GABAergic signalling in a neurogenic niche of the turtle spinal cord

Cecilia Reali¹, Anabel Fernández¹,³, Milka Radmilovich¹,², Omar Trujillo-Cenóz¹ and Raúl E. Russo¹

¹Neurofisiología Celular y Molecular, Instituto de Investigaciones Biológicas Clemente Estable, Avenida Italia 3318, CP11600, Montevideo, Uruguay
²Departamento de Histología y Embriología, Facultad de Medicina, Avenida Gral. Flores 2125, CP 11800, Montevideo, Uruguay
³Unidad Asociada Neuroanatomía Comparada, Facultad de Ciencias, UdelaR, Igua 4225, CP11400, Montevideo, Uruguay

Non-technical summary  Neurogenesis is tightly regulated by epigenetic factors that assure the correct assembly of neural circuits. Neurotransmitters play a fundamental role in this type of control. We show that GABA signals on progenitors and immature neurones within a neurogenic niche around the central canal (CC) of the turtle spinal cord. GABA depolarized progenitors whereas the effect on immature neurones varied from excitation to inhibition. In both cell types GABAergic receptor activation induced an increase in intracellular calcium. Our findings imply that GABAergic signalling around the CC shares fundamental properties with those in the embryo and adult neurogenic niches in the brain, suggesting that GABA is part of the mechanisms regulating the production and integration of neurones to already operational spinal circuits. Understanding the GABAergic modulation of progenitors and neuroblasts may provide useful clues about key mechanisms needed for functional neurogenesis in the spinal cord.

Abstract  The region that surrounds the central canal (CC) in the turtle spinal cord is a neurogenic niche immersed within already functional circuits, where radial glia expressing brain lipid binding protein (BLBP) behave as progenitors. The behaviour of both progenitors and neuroblasts within adult neurogenic niches must be regulated to maintain the functional stability of the host circuit. In the brain, GABA plays a major role in this kind of regulation but little is known about GABAergic signalling in neurogenic niches of the postnatal spinal cord. Here we explored the action of GABA around the CC of the turtle spinal cord by combining patch-clamp recordings of CC-contacting cells, immunohistochemistry for key components of GABAergic signalling and Ca²⁺ imaging. Two potential sources of GABA appeared around the CC: GABAergic terminals and CC-contacting neurones. GABA depolarized BLBP⁺ progenitors via GABA transporter-3 (GAT3) and/or GABA_A receptors. In CC-contacting neurones, GABA_A receptor activation generated responses ranging from excitation to inhibition. This functional heterogeneity appeared to originate from different ratios of activity of the Na⁺–K⁺–2Cl⁻ co-transporter (NKCC1) and the K⁺–Cl⁻ co-transporter (KCC2). In both progenitors and immature neurones, GABA induced an increase in intracellular Ca²⁺ that required extracellular Ca²⁺ and was blocked by the selective GABA_A receptor antagonist gabazine. Our study shows that GABAergic signalling around the CC shares fundamental properties with those in the embryo and adult neurogenic niches, suggesting that GABA may be part of the mechanisms regulating the production and integration of neurones within operational spinal circuits in the turtle.

(Received 17 June 2011; accepted after revision 9 September 2011; first published online 12 September 2011)

Corresponding author  R. E. Russo: Neurofisiología Celular y Molecular, Instituto de Investigaciones Biológicas, Clemente Estable, Avenida Italia 3318, CP 11600, Montevideo, Uruguay. Email: rrusso@iibce.edu.uy

Abbreviations  BLBP, brain lipid binding protein; CC, central canal; E_GABA, reversal potential for GABA-induced currents; E₀, equilibrium potential for K⁺; GAT1, GABA transporter-1; GAT3, GABA transporter-3; GAD, glutamic acid decarboxylase; KCC2, K⁺–Cl⁻ co-transporter; NKCC1, Na⁺–K⁺–2Cl⁻ co-transporter; RG, radial glia; V_m, membrane potential; SVZ, subventricular zone.
Introduction

Neurogenesis involves processes such as the proliferation of neurogenic progenitors and the migration, differentiation and integration of neuroblasts into developing or mature circuits. These events are tightly regulated not only by genetic programmes but also by epigenetic factors that assure the correct assembly of circuits (Spitzer, 2006; Ben-Ari & Spitzer, 2010). A key component of this epigenetic control is the activity-dependent regulation of both progenitors and neuroblasts by signalling via neurotransmitters (Nguyen et al. 2001; Ma et al. 2009). In the developing brain, GABA plays a central role in this type of regulation by providing an excitatory drive that controls various neurogenic steps (Ben-Ari, 2002; Owens & Kriegstein, 2002; Ben-Ari et al. 2007). Although recent studies suggest that GABA seem to have similar roles in neurogenic niches of the adult brain (Ge et al. 2006; Bordey, 2007), there is no information regarding GABAergic signalling in potential neurogenic niches of the spinal cord.

During early stages of spinal cord development, dynamic transcriptional codes in the neural tube define progenitor domains from which subtypes of spinal neurones originate (Lee & Pfaff, 2001). The cells lining the central canal (CC) of the adult spinal cord derive from the ventral part of the neural tube (Fu et al. 2003) and retained some properties of the primitive progenitors (Tanaka & Ferretti, 2009). Indeed in the mammalian spinal cord, ependymal cells are able to proliferate (Johansson et al. 1999; Meletis et al. 2008), although they fail to differentiate into neurones (Horner et al. 2000). However, under some conditions progenitors around the CC of mammals can generate new neurones (Danilov et al. 2006), as normally occurs in lower vertebrates (Tanaka & Ferretti, 2009). In the turtle spinal cord, a population of radial glia (RG) expressing brain lipid binding protein (BLBP) and the transcription factor Pax6 proliferate actively (Russo et al. 2008), suggesting they belong to a domain of neurogenic precursors (Pinto & Götz, 2007). These progenitors are intermingled with immature neurones with functional traits resembling different stages of maturation (Russo et al. 2004). Thus, the spinal cord of turtles represents a unique model system to study the signalling via neurotransmitters that may homeostatically regulate neurogenesis in a niche immersed within already functional circuits.

The presence of GABAergic terminals (Trujillo-Cenóz et al. 2007) around the CC and the fact that CC-contacting neurones receive functional GABAergic contacts (Russo et al. 2004) led us to hypothesize that GABA may signal on spinal progenitors and neuroblasts in a way similar to other neurogenic niches (Bordey, 2007). Here, we aimed at exploring GABAergic signalling around the CC by combining patch-clamp recordings of CC-contacting cells, immunohistochemistry and Ca\(^{2+}\) imaging. We found that GABA depolarized BLBP\(^+\) progenitors whereas the effect on CC-contacting neurones varied from excitation to inhibition. In both cell types GABA\(_A\) receptor activation induced an increase in [Ca\(^{2+}\)]. Our findings represent the first evidence that GABAergic signalling around the CC shares fundamental properties with those in the embryo and adult neurogenic niches in the brain, suggesting that GABA may be part of the epigenetic mechanisms regulating the production and integration of neurones to operational circuits in the turtle spinal cord.

Methods

Ethical approval

All experimental procedures were performed in accordance with the ethical guidelines established by our local Committee for Animal Care and Research at the Instituto de Investigaciones Biológicas Clemente Estable. Every precaution was taken to minimize animal stress and the number of animals used.

General

Data were obtained from 52 juvenile turtles (Trachemys dorbignyi; 5–7 cm carapace length). The animals were maintained in temperate aquaria (24–26°C) under natural illumination and fed daily with small earthworms. From a sensory-motor point of view, juvenile turtles are undistinguishable from adult specimens. Previous studies (Reali & Russo, 2005) showed that the electrophysiological phenotypes of neurones in juvenile animals are identical to those found in adult turtles (Russo & Hounsgaard, 1996a,b).

Slice preparation and electrophysiology

Turtles rendered torpid by hypothermia induced by immersion in crushed ice for 1.5–2 h (Melby & Altman, 1974; Alaburda et al. 2005) were decapitated and the blood was removed by intraventricular perfusion with Ringer solution (6°C). The cervical enlargement of the spinal cord was dissected out and transverse 300 μm-thick slices were cut, placed in a chamber (1 ml volume), and superfused (1 ml min\(^{-1}\)) with Ringer solution of the following composition (in mM): 96.5 NaCl, 2.6 KCl, 31.5 NaHCO\(_3\), 3 CaCl\(_2\), 2 MgCl\(_2\), and 10 glucose. The solution was saturated with 5% CO\(_2\) and 95% O\(_2\) to attain pH 7.6. All experiments were performed at room temperature (20–22°C). Cells were visualized with differential interference contrast optics (DM LFS; Leica, Wetzlar, Germany) with a 40× (0.8 numerical aperture) objective.

Patch-clamp whole-cell recordings were made with electrodes filled with the following solution (in mM): 122 potassium gluconate, 5 Na\(_2\)-ATP, 2.5 MgCl\(_2\), 0.003 CaCl\(_2\), 5634 C. Reali and others

© 2011 The Authors. Journal compilation © 2011 The Physiological Society
1 EGTA, 5.6 magnesium glucose, 5 K-Hepes, 5 H-Hepes, 10 biocytin and 0.25 Alexa 488, pH 7.4, 5–10 MΩ. To study the physiological effects of GABA, we made perforated patch recordings using the cation-selective ionophore gramicidin, which does not interfere with $[Cl^-]$ (Myers & Haydon, 1972). Gramicidin (Sigma-Aldrich, St Louis, MO, USA) was dissolved in DMSO (Sigma-Aldrich; 5 mg ml$^{-1}$, stored at $-20^\circ$C), diluted (5 μg ml$^{-1}$) in pre-filtered intracellular solution and used within 2 h after preparation. To facilitate the gigaseal formation, the tip of the electrode was filled with gramicidin free patch solution by brief (1–2 s) immersion of the back of the pipette into the solution. Stable perforated recordings (70 MΩ series resistances) were obtained in 15–30 min. Two methods were used to confirm that recordings were made in the perforated patch configuration: (1) using a patch solution containing Alexa 488 (250 μM) the dye would diffuse into the cell only when the perforated patch changed into a whole-cell configuration, and (2) using a high concentration of Cl$^-$ in the patch solution (122 mM KCl), rupture of the patch would be indicated by the appearance of a large inward current reversing around +6 mV, as predicted by the Nernst equation.

To estimate the cell membrane potential ($V_m$) without the error introduced by the shunt through the seal (Fricker et al. 1999; Verheugen et al. 1999; Wang et al. 2003), we measured the reversal potential of K$^+$ currents in cell-attached patches. With 158 mM K$^+$ in the pipette solution, which is close to the estimated $[K^+]_i$ in other cell types (Hille, 2001), the equilibrium potential for K$^+$ ($E_K$) across the patch would be around 0 mV and thus the holding potential at which the K$^+$ current reverses direction gives a non-invasive measure of $V_m$. To activate voltage-gated K$^+$ channels we applied voltage ramps (−140 to +200 mV). $E_K$ was measured from the intersection between the K$^+$ current and the fit to the linear component.

Current and voltage clamp recordings were performed with a Multiclamp 700B (Molecular Devices, Sunnyvale, CA, USA). Stimulation protocols and acquisition were done with pCLAMP10 (Molecular Devices), which was also used for further analysis. Series resistance and whole-cell capacitance were not compensated. In voltage clamp mode, cells were held at −70 mV (unless otherwise stated) and the resting membrane potential was estimated from the current–voltage relationship (at $I = 0$). Liquid junction potentials were determined and corrected (Barry and Diamond, 1970). Values are expressed as the mean ± SEM.

**Drug applications**

GABA (0.1–1 mM), baclofen (50 μM) and muscimol (30–60 μM) were dissolved in Ringer solution and puff applied using a Picospritzer III (Parker Instrumentation, Fairfield, NJ, USA) with a patch pipette (2 μm in tip diameter) placed at 15–20 μm from the soma of the recorded cell. In progenitor cells, the time of GABA application varied from 400 ms to 3 s to optimize the GABA-induced response. For CC-contacting neurones, 40–50 ms puffs of GABA were enough to elicit robust responses. In some experiments, the following drugs were added to the bath: tetrodotoxin (TTX, 1 μM; Alomone Labs, Jerusalem, Israel) to block Na$^+$ channels; gabazine (10–20 μM, Tocris Bioscience, Ellsville, MO, USA) and bicuculine (40 μM, Sigma-Aldrich) to block GABA$_A$ receptors; nipecotic acid (0.1–1 mM, Tocris) or (S)-SNAP 5114 (100 μM, Sigma-Aldrich) to block GABA transporter currents; bumetanide (10–20 μM, Sigma-Aldrich) and furosemide (50–200 μM, Sigma-Aldrich) to block cation chloride co-transporters, carbeneoxalone (100 μM, Sigma-Aldrich) to block gap junctions, 6,7-dinitro-quinoxaline-2,3-dione (DNQX; 20 μM; Tocris) to block AMPA receptors and DL-AP-5 (50 μM, Tocris) to block NMDA receptors.

**Morphological identification of recorded cells**

Cells were first visualized in living slices by injecting Alexa 488 and the resulting images were acquired with an FG7 frame grabber (Scion Instruments, Frederick, MD, USA) using NIH ImageJ. Slices were then fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB, pH 7.4) and to better analyse the morphology of cells, biocytin was revealed by using streptavidin conjugated with different fluorophores (Russo et al. 2008). In selected cases, slices were treated with different primary and secondary antibodies as described below. The slices were whole-mounted and examined with a confocal microscope (FV 300; Olympus, Tokyo, Japan).

**Ca$^{2+}$ imaging**

CC-contacting cells were filled with the calcium indicator Fluo-4 (Invitrogen, Carlsbad, CA, USA) by puff application (Picospritzer III, Parker Instrumentation) of the acetoxymethyl ester form (Fluo4-AM, 15–20 μM) dissolved in 0.007% pluronic acid (Invitrogen) and 0.3% DMSO (Sigma-Aldrich). Alternatively, single cell electroporation of the Fluo-4 K$^+$ salt (200–500 μM) dissolved in intracellular solution that lacked EGTA was performed by applying two voltage pulses (10 ms, 10 V) with a patch pipette placed on the surface of CC-contacting cells. Excitation was performed with a fluorescent source (EL 6000, Leica Microsystems, Wetzlar, Germany) equipped with a shutter controlled by TTL pulses. The filters for Ca$^{2+}$ imaging were as follows:
Excitation filter 470 ± 40 nm; dichromatic mirror 500 nm; emission filter 525 ± 50 nm (Leica Microsystems, Wetzlar; Germany). Time lapse imaging was performed with ImageJ (NIH) with a plug-in to use a frame grabber (LG7, Scion Instruments) attached to a CCD camera (TM-300, Pulnix, Hampshire, UK). Images were captured at 1–5 s intervals and adjusted to minimize background and avoid saturation. Intensity measurements were done with ImageJ (NIH) for defined regions of interest (ROI) and expressed as the change of fluorescence relative to background fluorescence ($\Delta F/F_0$).

### Immunohistochemistry

Animals were anaesthetized (50 mg kg$^{-1}$ pentobarbital; i.p.) and fixed by intracardiac perfusion with 4% paraformaldehyde in 0.1 M PB (pH 7.4) after removing the blood with isotonic saline solution (pH 7.4). For GABA immunostaining, turtles were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M PB (pH 7.4). Tissues were sectioned with a vibrating-blade microtome (60–80 μm) and placed in PB with 0.5% bovine serum albumin for 30 min and then incubated with the primary antibodies diluted in PB with 0.3% Triton X-100 (Sigma-Aldrich). The following primary antibodies were used: (1) anti-GABA (rabbit polyclonal, 1:100; no. 20094, Immunostar, Hudson, WI, USA); (2) anti-HuC/D (mouse monoclonal, 1:100; no. A-21271, Molecular Probes, Eugene, OR, USA); (3) anti-BLBP (rabbit polyclonal, 1:1000; no. AB9558, Millipore, Temecula, CA, USA); (4) anti-GAD-65/67 (C-20) (goat polyclonal antibody, 1:200; no. sc-7513, Santa Cruz Biotechnology, Santa Cruz, CA, USA); (5) anti-NKCC1 (T4, mouse monoclonal, 1:10; Developmental Studies Hybridoma Bank); (6) anti-KCC2 (rabbit polyclonal, 1:1000; no. 07–432, Millipore); (7) anti-GABA transporter-3 (GAT3, rabbit polyclonal, 1:1200; no. AB1574, Millipore); (8) anti-GABA transporter-1 (GAT1; rabbit polyclonal, 1:500; no. AB1570W, Millipore); and (9) anti-S100 (β subunit) (mouse monoclonal, 1:500, no. S2532, Sigma). After washing in PB, tissues were incubated in secondary antibodies conjugated with Alexa fluorophores (Invitrogen). The Zenon labelling kit (Invitrogen) was used for HuC/D when this antibody was combined with anti-NKCC1. In co-labelling experiments, Alexa 488 and 633 were used to avoid cross-talk. Control experiments were performed by omitting or replacing primary or secondary antibodies with pre-immune normal serum. In these experiments no detectable staining was observed. The histological material was visualized by confocal microscopy (FV 300; Olympus, Tokyo, Japan). The images were acquired with Fluoview 5 (Olympus) and then exported to Photoshop 7 or Corel Draw for image adjustment.

### Results

#### GABA around the CC

GABA released from neuroblasts (Wang et al. 2003; Hack et al. 2005) or from neighbouring GABAergic interneurones (Tozuda et al. 2005) regulates neurogenesis in the embryo and the adult brain (Bordey, 2007). To explore the possible source of GABA around the CC, we made immunohistochemistry for GABA and its synthesizing enzyme glutamic acid decarboxylase (GAD). We found numerous GAD$^+$ terminals around the CC (Fig. 1A). On the lateral aspects of the CC, many of the terminals were in close apposition with cell bodies (Fig. 1A, main panel and upper inset) and processes of BLBP$^+$ progenitors (Fig. 1A, main panel and lower inset). Immunoreactivity for GABA was absent or very weak in most CC-contacting cells. However, a few cells located on the dorso-lateral aspect of the CC were positive for GAD (Fig. 1A, arrowheads; Ba, arrows). GAD$^+$ cells were identified as neurones because they co-expressed the early neuronal marker HuC/D (Fig. 1Ba–c, arrows). In contrast to the weak expression of GAD, many CC-contacting cells stained positive for GABA (Fig. 1Ca). Most GABAergic cells around the CC (159 out of 177 cells) are identified as belonging to the neuronal lineage because they expressed HuC/D (Fig. 1Ca–c).

#### GABA-induced currents in progenitor cells

In the subventricular zone (SVZ), tonic activation of GABA receptors has an important role in the regulation of the proliferative capacity of progenitors (Liu et al. 2005; Bordey, 2007). Our immunohistochemical studies suggest that GABA may also signal on CC-contacting progenitors. To explore this possibility, we made patch-clamp recordings of progenitors on the lateral aspects of the CC and focally applied GABA. Figure 2A shows the typical electrophysiological phenotype of CC-contacting BLBP$^+$ progenitors which is dominated by leak currents (Fig. 2Aa; see Russo et al. 2008). These cells were electrically coupled as revealed by dye coupling of Alexa 488 (Fig. 2Ab). As expected, these clusters of cells were in close proximity with GAD$^+$ terminals (Fig. 2Ab, inset). In line with these findings, the majority of clustered progenitors (48 out of 59) responded to GABA generating small depolarizations from rest (Fig. 2Ba and Ca). In 26 out of 57 progenitors, GABA generated inward currents (Fig. 2Bb) that did not reverse at membrane potentials from −100 to +20 mV (Fig. 2Bc). In the remaining cases (31 out of 57), we found currents (Fig. 2Cb) that reversed between −75 and −27 mV (−49.1 ± 4.3 mV, n = 31; Fig. 2Cc). In all cases, GABA had a depolarizing effect because the resting membrane potentials of CC-contacting progenitors were more negative (−85.6 ± 2.5 mV, n = 31) than the reversal potential for GABA-induced currents ($E_{\text{GABA}}$).
Because CC-contacting progenitors are electrically coupled via gap junctions (Russo et al. 2008), GABA-induced currents may be contributed by the recorded cell and/or by their coupled neighbours. The gap junction blocker carbenoxolone (100 μM) uncoupled the recorded cell (Fig. 2Da) and reduced the amplitude of GABA currents, producing a shift of $E_{\text{GABA}}$ toward hyperpolarized potentials (Fig. 2Db, 13.2 ± 2.6 mV, $n = 5$). In a minority of cases (2 out of 8), carbenoxolone suppressed non-reversing GABA-induced currents (Fig. 2Dc), indicating the current originated from neighbours coupled to the recorded cell. In the remaining cases the amplitude of the transporter current decreased (4 out of 8) or unmasked a small reversing GABA-induced current (2 out of 8, data not shown).

The data in Fig. 2 suggest different mechanisms for GABA-induced currents. To explore the mechanisms of GABA-induced currents in CC-contacting progenitors, we bath applied nipecotic acid – a non-selective blocker of GABA transporters (GAT) – and the selective GABA$_A$ receptor antagonists gabazine and bicuculline. In 7 out of 15 clustered progenitors, GABA-induced currents had components mediated by GAT and GABA$_A$ receptors since the current was reduced by nipecotic acid (1 mM) and gabazine (20 μM) and abolished when both drugs were applied together (Fig. 3A). In other cases (4 out of 15 cells), the currents were mediated only by GAT because they were blocked by nipecotic acid (1 mM) but not by gabazine (20 μM) (Fig. 3B). In the remaining cells (4 out of 15) GABA-induced currents were abolished by gabazine (20 μM) or bicuculline (40 μM) (Fig. 3C).

Progenitor cells in the adult SVZ express GAT3/4 transporters (Bolteus & Bordey, 2004). Thus, to test whether GABA-induced currents sensitive to nipecotic acid may be mediated by GAT3 we used the selective antagonist SNAP 5114. We found that SNAP 5114 (100 μM) abolished non-reversing GABA-induced currents (Fig. 3Da, $n = 4$). In line with these results, immunohistochemistry against GAT3 and the glial and ependymal cell marker S100 –which is also expressed in BLBP$^+$ cells (Trujillo-Cenoz et al. 2007) – showed that on the lateral aspects of the CC, cells immunoreactive for S100 also expressed GAT3 (Fig. 3Db, arrows).

The fact that $E_{\text{GABA}}$ values were more depolarized than expected (–78 mV, calculated from the Nernst equation) suggested a robust mechanism for Cl$^-$ accumulation in BLBP$^+$ progenitor cells. Because the excitatory effect of GABA during development is mostly due to the activity

![Figure 1. Source of GABA around the CC](https://example.com/figure1.png)

**Figure 1. Source of GABA around the CC**

A, immunohistochemistry for GAD-65/67 (red) and brain lipid binding protein (BLBP, green). A plexus of GAD$^+$ terminals surrounds the CC. A few CC-contacting cells on the dorso-lateral aspect of the CC stained positive for GAD (arrowheads). Many GAD$^+$ terminals were in close apposition with either proximal (upper inset, arrows) or distal (lower inset, arrow) processes of BLBP$^+$ cells. B, immunohistochemistry for GAD shows strongly reactive terminals around the CC and a weaker expression of GAD in the cytoplasm of a few cells located on the dorsal aspect of the CC (arrows in a). These GAD$^+$ cells expressed the early neuronal marker HuC/D (arrows in b). However, notice that most HuC/D$^+$ cells around the CC were GAD$^-$ (a–c). C, in contrast to GAD expression, the CC is surrounded by a large number of cells containing GABA (a) which also expressed HuC/D (b and c). Main panel in A, B and C are confocal optical sections. Scale bars: A, main panel 20 μm, upper and lower insets, 10 μm; B and C, 10 μm. CC: central canal.
of a Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter (NKCC1) (Owens & Kriegstein, 2002; Spitzer, 2010), we carried out immunohistochemistry for NKCC1. The antibody we used (T4, DSHB) specifically recognizes NKCC1 in the central nervous system because blocking NKCC1 expression by RNA interference eliminates the immunostaining in both newborn dentate granule cells (Ge et al. 2006) and immature cortical neurones (Wang & Kriegstein, 2008). In addition, immunostaining with this antibody in the retina is present in wild-type but not in NKCC1 knockout mice (Li et al. 2008). As shown in Fig. 3Ea, NKCC1 expressed around the CC and particularly in the endfeet of BLBP\(^+\) progenitors in contact with the CC lumen. In agreement with this, the selective NKCC1 antagonist bumetanide (20 \(\mu\)M) shifted \(E_{\text{GABA}}\) toward hyperpolarized potentials (Fig. 3Eb and Ec, average shift –9.4 \(\pm\) 1.8 mV, \(n = 7\)).

GABA-induced currents in CC-contacting neurones

We speculated that as the region surrounding the CC is a neurogenic niche (Fernández et al. 2002; Russo et al. 2004, 2008), GABA signalling should reflect the different degrees of maturation of CC-contacting neurones. To test this idea, we analysed the responses of CC-contacting neurones to focal application of GABA. Conventional whole-cell patch-clamp recordings showed that GABA (\(n = 11\)) or the GABA\(_A\) selective agonist muscimol (\(n = 10\), data not shown) generated currents that reversed between –81 and –49 mV (–65.5 \(\pm\) 4.2, \(n = 18\)) in all CC-contacting neurones (Fig. 4A–C). GABA-induced currents were mediated entirely by GABA\(_A\) receptor activation because they were abolished by gabazine (Fig. 4D; 20 \(\mu\)M, \(n = 3\)) or bicuculline (40 \(\mu\)M, \(n = 2\); data not shown). Focal application of 50 \(\mu\)M baclofen – a GABA\(_B\) receptor agonist – did not produce any effect on CC-contacting neurones (\(n = 8\), data not shown). In neuroblasts of the adult mammalian brain, GABA\(_A\) receptors are tonically activated by ambient GABA (Ge et al. 2006; Bordey, 2007). Similarly, we found that application of gabazine (20 \(\mu\)M) blocked a tonic current in CC-contacting neurones (\(n = 11\); Fig. 4E).

GABA acts as an excitatory transmitter in immature neurones, shifting to an inhibitory action as neurones complete their differentiation (Ben-Ari, 2002; Owens & Kriegstein, 2002; Cherubini et al. 2010). If CC-contacting neurones are in different stages of maturation, then it is reasonable to speculate that GABA should produce heterogeneous effects. To test the actual effect of GABA on

**Figure 2. GABA effects on CC-contacting progenitors**

Aa, current responses to a series of voltage steps recorded in whole-cell mode (a). After recording, the cell appeared dye coupled with neighboring cells (b). Notice the close proximity of GAD terminals to the cluster of dye coupled cells (b, main panel and inset). B, progenitors were depolarized by transient application of GABA (400 ms, 1mM; a). In some progenitors GABA-induced inward currents (b) did not reverse at membrane potentials from –100 to +20 mV (c). C, in other clusters of progenitors, the depolarization induced by GABA (a) was generated by currents (b) that had clear reversal potentials (c). D, the gap junction blocker carbenoxolone (100 \(\mu\)M) increased the apparent input resistance (a) and decreased a GABA-induced current, shifting the reversal from –49 to –60 mV (b). In a few cases, the GABA-induced current disappeared in the presence of carbenoxolone (c). Holding potential in Db and c; –100 mV. Ab, confocal optical section. Scale bar in Ab: main panel, 20 \(\mu\)m; inset, 3 \(\mu\)m.

© 2011 The Authors. Journal compilation © 2011 The Physiological Society
CC-contacting neurones, we made gramicidin perforated patch-clamp recordings. GABA-induced currents in CC-contacting neurones (Figs 5Aa and 6Aa) reversed between −34 and −81 mV (−56.1 ± 1.9 mV, n = 41, see Figs 5Ab and 6Ab) and as expected, were abolished by gabazine (n = 9, Figs 5Ac, inset and 6Ac lower trace). In 18 out of 46 cells, GABA produced a depolarization from rest (Fig. 5Ac) that in some cases was strong enough to generate spike firing (Fig. 5Ca, 6 out of 18). GABA hyperpolarized from rest the remaining cells recorded with the perforated patch-clamp technique (28 out of 46, Fig. 6Ac).

To confirm the recordings were done in the perforated patch configuration, we routinely excited the Alexa 488 contained in the pipette and observed no diffusion into the cell (Fig. 5Ba). After rupturing the patch the dye quickly filled the recorded cell and confirmed it contacted the CC lumen (Fig. 5Bb). As an alternative approach to evaluate GABA-induced excitation, we applied GABA in the cell-attached configuration. Under these conditions, application of GABA generated spike firing in 4 out of 12 cells (Fig. 5Cb). The complete blockade of GABA-induced currents by gabazine suggested that, unlike progenitors, there are no GABA transporters in CC-contacting neurones. In support of this interpretation, the neuronal GABA transporter GAT1 was expressed in the ventral and dorsal horn but not in the region surrounding the CC (data not shown).

Most CC-contacting neurones hyperpolarized by GABA (18 out of 25) fired action potentials repetitively (Fig. 6Aa and c), suggesting they may be cells in more advanced stages of differentiation (see discussion). In line with this, some CC-contacting neurones inhibited by GABA expressed GAD (Fig. 6Ba–c, arrow), another element favouring a more differentiated stage for these cells.

Because the input resistance of CC-contacting neurones (∼1–4 GΩ) is close to the resistance of the seal (∼4–18 GΩ), it is likely that the resting membrane

---

**Figure 3. The nature of GABA-induced currents in CC-contacting progenitors**

*Panel A* GABA-induced currents with components sensitive to the GABA transporter blocker nipecotic acid (1 mM, nip. ac.) and gabazine (20 μM). Application of nipecotic acid and gabazine together blocked the response. *Panel B*, in cells in which the GABA-induced currents had no reversal, the response was blocked by nipecotic acid (1 mM) and not affected by gabazine (20 μM). *Panel C*, in a third class of progenitors, the GABA-induced currents were abolished by the GABA<sub>A</sub> receptor antagonists gabazine (20 μM) or bicuculline (40 μM). *Panel D*, addition of the specific GAT3 antagonist SNAP 5114 (100 μM) blocked a non-reversing GABA-induced current (a). Double immunostaining showed expression of GAT3 (b) in S100 cells (arrows in b) close to the CC lumen. *Panel E* NKCC1 (a) was strongly expressed in the apical processes of BLBP (b) immunoreactive cells. The NKCC1 blocker bumetanide (20 μM) shifted the reversal of GABA-induced currents toward hyperpolarized potentials (b and c). All the recordings were made in whole-cell mode at a holding potential of −100 mV. *Db* and *Ea*, stacks of 10 optical sections. Scale bars: 5 μm.

© 2011 The Authors. Journal compilation © 2011 The Physiological Society
potential was underestimated in our study due to the shunt to ground through the seal (Tyzio et al. 2003). To overcome this problem, we measured the reversal potential of K+ currents in cell-attached mode (assuming 150 mM K+ in the patch solution gives a symmetric K+ concentration) to estimate the resting membrane potential (Fig. 7A; see Wang et al. 2003). We found that the resting potential was more hyperpolarized (19.5 ± 2.7 mV, n = 6) than that measured with perforated patch recordings implying that in most cases GABA depolarizes CC-contacting neurones from rest (Fig. 7B).

The depolarizing action via GABA_A receptors is due to a high [Cl−], attained by the co-transporter NKCC1 which is counteracted by the efflux of Cl− by KCC2 (Ben-Ari, 2002; Spitzer, 2010). Thus, the actual effect of GABA would be determined by the balance of NKCC1 and KCC2 in CC-contacting neurones. Figure 7C shows that in many cells (14 out of 17) the selective NKCC1 antagonist bumetanide (20 μM) shifted E_{GABA} to more hyperpolarized potentials (−56.5 ± 2.4 mV to −64.7 ± 2.2 mV; P < 0.004, Wilcoxon’s matched pairs test, n = 14; Fig. 7Ca–c). To check whether KCC2 could be active in cells with relatively hyperpolarized E_{GABA} (≥−55 mV), we applied the non-selective antagonist of cation chloride co-transporters furosemide (Payne et al. 2003). In 5 out of 8 cells, E_{GABA} shifted to depolarized membrane potentials (−72 ± 3.3 mV to −59.65 ± 2.8 mV; P < 0.03, Wilcoxon’s matched pairs test, n = 5) in the presence of furosemide (50–200 μM, Fig. 7Ca–c) suggesting a high KCC2:NKCC1 ratio (Woodin et al. 2003). Immunohistochemistry for these transporters showed a high expression of NKCC1 in most CC-contacting neurones (Fig. 7D) whereas the expression of KCC2 was less robust and mostly confined to cells located dorso-laterally (Fig. 7E, arrowheads). However, the expression of KCC2 in neurones outside the ependyma was conspicuous (Fig. 7Eb and c; arrows).

As a functional correlate of KCC2 immunohistochemical data, we tested the activity of KCC2 in CC-contacting neurones with different E_{GABA} using a strategy described by Ben-Ari et al. (2011) in the hippocampus. In gramicidin perforated patch recordings we first applied GABA at a holding potential equal to E_{GABA} and then shifted to a depolarized potential (0 mV, 1 min), where GABA generated large outward currents due to the influx of Cl− via GABA_A receptors (Fig. 7Fa and b, n = 13). After returning to the initial holding potential, GABA generated inward currents because of the shift in E_{GABA} induced by the load of Cl− (Fig. 7Fa and b). The time course of recovery of E_{GABA} reflects the dynamic removal of Cl− by KCC2 (Ben-Ari et al. 2011; Nardou et al. 2011). We found that in cells with depolarized E_{GABA} the recovery was slow, taking up to 10 min (Fig. 7Fa and c). However, in CC-contacting neurones with a hyperpolarized E_{GABA} (Fig. 7Fb, E_{GABA} = −78 mV), the inward GABA-induced current disappeared with a faster time course (Fig. 7Fc). In these cells (E_{GABA} ≥ −68 mV), the half-time decay of the recovery was increased by furosemide (Fig. 7Fc, inset; 4 out of 4 cells, average increase 61.7 ± 24.7 s) suggesting KCC2 contributed to the clearance of intracellular Cl−. Overall, these results strengthen the interpretation that the depolarized E_{GABA} observed in more immature CC-contacting neurones arises from low levels of KCC2 activity.

**Intracellular Ca²⁺ responses induced by GABA**

Many regulations exerted by GABA on progenitors and neuroblasts in neurogenic niches are mediated by a rise in intracellular Ca²⁺ (Owens & Kriegstein, 2002; Ge et al. 2006; Bordey, 2007). To check whether GABA signalling around the CC also produce changes in [Ca²⁺]i, we performed Ca²⁺ imaging of CC-contacting cells. BLBP cells on the lateral aspects of the CC were loaded with the Ca²⁺ imaging indicator Fluo4 either by puff application of Fluo4-AM or by electroporation of the K+ salt of Fluo4. As shown in Fig. 8Aa, GABA produced a slow increase in [Ca²⁺]i (15.4 ± 3.7%ΔF/F₀; time to peak 165.5 ± 24.2 s, n = 25) in some of the Fluo4-loaded cells lying on the ependyma.
lateral aspect of the CC (25 out of 40 cells). Although we did not characterize the time course of the decay of the Ca\(^{2+}\) response to avoid bleaching of the dye, the time needed for recovery of [Ca\(^{2+}\)]\(_i\) before GABA application could last several minutes (up to 13 min, data not shown). Immunohistochemistry against BLBP confirmed that Fluo4 loaded cells belonged to the domain of BLBP\(^+\) RG (Fig. 8Ab). The increase in [Ca\(^{2+}\)]\(_i\) induced by GABA involved GABA\(_A\) receptors and required extracellular Ca\(^{2+}\) because it was strongly decreased by adding gabazine \((n = 8)\) or lowering Ca\(^{2+}\) \((n = 5)\) in the bath to 0.2 mM (Fig. 8B and C).

We also explored the Ca\(^{2+}\) response to GABA application in CC-contacting neurones. Immature

![Figure 5. GABA as an excitatory transmitter](image)

**Figure 5.** GABA as an excitatory transmitter

A, response of a CC-contacting neurone to a current step (gramicidin perforated patch-clamp technique) elicits two small spikes (a). The current–voltage relationship of the peak GABA-induced current (b) revealed an \(E_{\text{GABA}}\) close to –40 mV. In current clamp mode, GABA depolarized the cell (c, left trace) but did not produce spiking even when the membrane was depolarized with a current pulse (c, right trace). The inward GABA-induced current was blocked by gabazine \((10 \mu M; c, \text{inset})\). B, Alexa 488 \((250 \mu M)\) in the pipette did not fill the cell in the perforated patch configuration. After attaining the whole-cell configuration the cell was filled with Alexa 488 revealing an apical process contacting the CC. C, in some cases the GABA-induced depolarization generated spikes (a, gramicidin perforated patch; b, cell-attached mode). The GABA-induced excitation was blocked by gabazine \((20 \mu M, a)\).

![Figure 6. GABA as an inhibitory transmitter](image)

**Figure 6.** GABA as an inhibitory transmitter

A, repetitive spiking of a CC-contacting neurone (a) generated by a depolarizing current pulse (gramicidin perforated patch technique). The \(I-V\) plot for GABA-induced currents in this cell showed a reversal at –66 mV (b). A puff of GABA hyperpolarized the cell and inhibited spike firing (c, upper trace), an effect blocked by gabazine \((10 \mu M)\) (c, lower trace). B, a repetitive spiking CC-contacting neurone inhibited by GABA (a, main panel and inset). The recorded cell was reactive for GAD (b and c, arrows). Confocal optical sections. Scale bars: B, main panels, 10 \(\mu M\); inset, 10 mV and 400 ms. A and B from the same cell.
neurones loaded with Fluo4 were easily identified by their pear-like shape. In 19 of 32 cells, GABA produced a substantial $[\text{Ca}^{2+}]_i$ increase ($17.6 \pm 8.8\% \Delta F/F_0$; time to peak $74.3 \pm 13.8$ s; Fig. 9Aa and Ba). In the developing spinal cord of *Xenopus* embryos, GABA generates a rise in $[\text{Ca}^{2+}]_i$ mediated by activation of metabotropic receptors (Root et al. 2008). However, the increase in $[\text{Ca}^{2+}]_i$ in CC-contacting neurones was blocked by gabazine, implying GABA$_A$ receptors (Fig. 9Ab and B; $n = 6$). Similar to progenitor cells, the rise in $[\text{Ca}^{2+}]_i$ in CC-contacting neurones was blocked in low (0.2 mM) extracellular $\text{Ca}^{2+}$ ($n = 6$, data not shown). The neuronal nature of the cell shown in Fig. 9 was confirmed by immunohistochemistry for HuC/D (Fig. 9C).

---

**Figure 7. Mechanisms of GABA actions in CC-contacting neurones**

A, a voltage ramp induced a current in cell-attached mode that reversed at 90 mV in a symmetric [K+] condition. B, histogram showing the $E_{\text{GABA}}$ measured in gramicidin perforated patch recordings. The average resting potentials estimated in cell-attached (RMP CA) and perforated patch (RMP PP) modes are indicated by arrows. C, currents induced by GABA at $-70$ mV (1) in control (top trace) and in the presence of bumetanide (20 μM, second trace from top). Notice that in the presence of bumetanide, GABA generated an outward current, an effect that reversed upon wash-out (third trace from top). Addition of furosemide (100 μM, forth trace from top) increased the amplitude of the inward GABA-induced current. The I–V plots of GABA-induced currents shows the shifts in $E_{\text{GABA}}$ induced by bumetanide and furosemide (b). The scatter plot in c shows the changes in $E_{\text{GABA}}$ induced by bumetanide and furosemide for different cells. D, most HuC/D cells (a) express NKCC1 (b and c). E, double immunohistochemistry for HuC/D (a) and KCC2 (b). KCC2 was strongly expressed in neurones outside the CC region (a–c arrows) whereas in the ependyma was only weakly expressed in some HuC/D$^+$ cells located dorsally (a–c, arrowheads). F, kinetics of $\text{Cl}^-$ removal in CC-contacting neurones recorded in perforated patch mode. GABA (50 ms, 100 μM; arrows) was applied in the presence of DNQX (20 μM) and AP-5 (50 μM) first at a holding potential equal to $E_{\text{GABA}}$ ($-35$ mV, a) and then at 0 mV during 1 min where GABA induced outward currents. Upon returning to the initial holding potential, GABA induced inward currents that decayed slowly with a half-decay time of 168 s (a, inset). In a cell with a more hyperpolarized $E_{\text{GABA}}$ ($-78$ mV, b) the GABA-induced inward currents recovered with a faster kinetic (half-decay time 32 s). Addition of furosemide (200 μM) slowed down the time course of recovery (b, inset). The scatter plot in c shows the relationship between $E_{\text{GABA}}$ and the half-decay time for different CC-contacting neurones. Notice that depolarized $E_{\text{GABA}}$ values correlate with longer half-decay times. D and E, confocal optical sections. Scale bars: D and E, 20 μm.
**Discussion**

Neurogenesis within already operating circuits must be homoeostatically regulated to preserve function. In the brain, GABA is a key component of this type of regulation (Ben-Ari, 2002). We find here that GABA signals on BLBP$^+$ spinal progenitors and CC-contacting neurones in a way similar to other neurogenic niches (Bordey, 2007). The effects of GABA on CC-contacting neurones depended on the balance between NKCC1 and KCC2 suggesting various stages of neuronal maturation. GABA$_A$ receptor activation led to an increase in $[\text{Ca}^{2+}]_i$, which may provide a link between activity and gene transcription (Ben-Ari & Spitzer, 2010).

**The sources of GABA around the CC**

Two potential sources of GABA appeared around the CC: (a) a plexus of GAD$^+$ terminals and (b) a population of GABAergic neurones. The numerous GAD$^+$ terminals around the CC suggest a source for phasic, synaptically driven GABA signalling (Bordey, 2007). Indeed, phasic GABA signalling in CC-contacting neurones is supported by our previous studies showing spontaneous GABAergic synaptic potentials (Russo et al. 2004). However, in contrast to hippocampal progenitors (Tozuka et al. 2005), phasic GABA$_A$ receptor activation seems unlikely for spinal progenitors because we never observed spontaneous synaptic events in BLBP$^+$ cells. Unlike the abundant GAD$^+$ terminals, GAD immunoreactivity in CC-contacting cells was faint and confined to a few cells. However, as in the adult SVZ (Wang et al. 2003) most CC-contacting neurones contained GABA. It remains to be determined whether CC-contacting GABAergic cells may locally release GABA like their counterparts in the SVZ (Liu et al. 2005). The tonic activation of GABA$_A$ receptors reported here supports this possibility. Alternatively, synaptically released GABA may spill over contributing a tonic GABA$_A$-induced current.

The mismatch between the GABAergic nature and GAD immunoreactivity of CC-contacting neurones suggests

---

**Figure 8. GABA induces intracellular Ca$^{2+}$ increases in BLBP$^+$ progenitors**

A, multiple cells on the lateral aspects of the CC loaded with Fluo4 (a) responded with an increase in $[\text{Ca}^{2+}]_i$ to puff application of GABA (10 s). Combination with immunohistochemistry for BLBP showed that many Fluo4 loaded cells belonged to the domain of BLBP$^+$ progenitors (b). The $\text{Ca}^{2+}$ response induced by GABA in CC-contacting progenitors was abolished by addition of gabazine (20 μM). C, plot showing the time course of the increase in $[\text{Ca}^{2+}]_i$, induced by GABA. The response was abolished in low $\text{Ca}^{2+}$ Ringer solution (0.2 mM). Scale bars: A and B, 10 μm.

**Figure 9. GABA increases Ca$^{2+}$ in CC-contacting neurones**

A, a cell loaded with Fluo4 (puff application of the Fluo4-AM ester) responded with an increase in $[\text{Ca}^{2+}]_i$ to GABA (a). GABA failed to increase $[\text{Ca}^{2+}]_i$ in the presence of gabazine (b, 20 μM). B, time course of $[\text{Ca}^{2+}]_i$ increase shown in A in control and after addition of gabazine (20 μM). C, immunohistochemistry for HuC/D confirmed that the cell shown in A was a CC-contacting neurone. Scale bars: A and C, 10 μm.
that GABA may be synthesized by a pathway different from glutamate conversion by GAD (Martin & Rimvall, 1993). Some reports show that SVZ GABAergic neuroblasts express GAD (Wang et al. 2003) whereas others reported lower levels of GAD until SVZ neuroblasts reach the olfactory bulb (De Marchis et al. 2004), and proposed that GABA may be produced from putrescine (Sequerra et al. 2007). In the developing retina, GABA synthesis is first performed by ornithine decarboxylase and then taken over by GAD as its initial low expression increases during development (Yamasaki et al. 1999). A similar scenario may take place around the CC. The fact that GAD was expressed by a subset of CC-contacting cells may indicate they are in more advanced stages of differentiation, as suggested by their functional traits of maturity such as inhibition by GABA and repetitive firing (Spitzer et al. 2000).

**GABA signalling in spinal progenitors**

In most clusters of electrically coupled BLBP⁺ progenitors, GABA-induced currents had sizeable components mediated by GAT and GABA₄ receptors whereas a few were dominated either by GAT or GABA₃ components. The recordings obtained in the presence of carbonoxolone suggest that within a cluster progenitors react differently to GABA. The heterogeneity of GABA responses resembles the variable expression of K⁺ delayed rectifier currents among BLBP/Pax6⁺ progenitors (Russo et al. 2008), and may reflect different functional roles or developmental stages.

In the SVZ, GABA uptake into precursors by GAT3/4 plays an important role in clearing GABA released from neuroblasts, thereby regulating their migration (Bolteus & Bordey, 2004) and the proliferation of progenitors (Liu et al. 2005). CC-contacting progenitors expressed functional GAT3 whereas the neuronal GAT1 was absent around the CC, indicating the removal of GABA in the ependyma relies on uptake via GAT3 by progenitors. The tridimensional network of BLBP/Pax6 progenitors around HuC/D⁺ cells (Russo et al. 2008) is a geometric arrangement similar to that of the SVZ which appears well suited to spatially control extracellular GABA levels (Bolteus & Bordey, 2004; Plate et al. 2008).

As in the mammalian brain (Owens & Kriegstein, 2002), many BLBP⁺ progenitors had functional GABA₄ receptors. The mismatch between the recorded \( E_{GABA} \) in many clusters of progenitors and the theoretical equilibrium potential for Cl⁻ imposed by the pipette solution may be explained by the lack of space clamp of electrically coupled cells far from the recording site. The shift of \( E_{GABA} \) toward hyperpolarized potentials produced by carbonoxolone supports this interpretation. However, the \( E_{GABA} \) in cells uncoupled with carbonoxolone was still more depolarized than predicted by \( [\text{Cl}^-]_i \), suggesting an efficient mechanism for Cl⁻ accumulation. This possibility is supported by the strong expression of NKCC1 in the apical process of BLBP⁺ cells.

The GABA-induced depolarization in CC-contacting progenitors resembles GABA actions on progenitors in the developing brain (Lo Turco et al. 1995) and postnatal neurogenic niches (Bolteus & Bordey, 2004; Liu et al. 2005). In the SVZ, GABA released by neuroblasts decreases the proliferation of progenitors (Lo Turco et al. 1995; Haydar et al. 2000; Liu et al. 2005). It is tempting to speculate that GABA released from CC-contacting neurones regulates the proliferation of BLBP⁺ progenitors. In addition, the intimate relationship between GABAergic terminals and BLBP⁺ cells raises the possibility that spillover from synaptically released GABA may influence progenitors via GABA₄ receptors. GAD⁺ terminals around the CC are likely to originate from neighbouring GABAergic interneurones because CC-contacting neurones lack local axon collaterals (Russo et al. 2004; Trujillo-Cenóz et al. 2007). Thus, GABA released from interneurones may help regulating the behaviour of CC-contacting progenitors according to the activity of spinal circuits. In vivo experimental approaches are needed to test these possibilities.

**GABA: a signal for maturation of CC-contacting neurones?**

CC-contacting neurones had functional GABA₄ receptors as indicated by the full blockade of GABA-induced currents by gabazine. In gramicidin perforated patch recordings, GABA₄ receptor activation generated responses ranging from depolarizations that elicited spike firing to hyperpolarizations from rest. Taking into account the resting membrane potential estimated from cell-attached recordings, GABA depolarized from rest most CC-contacting neurones as reported for migrating neuronal progenitors in the SVZ and rostral migratory stream (Wang et al. 2003). The depolarized \( E_{GABA} \) implies a \( [\text{Cl}^-]_i \) higher than in mature neurones and is in agreement with the robust expression of NKCC1 in HuC/D⁺ cells. Although KCC2 strongly expressed in spinal neurones outside the ependyma, it only expressed weakly in a few CC-contacting HuC/D⁺ cells. Thus, our electrophysiological and immunohistochemical findings suggest a predominance of NKCC1 over KCC2 rendering a depolarized \( E_{GABA} \) in most CC-contacting neurones. This interpretation is supported by the shift of \( E_{GABA} \) towards hyperpolarized potentials by the NKCC1 antagonist bumetanide whereas furosemide, a blocker of both NKCC1 and KCC2 (Payne et al. 2003), had the opposite effect on cells with relatively hyperpolarized \( E_{GABA} \). The functional assay of KCC2 (see Fig. 7F) implies that the
more hyperpolarized $E_{\text{GABA}}$ in some CC-contacting neurones results from the appearance of some degree of KCC2 activity.

Both in the SVZ and the dentate gyrus, GABA provides an excitatory drive to newborn neurones because of high activity of NKCC1 (Bordey, 2007). As neurones mature, GABA action switches from excitation to inhibition because down-regulation of NKCC1 and/or increase of KCC2 activity (Rivera et al. 1999; Ganguly et al. 2001). Our data suggest that HuC/D$^{+}$ cells in the ependyma are neurones in different stages of maturation with variable NKCC1:KCC2 ratios that determine GABA actions from excitation to inhibition. In agreement, HuC/D$^{+}$ cells hyperpolarized by GABA generally spiked repetitively – an electrophysiological phenotype of more differentiated neurones (Russo & Hounsgaard, 1999; Spitzer et al. 2000) – and had measurable KCC2 activity.

**GABA and Ca$^{2+}$: a tandem in neurogenic niches**

Most GABA actions on neurogenesis are mediated by an increase in [Ca$^{2+}$], induced by membrane depolarization (Ge et al. 2006; Bordey, 2007). In many CC-contacting progenitors and neurones, GABA produced a prolonged increase in [Ca$^{2+}$], that required extracellular Ca$^{2+}$ and GABA$_{A}$ receptor activation. The time course of Ca$^{2+}$ signals was similar to spontaneously occurring slow Ca$^{2+}$ waves in the developing spinal cord of *Xenopus* (Gu et al. 1994) and to GABA-induced Ca$^{2+}$ signals in cortical progenitors (LoTurco et al. 1995). The increase in [Ca$^{2+}$], induced by GABA is commonly attributed to the opening of voltage-gated Ca$^{2+}$ channels (Ben-Ari, 2002). Whereas this may be a plausible mechanism for CC-contacting neurones, it is unlikely that the small depolarization observed in many cells would be enough to open voltage-gated Ca$^{2+}$ channels. Moreover, BLBP$^{+}$ progenitors lack inward voltage-gated currents (Russo et al. 2008). Alternative mechanisms such as activation of Ca$^{2+}$-permeable transient receptor potential channels gated by osmotic changes may participate in the GABA-induced [Ca$^{2+}$], increase described here (Chavas et al. 2004). The detailed mechanisms and functional relevance of GABA-induced [Ca$^{2+}$], increases in CC-contacting cells remains to be clarified.

**Concluding remarks**

GABAergic signalling appears to play a key part in matching the activity of neural circuits with different steps of neurogenesis in the brain (Ben-Ari & Spitzer, 2010). We present here the first evidence that the region surrounding the CC has various components of GABAergic signalling characteristic of adult neurogenic niches, suggesting that GABA may similarly regulate neurogenesis in the turtle spinal cord. Some elements of GABA signalling described here may still be at work in the rat ependyma. Although the mammalian spinal cord seem to have lost the ability for neurogenesis (Horner et al. 2000), there are cells in the rat ependyma that display molecular and electrophysiological features of neuroblasts which, as described here, respond heterogeneously to GABA (Marichal et al. 2009). Interestingly, a recent study in the spinal cord of adult mice reported neurogenesis enhanced by sensory stimulation, but newborn neurones fail to mature and integrate into existing spinal circuits (Shechter et al. 2010). It is possible that progression of these neuroblasts to mature neurones does not occur because the lack of key components of GABAergic signalling. Understanding the GABAergic modulation of progenitors and neuroblasts in different animal models may give useful clues about key mechanisms needed for functional neurogenesis in the spinal cord.

**References**

Alaburda A, Russo R, MacAulay N & Hounsgaard J (2005). Periodic high-conductance states in spinal neurons during scratch-like network activity in adult turtles. *J Neurosci* **25**, 6316–6321.

Barry PH & Diamond JM (1970). Junction potentials, electrode standard potentials, and other problems in interpreting electrical properties in membranes. *J Membr Biol* **3**, 93–122.

Ben-Ari Y (2002). Excitatory actions of GABA during development: the nature of the nurture. *Nat Rev Neurosci* **3**, 728–739.

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.

Ben-Ari Y & Spitzer NC (2010). Phenotypic checkpoints regulate neuronal development. *Trends Neurosci* **33**, 485–492.

Ben-Ari Y, Tyzio R & Nehlig A (2011). Excitatory action of GABA on immature neurons is not due to absence of ketone bodies metabolites or other energy substrates. *Epilepsia* **52**, 1544–1558.

Bolteus AJ & Bordey A (2004). GABA release and uptake regulate neuronal precursor migration in the postnatal subventricular zone. *J Neurosci* **24**, 7623–7631.

Bordey A (2007). Enigmatic GABAergic networks in adult neurogenic zones. *Brain Res Brain Res Rev* **53**, 124–134.

Chavas J, Forero ME, Collin T, Llano I & Marty A (2004). Osmotic tension as a possible link between GABA$_{A}$ receptor activation and intracellular calcium elevation. *Neuron* **44**, 701–713.

Cherubini E, Griguoli M, Safiulina V & Lagostena L (2010). The depolarizing action of GABA controls early network activity in the developing hippocampus. *Mol Neurobiol* **43**, 97–106.

Danilov AI, Covacu R, Moe MC, Langmoen IA, Johansson CB, Olsson T & Brundin L (2006). Neurogenesis in the adult spinal cord in an experimental model of multiple sclerosis. *Eur J Neurosci* **23**, 394–400.
De Marchis S, Temoney S, Erdelyi F, Bovetti S, Bovolin P, Szabo G & Puche AC (2004). GABAergic phenotypic differentiation of a subpopulation of subventricular derived migrating progenitors. Eur J Neurosci 20, 1307–1317.

Fernández A, Radmilovich M & Trujillo-Canéz O (2002). Neurogenesis and gliogenesis in the spinal cord of turtles. J Comp Neurol 453, 131–44.

Fu H, Qi Y, Tan M, Cai J, Hu X, Liu Z, Jensen J & Qiu M (2003). Molecular mapping of the origin of postnatal spinal cord ependymal cells: evidence that adult ependymal cells are derived from Nkx6.1+ ventral progenitor cells. J Comp Neurol 456, 237–244.

Fricker D, Verheugen JA & Miles R (1999). Cell-attached measurements of the firing threshold of rat hippocampal neurones. J Physiol 517, 791–804.

Ganguly K, Schröder AF, Wong ST & Poo M (2001). GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. Cell 105, 521–532.

Ge S, Goh EL, Sailor KA, Kitabatake Y, Ming GL & Song H (2006). GABA regulates synaptic integration of newly generated neurones in the adult brain. Nature 439, 589–593.

Gu X, Olson EC & Spitzer NC (1994). Spontaneous neuronal calcium spikes and waves during early differentiation. J Neurosci 14, 6325–6335.

Hack MA, Saghatelayan A, de Chevigny A, Pfeifer A, Ashery-Padan R, Lledo PM & Gottz M (2005). Neuronal fate determinants of adult olfactory bulb neurogenesis. Nat Neurosci 8, 865–872.

Haydar TF, Wang F, Schwartz ML & Rakic P (2000). Differential modulation of proliferation in the neocortical ventricular and subventricular zones. J Neurosci 20, 5764–5774.

Hille B (2001). Ionic Channels of Excitable Membranes, pp. 1–20. Sinauer Associates Inc., Sunderland, MA, USA.

Horner PH, Power AE, Kempermann G, Kuhn GH, Palmer TD, Winkler J, Thal LJ & Gage FH (2000). Proliferation and differentiation of progenitor cells throughout the intact adult rat spinal cord. J Neurosci 20, 2218–2228.

Johansson CB, Momma S, Clarke DL, Risling M, Lendahl U & Frisen J (1999). Identification of a neural stem cell in the adult mammalian central nervous system. Cell 96, 25–34.

Lee SK & Pfaff SL (2001). Transcriptional networks regulating neuronal identity in the developing spinal cord. Nat Neurosci 4, 1183–1191.

Li B, McKernan K & Shen W (2008). Spatial and temporal distribution patterns of Na-K-2Cl cotransporter in adult and developing mouse retinas. Vis Neurosci 25, 109–123.

Liu X, Wang Q, Haydar TF & Bordey A (2005). Nonsynaptic GABA signaling in postnatal subventricular zone controls proliferation of GFAP-expressing progenitors. Nat Neurosci 8, 1179–1187.

LoTurco JJ, Owens DF, Heath MJ, Davis MB & Kriegstein AR (1995). GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. Neuron 15, 1287–1298.

Ma DK, Kim WR, Ming GL & Song H (2009). Activity-dependent extrinsic regulation of adult olfactory bulb and hippocampal neurogenesis. Ann N Y Acad Sci 1170, 664–673.

Marichal N, García G, Radmilovich M, Trujillo-Canéz O & Russo RE (2009). Enigmatic central canal contacting cells: immature neurones in “standby mode”? J Neurosci 29, 10010–1024.

Martin DL & Rimvall K (1993). Regulation of glycineaminobutyric acid synthesis in the brain. J Neurochem 60, 395–407.

Melby ECJ & Altman NH (1974). Handbook of Laboratory Animal Science. CRC, Cleveland.

Meletis K, Barnabé-Heider F, Carlen M, Evergren E, Tomilin N, Shupliakov O & Frisen J (2008). Spinal cord injury reveals multilineage differentiation of ependymal cells. PLoS Biol 6, 1494–1507.

Myers VB & Haydon DA (1972). Ion transfer across lipid membranes in the presence of gramicidin A. II. The ion selectivity. Biochim Biophys Acta 274, 313–322.

Nardou R, Yamamoto S, Chazal G, Bhar A, Ferrand N, Dulac O, Ben-Ari Y & Khalilov I (2011). Neuronal chloride accumulation and excitatory GABA underlie aggravation of neonatal epileptiform activities by phenobarbital. Brain 134, 987–1002.

Nguyen L, Rigo JM, Rocher V, Belachew S, Malgrange B, Register B, Leprince P & Moonen G (2001). Neurotransmitters as early signals for central nervous system development. Cell Tissue Res 305, 187–202.

Owens DF & Kriegstein AR (2002). Is there more to GABA than synaptic inhibition? Nat Rev Neurosci 3, 715–727.

Payne JA, Rivera C, Voipio J & Kaila K (2003). Cation-chloride co-transporters in neuronal communication, development and trauma. Trends Neurosci 26, 199–206.

Pinto L & Götz M (2007). Radial glial cell heterogeneity—the source of diverse progeny in the CNS. Prog Neurobiol 83, 2–23.

Platel JC, Dave KA & Bordey A (2008). Control of neuroblast production and migration by converging GABA and glutamate signals in the postnatal forebrain. J Physiol 586, 3739–3743.

Reali C & Russo RE (2005). An integrated spinal cord-hindlimbs preparation for studying the role of intrinsic properties in somatosensory information processing. J Neurosci Methods 142, 317–326.

Rivera C, Voipio J, Payne JA, Rusuuvuori E, Lahtinen H, Lamsa K, Pirvola U, Saarma M & Kaila K (1999). The K+Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature 397, 251–255.

Root CM, Velázquez-Ulloa NA, Monsalve GC, Minakova E & Spitzer NG (2008). Embryonically expressed GABA and glutamate drive electrical activity regulating neurotransmitter specification. J Neurosci 28, 4777–4784.

Russo RE, Fernández A, Reali C, Radmilovich M & Trujillo-Canéz O (2004). Functional and molecular clues reveal precursor-like cells and immature neurones in the turtle spinal cord. J Physiol 560, 831–838.

Russo RE & Hounsgaard J (1996a). Plateau-generating neurones in the dorsal horn in an in vitro preparation of the turtle spinal cord. J Physiol 493, 39–54.

Russo RE & Hounsgaard J (1996b). Burst-generating neurones in the dorsal horn studied in an in vitro preparation of the turtle spinal cord. J Physiol 493, 55–66.
GABAergic signalling around the central canal

Russo RE & Hounsgaard J (1999). Dynamics of intrinsic electrophysiological properties in spinal cord neurones. Prog Biophys Mol Biol 72, 329–365.

Russo RE, Reali C, Radmilovich M, Fernández A & Trujillo-Cenóz O (2008). Conexin 43 define functional domains of neurogenic precursors in the spinal cord. J Neurosci 28, 3298–3309.

Sequerra EB, Gardino P, Hedin-Pereira C & de Mello FG (2007). Putrescine as an important source of GABA in the postnatal rat subventricular zone. Neuroscience 146, 489–493.

Shechter R, Baruch K, Schwartz M & Rolls A (2010). Touch gives new life: mechanosensation modulates spinal cord adult neurogenesis. Mol Psychiatry 16, 342–52.

Spitzer NC (2006). Electrical activity in early neuronal development. Nature 444, 707–712.

Spitzer NC (2010). How GABA generates depolarization. J Physiol 588, 757–758.

Spitzer NC, Vincent A & Lautermilch NJ (2000). Differentiation of electrical excitability in motoneurons. Brain Res Bull 53, 547–552.

Tanaka EM & Ferretti P (2009). Considering the evolution of regeneration in the central nervous system. Nat Rev Neurosci 10, 713–723.

Touzaki Y, Fukuda S, Namba T, Seki T & Hisatsune T (2005). GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. Neuron 47, 803–815.

Trujillo-Cenóz O, Fernández A, Radmilovich M, Reali C & Russo RE (2007). Cytological organization of the central gelatinosa in the turtle spinal cord. J Comp Neurol 502, 291–308.

Tyzio R, Ivanov A, Bernard C, Holmes GL & Ben-Ari Y, Khazipov R (2003). Membrane potential of CA3 hippocampal pyramidal cells during postnatal development. J Neurophysiol 90, 2964–2972.

Verheugen JA, Fricker D & Miles R (1999). Noninvasive measurements of the membrane potential and GABAergic action in hippocampal interneurons. J Neurosci 19, 2546–2555.

Wang DD & Kriegstein AR (2008). GABA regulates excitatory synapse formation in the neocortex via NMDA receptor activation. J Neurosci 28, 5547–5558.

Wang DD, Krueger DD & Bordey A (2003). GABA depolarizes neuronal progenitors of the postnatal subventricular zone via GABA_A receptor activation. J Physiol 550, 785–800.

Woodin MA, Ganguly K & Poo MM (2003). Coincident pre- and postsynaptic activity modifies GABAergic synapses by postsynaptic changes in Cl^- transporter activity. Neuron 39, 807–820.

Yamasaki EN, Barbosa VD, De Mello FG & Hokoc JN (1999). GABAergic system in the developing mammalian retina: dual sources of GABA at early stages of postnatal development. Int J Dev Neurosci 17, 201–213.

Authors contributions

The study was done in the department of Neurofisiología Celular y Molecular, Instituto de Investigaciones Biológicas Clemente Estable. C.R.: conception and design of the experiments; collection, analysis and interpretation of electrophysiological data and Ca^{2+} imaging; drafting of the manuscript. A.F. and M.R.: collection, analysis and interpretation of immunohistochemical data. O.T.C.: analysis and interpretation of immunohistochemical data; revising the manuscript critically for important intellectual content. R.E.R.: conception and design of the experiments; analysis and interpretation of data; writing of the manuscript. All authors approved the final version of the manuscript.

Acknowledgements

We thank G. Fabbiani and M. I. Rehermann for technical assistance and Dr Antonia Marin-Burgin for advice on Ca^{2+} imaging. The Na^+-K^+-Cl^- co-transporter (NKCC1) monoclonal antibody developed by Drs C. Lytle and B. Forbush III was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA, USA. The work described here was supported by Grant no. FCE_2920 from ANII and Grant no. R01NS048255 from the National Institute of Neurological Disorders and Stroke to R.E.R. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Neurological Disorders and Stroke or the National Institutes of Health.