synaptic currents in GFAP-expressing cells. The persistence of evoked GABA_A currents in GFAP-expressing cells following BoNT treatments suggests that GABA release is independent of a classical SNARE complex. Taken together, these results demonstrate that GABA is nonsynaptically, SNARE-independently released in the postnatal mammalian SVZ. Such nonsynaptic, Q-SNARE-independent GABAergic communication has been observed in embryonic and neonatal hippocampal neurons¹⁹. However, in contrast with GABAergic signaling in the neonatal hippocampus, we found that GATs control ambient GABA levels and limit the diffusion of released GABA. GAT3/4, and to a lesser extent GAT1 transporters, played a major role in regulating ambient GABA levels in the SVZ.

GABA is present in neuroblasts¹¹ but not in GFAP-expressing cells. We cannot rule out that GABA is also contained in glioblasts but this population is a minority at the ages studied^{11, 32,33}. It was previously reported by us and others that SVZ neuroblasts express glutamic acid decarboxylase^{10,34,35}. Neuroblasts displayed spontaneous depolarizations and our paired recordings indicate that neuroblasts release GABA spontaneously and upon depolarization. Collectively these data strongly suggest that GABA is spontaneously released from neuroblasts, diffuses and activates GABA_ARs on neighboring GFAP-expressing cells known to ensheath neuroblasts¹. While further studies are required to define the mechanisms of GABA

release from neuroblasts, the two following mechanisms can be ruled out: (1) GABA transport reversal although release via GAT2 transport reversal cannot be ruled out; (2) Release through hemichannels as observed for ATP and glutamate^{36,37} since MFA is a blocker of hemichannels²⁶. One possible mechanism of GABA release is via unconventional exocytosis triggered by Ca^{2+} release from intracellular stores as buffering intracellular Ca^{2+} prevented the induction of evoked GABA_A currents. Furthermore, neuroblasts display spontaneous Ca^{2+} transients (Huang F and Bordey A, unpublished observations), contain synapsin III, a protein associated with synaptic vesicles^{38,39}.

Function of GABAergic signaling between SVZ progenitors

Our data indicate that in the SVZ neuroblasts communicate with GFAP-expressing cells by releasing GABA that activates GABA_ARs in GFAP-expressing cells (Supplemental Fig. 3). Furthermore, we show that GABA_AR activation in GFAP-expressing cells limits their progression through the cell cycle. As more neuroblasts are generated, it is possible that more GABA is released in the extracellular space resulting in increased ambient GABA levels and GABA_AR activation in GFAP-expressing cells. Because a subset of GFAP-expressing cells are thought to generate neuroblasts⁶⁻⁸, increase in the number of neuroblasts serves as a negative feedback to decrease GFAP-expressing cell proliferation and neuroblast production by activating GABA_ARs. This is consistent with earlier studies showing that GABA alters proliferation^{14,15,18}, and in particular reduces the proliferation of embryonic SVZ cells¹³, which are thought to be lineally related to GFAP-expressing cells in the postnatal SVZ⁴⁰. Thus, with respect to ambient GABA's proliferative action, there is a nice parallel between results from embryonic and postnatal SVZ cells. Such a feedback mechanism may also apply for glioblast production from GFAP-expressing cells or radial glia in the neonatal SVZ assuming that glioblasts contain GABA^{32,33,41}. Furthermore, the feedback mechanism provided by neuroblasts on GFAP-expressing cell proliferation fits well with the constant migration of neuroblasts away from the SVZ to the olfactory bulb^{5,42,43}, which would limit ambient GABA accumulation. This feedback also fits well with the increased proliferation of GFAP-expressing cells following elimination of neuroblasts⁹. In conclusion, changes in GABA levels due to neuroblast migration or production provide a mechanism to control the proliferation of GFAP-expressing cells and maintain a balance between neuroblast production and mobilization.

METHODS

Slice preparation and electrophysiology

Coronal (250 μm -thick) brain slices from 16- to 41-day old transgenic mice (Jackson laboratory)²¹ were prepared as previously described^{10,24}. Experimental procedures were in accordance with the animal welfare guidelines of Yale University. The standard external solution contained (in mM): NaCl 125, KCl 2.5, CaCl_2 2, MgCl_2 1, NaH_2PO_4 1.25, NaHCO_3 25, glucose 10, pH 7.4 when equilibrated with 95% O_2 /5% CO_2 . Whole-cell and gramicidin perforated patch-clamp recordings were obtained as previously described^{10,22,44} and as detailed in Supplementary Method. GFAP-expressing cells had a mean capacitance of 17.7 ± 0.5 pF ($n = 111$) with 100 μM MFA. Electrical stimulation was performed using a 6-8 M Ω patch pipette (tip diameter < 1 μm) filled with ACSF and surrounded with a silver wire. For BoNT treatment, acutely prepared slices were transferred to tissue culture inserts (0.4 μm -membrane, Becton Dickinson) in 35 mm 6-well plates, which contained external solution (1 ml/well) with antibiotics, and with or without 100 ng/ml BoNT/A or BoNT/B for 18 hrs at 37°C in a 95% O_2 /5% CO_2 -saturated atmosphere.

Immunohistochemistry and proliferation assay

Slices were fixed for 3 hr in 4% paraformaldehyde (PAF) in PBS containing 4% sucrose (PBSS). Immunostaining was performed as previously described^{10,11}. The primary antibodies

included mouse anti-nestin IgG1 antibody (1:1, Rat-401, Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa) and rabbit anti-GABA antibody (1:500, Sigma), mouse Alexa 594-conjugated anti-BrdU IgG1 antibody (1:1000, Molecular Probes) and rabbit anti-GFP antibody (1:200, Abcam). Secondary antibodies were the appropriate Alexa Fluor (1:1000, Molecular Probes). Stained sections were viewed on a confocal scanning laser microscope (Biorad MRC600) or on an epifluorescence microscope (Olympus BX51) using standard procedures. Proliferation experiments were exactly as previously described⁴⁵ and as detailed in Supplementary Method.

Data analysis

Data acquisition and analysis were performed using PClamp 8 (Axon Instruments, CA). For analysis of cell proliferation, confocal z-stacks (10 sections spaced by 1 μ m) were obtained in 2-3 fields of the proximal RMS or anterior SVZ per slice using a LSM 510 META NLO confocal microscope (Carl Zeiss, Inc). The number of GFP⁺ cells per field and then the number of cells that were both GFP⁺ and BrdU⁺ were counted using Adobe Photoshop. None of the control proliferation data (for SNAP5114 and the bicuculline experiments) were significantly different. We combined the two control data for SNAP5114 and bicuculline that were performed with glutamate receptor blockers. Data are given as mean $\pm$ SEM, n being the number of cells or slices when noted. Levels of significance were determined by Student's *t*-test (Statview, Inc).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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BordeyFig1

Figure 1.

GFAP-expressing cells in the SVZ express functional GABA_ARs. (a) Photograph of a cell recorded at the edge of the SVZ (upper). The recorded cell was GFP⁺. Photograph of the lucifer yellow (LY)-fill of the recorded cell (below). (b) Photograph of a LY-filled GFP⁺ cell (green) immunostained for nestin (red), a marker of immature cells. (c) GABA-induced currents before, and during application of MFA (100 μM). (d) Currents in response to a 10-mV hyperpolarizing pulse before and during MFA application from the same cell as in (C). (e) GABA-induced currents before and during application of the GABA_AR antagonist, gabazine (50 μM). (f) Current-voltage curve of GABA responses obtained by applying a ramp protocol (from -120 to 0 mV in 200 ms) near the peak of the current. GABA response reverses at -55 mV as expected for a Cl⁻-carried current in our recording conditions ($E_{Cl^-} = -54$ mV). GFP⁺ cells were recorded at a holding potential of -80 mV.

BordeyFig2

Figure 2.
GABA and picrotoxin affinities for GABA_ARs in GFAP-expressing cells of the SVZ.
 (a) GABA responses induced by increasing GABA concentrations. (b-c) Mean dose-response curve for GABA (b) and picrotoxin (PTX, c) in GFAP-expressing cells gave an EC_{50} of 15.1 μM ($n = 5$) and an IC_{50} of 11.9 μM ($n = 4$), respectively. Four to six GABA concentrations were successively bath applied to each cell. The resulting averaged dose-response curve was fitted with a classical Hill equation. Errors bars represent s.e.m.

BordeyFig3

Figure 3.
Electrical stimulation of SVZ progenitors evoked nonsynaptic GABA_A currents in GFAP-expressing cells. (a) Repetitive focal electrical stimulation in the SVZ (1, 3 and 5 pulses of 200 μ s, 50 Hz) induces inward currents in GFAP-expressing cells. (b) Currents evoked by a 5-pulse stimulus are reversibly blocked with bicuculline (BIC, 100 μ M). (c) Evoked currents develop and decay slowly (Rise time: 680 ms and mono-exponential decay time constant: 1.7 s). Inset: trace at higher time scale. (d) Spontaneous synaptic events are observed in striatal neurons (top two traces) but not in GFAP-expressing cells (bottom trace) during 1 nM α -latrotoxin applications. Transient currents represent responses to 10-mV depolarizing pulses. Scale bars: 50 pA and 20 s (neuron), and 20 pA and 20 s (GFAP⁺ cell). (e) GABA_A currents

evoked by electrical stimulation at positions a, b and c shown (arrows) in the photograph. The arrowhead points to the recorded cell. **(f)** Plots of the normalized amplitudes and rise-times of evoked GABA_A currents against the distance between the stimulating electrodes and recorded cells. **(g)** Evoked currents recorded with 1 μ M TTX (left), in a 0-Ca²⁺ solution (30 min, middle), and in Cd²⁺ and Ni²⁺ for 30 min (each at 100 μ M, right). **(h)** Failure to evoke currents recorded in slices incubated (30 min prior recording) in a Ca²⁺-free solution containing BAPTA-AM. **(i)** Focal electrical stimulation in the SVZ induces inward currents in GFAP-expressing cells recorded midway in the SVZ while no current was induced with an identical stimulation in the striatum.

BordeyFig4

Figure 4.

GABA release from SVZ progenitors is SNARE-independent. (a) Spontaneous synaptic currents in a striatal neuron under control conditions and after 30 min application of 200 ng/ml BoNT/B. BoNT/B did not affect series resistance or cell capacitance measured by applying a 10 mV-hyperpolarizing pulse before and after BoNT/B application. (b) Evoked GABA_A currents in GFAP-expressing cells after a 30 min-application of BoNT/B. (c) Records of synaptic activity in striatal neurons in slices incubated for 18 hrs in a solution with or without 100 ng/ml BoNT/B. (d) Evoked GABA_A currents in GFAP-expressing cells in slices treated with BoNT/B for 18 hrs.

BordeyFig5

Figure 5.

GABA uptake controls GABA_AR activation in GFAP-expressing cells of the SVZ. (a) Bath application of GABA transporter antagonists, 50 μ M NO-711 (for GAT1) and 50 μ M SNAP5114 (for GAT3/4) enhances the amplitude and prolongs the decay time of PTX-sensitive currents evoked by tetanic stimulation in the SVZ. (b) Percentage increases of the evoked GABA_A current amplitude and decay time constant in the presence of NO-711 and SNAP5114. Errors bars represent s.e.m. (c) NO-711 and SNAP5114 (50 μ M each) reversibly induce an inward shift of the holding current in an GFAP-expressing cell. (d) Inward currents evoked by pressure applications of GABA (100 μ M, 100 ms, every 20 s) before and during bath application of NO-711 (50 μ M) and SNAP5114 (50 μ M). Note that GABA responses were preceded by short 10 mV-depolarizations (transients on the records) to monitor the series and input resistance. In addition, NO-711 and SNAP5114 induced inward currents, suggesting that GABA transporter inhibition induced an increase in ambient GABA levels. (e) Average trace of three successive GABA responses at a higher time scale under control (a), in the presence of NO-711 (b) or SNAP5114 (c) and after washout (d). (f) Superimposed GABA responses (averaged) under control (a) and with NO-711 (b) or SNAP5114 (c). (g) Mono-exponential decay time constant of GABA response against the recording time.

BordeyFig6

Figure 6.

Near-physiological depolarization of SVZ progenitors induces activation of GABA_ARs in GFAP-expressing cells that are also tonically activated. (a) Bath application of 7.5 mM K⁺ induces BIC-sensitive inward currents in an GFAP-expressing cell recorded in a Ca²⁺-free solution. (b) Focal pressure application of 12.5 mM K⁺ to a cluster of putative neuroblasts induced PTX-sensitive currents in GFAP-expressing cells. High K⁺ applications are not accompanied by changes in series (access) resistance or capacitance calculated in response to a 10 mV-depolarizing pulse applied before and during PTX application. (c) Spontaneous depolarizations of 15-20 mV in neuroblasts at -60 mV. (d) 100 μM BIC induces a 5 pA-outward shift of the holding current in an GFAP-expressing cell without affecting series resistance or cell capacitance measured by applying a 10 mV-depolarizing pulse before, during and after BIC application. The extracellular solution contained 2.5 mM K⁺. Scale bar: 100 pA, 20 ms. (e) 50 μM PTX induces a 20 pA-outward shift of the holding current in an GFAP-expressing cell in the presence of NO-711 and SNAP5114 (50 μM each).

BordeyFig7

Figure 7.

Neuroblasts release GABA spontaneously and upon depolarization. (a) GABA immunostaining (red) in the SVZ of GFAP^P-GFP mice. (b) Photograph of a neuroblast recorded in cell-attached above the slice. The second patch electrode contains an outside-out patch from a GFP⁺ cell (or a striatal neuron as in C) positioned < 5 μm from the simultaneously recorded neuroblast. (c) Simultaneous recordings from a cell-attached neuroblast (V_{pip} of 0 mV) and an outside-out patch from a striatal neuron held at -80 mV. No single channel activity is detected when the patch is 50 μm away from the neuroblast but channel activity is observed when the patch is < 5 μm from the neuroblast shown in (b) and is blocked by 20 μM gabazine. Insets: Higher time scale of the trace between the arrows and amplitude histogram of middle

records. **(d-e)** Simultaneous recordings from a cell-attached neuroblast and an outside-out patch from a GFP⁺ cell positioned < 5 μm from the neuroblast. **(d)** No channel activity is observed when recording with 20 μM gabazine but single channel activity develops upon gabazine removal. **(e)** Spontaneous channel activity develops after a 5 s-long depolarization of the cell-attached neuroblast (V_{pip} from 0 to -120 mV). Insets: Traces between the arrows displayed at higher time scale. In (e), amplitude histogram of depolarization-induced channels. Scale bar in insets: 200, 500 and 500 μs in c, d and e, respectively. These experiments were performed in the presence of 20 μM DNQX and 50 μM D-APV but without MFA.

BordeyFig8

Figure 8.

GABA_A receptor activation limits the proliferation of GFAP-expressing cells. (a) Reconstructions of 10 μ m z-stacks of BrdU (red) and GFP (green) immunostaining in control and BIC-treated slices. White arrows indicate some GFP⁺ cells that are BrdU⁺. The reconstructed stack in the BIC-treated slice included a region outside the SVZ. BrdU⁺ cells were within the SVZ. (b) Percentage of GFP⁺ cells that are BrdU⁺ in control and in 50 μ M BIC-treated slices. (c) Percentage of GFP⁺ cells that are BrdU⁺ in control in the presence of AMPA/kainate and NMDA receptor blockers (20 μ M DNQX and 50 μ M D-APV, respectively) and in 50 μ M BIC- or 100 μ M SNAP5114 (SNAP)-treated slices. SNAP-5114 is a non-transportable blocker of GAT4 transporters. *: $P < 0.05$, **: $P < 0.001$ (unpaired t -test with unequal variance), box: 25th and 75th percentiles, upper and lower bars: 5th and 95th percentiles, •: minimum and maximum, □: mean, middle bar: median.