Induction of NMDA and GABA$_A$ receptors-mediated Ca$^{2+}$ oscillations with KCC2 mRNA downregulation in injured facial motoneurons

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Running title: Axotomy induces GABA-mediated Ca$^{2+}$ Oscillation

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ABSTRACT

To clarify the changes that occur in γ-aminobutyric acid type A (GABA<sub>A</sub>) receptor-mediated effects and contribute to alterations in the network activities after neuronal injury, we studied intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) dynamics in a rat facial-nerve-transection model. In facial motoneurons, an elevation of the resting [Ca<sup>2+</sup>]<sub>i</sub>, GABA-mediated [Ca<sup>2+</sup>]<sub>i</sub> transients, enhancement of the glutamate-evoked [Ca<sup>2+</sup>]<sub>i</sub> increases, and spontaneous [Ca<sup>2+</sup>]<sub>i</sub> oscillations were induced by axotomy. All these axotomy-induced modifications were abolished by the GABA<sub>A</sub>-receptor antagonist bicuculline and NMDA-receptor antagonist D-AP5. A downregulation of K<sup>+</sup>-Cl<sup>-</sup> cotransporter (KCC2) mRNA, an increase in intracellular Cl<sup>-</sup> concentration ([Cl<sup>-</sup>]<sub>i</sub>) and transformation of GABAergic hyperpolarization to depolarization were also induced by axotomy. We suggest that in axotomized neurons KCC2 downregulation impairs Cl<sup>-</sup> homeostasis and makes GABA act depolarizing, resulting in endogenous GABA inducing [Ca<sup>2+</sup>]<sub>i</sub> oscillations via facilitation of NMDA-receptor activation. Such GABA<sub>A</sub>-receptor-mediated [Ca<sup>2+</sup>]<sub>i</sub> oscillations may play a role in neural survival and regeneration.

Key Words: chloride homeostasis, NKCC1, regeneration, intracellular calcium, Cl<sup>-</sup> transporter
INTRODUCTION

γ-Aminobutyric acid (GABA), the principal inhibitory neurotransmitter in the brain, decreases neural activity by hyperpolarizing the membrane potential via Cl\(^-\) influx through GABA\(_A\) receptor channels. However, early in development, GABA seems to depolarize and excite the neuronal membrane potential and increase the [Ca\(^{2+}\)]\(_i\) (Ben-Ari et al. 1989, 1997; Ben-Ari 2002; Chen et al. 1996; Cherubini et al. 1990; Khazipov et al. 1997; Leinekugel et al. 1995; LoTurco et al. 1995; Owens et al. 1996; Yuste and Katz 1991). The more depolarized equilibrium potential for Cl\(^-\), effected by the intracellular Cl\(^-\) increase, may be involved in this immature action of GABA (Chen et al. 1996; Cherubini et al. 1990; Owens et al. 1996; Serafini et al. 1995).

Cation-chloride cotransporters are considered to play a critical role in intracellular Cl\(^-\) homeostasis (Kaila 1994). Under physiological conditions, K\(^+\)-Cl\(^-\) cotransporters (KCC2) appear to extrude Cl\(^-\) from the neuron (Jarolimek et al. 1999; Kakazu et al. 1999; Rivera et al. 1999), while NKCC1, a Na\(^+\),K\(^+\)-2Cl\(^-\) cotransporter, is a candidate for the promotion of Cl\(^-\) accumulation within the cell (Plotkin et al. 1997; Sun and Murali 1999; but see DeFazio et al. 2000). In immature neurons, downregulated KCC2 and upregulated NKCC1 expression may thus be responsible for depolarizing and excitatory action of GABA (Kakazu et al. 1999; Yamada et al. 2002).

Network-driven membrane potential and Ca\(^{2+}\) oscillations have been reported in immature hippocampus (Ben-Ari et al. 1989, 1997; Ben-Ari 2002; Cherubini et al. 1990; Khazipov et al. 1997; Leinekugel et al. 1995) and neocortex (Garaschuk et al. 2000; Yuste and Katz 1991). GABAergic excitation and secondary activation of NMDA receptors have
been postulated as an underlying mechanism of these Ca\textsuperscript{2+} oscillations (Ben-Ari et al. 1997; Ben-Ari 2002; Khazipov et al. 1997; Leinekugel et al. 1997). It has been reported that axotomy of vagal motoneurons can transform the GABAergic effect from inhibitory to excitatory by increasing [Cl\textsuperscript{−}], via downregulation of KCC2, so that exogenously applied GABA then evokes a transient [Ca\textsuperscript{2+}]\textsubscript{i} increase (Nabekura et al. 2002). However, since this study has been done by using acutely isolated neurons, it is yet to be studied whether this [Ca\textsuperscript{2+}]\textsubscript{i} increase is related to induction of network-driven Ca\textsuperscript{2+} oscillations accompanied by an alteration in endogenous GABAergic and NMDA receptor-mediated functions.

To clarify the changes in endogenous GABA\textsubscript{A} receptor-mediated effects resulting from \textit{in vivo} neural injury and the underlying mechanism, we used a facial-nerve-transection model, since GABA-containing vesicles in presynaptic terminals on facial motoneurons are enlarged after axotomy (Vaughan 1994) and axotomized motoneurons are considered a model of regeneration (Streit and Graeber 1993). We studied changes in the [Ca\textsuperscript{2+}]\textsubscript{i} responses induced by either endogenous or exogenous GABA and glutamate, which enabled us to evaluate functional alterations in local neural circuits following axotomy. We also evaluated the molecular and physiological basis for those changes.

**METHODS**

All experiments conformed to the guidelines for animal experimentation at Hamamatsu University School of Medicine on the ethical use of animals and all efforts were made to minimize the number of animals used and their suffering.

\textit{In situ} hybridization histochemistry
We used adult male Wistar rats weighing about 150 g as well as young ones of either sex at postnatal day (P) 10 (Japan SLC, Shizuoka, Japan). Under pentobarbital anesthesia (50 mg/kg, i.p.), we transected the right facial nerve just distal to the posterior auricular branch and removed about 5 mm of the distal nerve. At 1, 3, 7, 14, 21, 28, 42, 56 and 112 days after this operation, they (n = 5 at each time-point) were deeply anesthetized with ether and killed. Brains were quickly removed and frozen on powdered dry ice. Frozen sections (16 µm thick) were cut on a cryostat, thaw-mounted onto silane-coated slides, then stored at -80°C.

The in situ hybridization histochemical technique used for KCC2 and NKCC1 is described in detail elsewhere (Kanaka et al. 2001). Briefly, hybridization was performed by incubating paraformaldehyde-fixed sections for 24 h at 42°C in a buffer of the following composition: 0.6 M NaCl and 0.06 M sodium citrate, 50% deionized formamide, 0.12 M phosphate buffer, 2.5% tRNA, 10% dextran sulfate in Denhardt's solution, containing \(^{35}\)SdATP (37-55.5 TBq/mmol; New England Nuclear, Boston, MA, USA)-labelled probes (1-2 x 10\(^{7}\) dpm/ml, 0.2 ml/slide). The sections were coated with Kodak NBT-2 emulsion, kept at 4°C for 2-3 weeks, then developed in D-19 developer. Since the expression levels of KCC2 and NKCC1 mRNAs were different, we used the exposure times of 2 weeks for KCC2 and 3 weeks for NKCC1 for emulsion autoradiography. The sections were counterstained with thionin solution to allow morphological identification.

The probes for KCC2 and NKCC1 mRNAs (Kanaka et al. 2001) were complementary to the bases 2981-3016 and 2914-2949, respectively, of these mRNAs (Moore-Hoon and Turner 1998; Payne et al. 1996). The specificity of the probes has been already confirmed (Kanaka et al. 2001). For semi-quantitative analysis of labelled neurons,
four sections were randomly chosen from 3 animals killed at each of the nine post-surgical time-points. Neurons with three times more grains than the background level were considered to be positively labelled. Motoneurons were counted on thionin-stained sections.

**Preparation of brain slices**

The young rats were subjected to right-facial-nerve transection, after 3 days (P10-12), they were deeply anesthetized and killed. A block of brain including the facial nucleus was quickly removed and placed in cold (4°C), oxygenated, modified artificial cerebrospinal fluid (ACSF). The solution contained the following (in mM): 170 sucrose, 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 12.0 MgSO_4, 26.0 NaHCO_3, 30.0 glucose. Coronal slices (400 µm) through the facial nucleus were cut in the modified ACSF using a vibratome (Leica VT-100, Germany). Slices were allowed to recover for 60 min on nylon meshes (with 1 mm pores) which were submerged in dishes containing standard ACSF consisting of (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2.0 MgSO_4, 2.0 CaCl_2, 26.0 NaHCO_3, 20.0 glucose. The dishes were then placed in a tightly sealed box filled with 95% O_2-5% CO_2 at a pressure of 50 kPa at room temperature.

**Gramicidin-perforated patch-clamp recordings**

Gramicidin-perforated patch-clamp recording was carried out as previously described (Ebihara et al. 1995). Patch electrodes were made from borosilicate capillary tubing (diameter, 1.5 mm; Garner Glass Company, USA) using a Narishige PP-83 vertical puller (Narishige, Japan). The electrode resistance was within the range 3 to 4.5 MΩ. The pipette solution contained (in mM): 130 KCl, 5 NaCl, 0.4 CaCl_2, 1.0 MgCl_2, 1.1 EGTA, 10 HEPES (pH 7.3 with KOH). Gramicidin (50 mg/ml) was dissolved in the pipette
solution just before the experiment. Facial motoneurons in slices were viewed on a monitor via a 40x water-immersion objective lens with the aid of an infrared differential interference-contrast (IR-DIC) filter and a CCD-camera (C2400-79; Hamamatsu Photonics, Japan). Real-time video images were contrast-enhanced by a video processor (Argus-20; Hamamatsu Photonics, Japan). Membrane currents and potentials were recorded using an Axopatch 1D amplifier (Axon Instruments, USA). Data were digitized using an A/D converter (Digidata 1200, Axon Instruments) and analysed by means of pCLAMP8 software.

To measure $E_{\text{GABA}}$, voltage steps were applied and GABA (10 $\mu$M) was pressure-applied through the patch pipette to the soma of the neurons at each membrane potential. All experiments were carried out at 30°C.

**Ca$^{2+}$-imaging using fura-2**

The methods used for Ca$^{2+}$-imaging were similar to those described previously (Fukuda et al. 1998a). Neurons were loaded with the Ca$^{2+}$-indicator, fura-2, by incubating slices for 60 min with fura-2 acetoxy methyl (10 $\mu$M) in ACSF containing 0.01% pluronic F127. Slices were then laid on the glass bottom of a submerged-type chamber and this was placed on a microscope stage and continuously perfused with standard ACSF gassed with 95% $O_2$-5% $CO_2$ at a rate of 2-3 ml/min. In some experiments, the concentration of Mg$^{2+}$ in the standard ACSF was reduced to 1 mM or 0.5 mM (low-Mg$^{2+}$). The bathing solution was maintained at 30°C and had a pH of 7.4.

Fura-2 fluorescence was excited using a multi-wavelength monochrometer (C6789; Hamamatsu Photonics, Japan) and the emitted light was filtered using a band-pass filter (510 nm). Fluorescence images were obtained using a 40x objective lens (Plan Fluor, N.A. 0.75; Nikon, Japan) via a cooled-CCD camera (C6790-81; Hamamatsu Photonics, Japan) fitted to
an upright microscope (E600-FN; Nikon, Japan). Data were stored for off-line analysis by means of image-processing software (Aqua Cosmos; Hamamatsu Photonics, Japan). 

\([Ca^{2+}]_i\) was expressed as the ratio of the fura-2 fluorescence intensities excited at 340 nm and 380 nm (RF340/F380). Changes with time in RF340/F380 were monitored in facial motoneurons by taking measurements every 10 s. These were converted into \([Ca^{2+}]_i\), using the following equation: 

\[ [Ca^{2+}]_i = K_d \left[ \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)} \right] \beta \]

(Gryniewicz et al. 1985) in which \(K_d\) is the effective dissociation constant of fura-2 and \(\beta\) is the ratio of fluorescence intensities at 380 nm excitation for fura-2/(fura-2 + Ca\(^{2+}\)). 

\[ R_{\text{min}} = 0.2, \quad R_{\text{max}} = 7.2, \quad \beta = 3.3 \] and \(K_d = 146\) nM were obtained using the calibration method (Williams et al. 1985). All drugs were applied by bath-perfusion.

**Cl\(^-\) imaging using 6-methoxy-N-ethylquinolinium iodide (MEQ)**

The devices and materials used for Cl\(^-\) imaging were similar to those described above for Ca\(^{2+}\) imaging and the techniques used for Cl\(^-\) imaging were as described previously (Fukuda et al. 1998b; Schwartz and Yu 1995). Briefly, prior to bath-loading of the slices, MEQ was reduced to a cell-permeable form, diH-MEQ (Biwersi and Verkman 1991). This reduction of MEQ (2 mg/100 µl) was achieved by addition of 15 µl of 12% NaBH\(_4\) solution and bubbling with N\(_2\) for 30 min. DiH-MEQ was extracted from the reaction mixture as a yellow organic layer, a portion of which was added to ACSF to yield a final concentration of 360 µM.

Neurons were loaded with MEQ by incubating slices with diH-MEQ for 60 min. MEQ was excited at 355 nm to emit fluorescence and this was filtered at 460 nm. The perfusion medium was changed from standard ACSF to a calibration solution containing 0 mM Cl\(^-\), in which NaCl was substituted by equimolar methylsulfuric acid potassium salt, and
to which tributyltin, a Cl⁻-OH antiporter (20 µM), and nigericin, a K⁺-H⁺ antiporter (14 µM), were added (Simchowitz et al. 1991). When in the presence of these reagents for > 20 min, [Cl⁻]ᵢ can be assumed to have equilibrated across the plasma membrane of the neurons in the slice. At the end of the procedure, total quenchable intracellular-MEQ fluorescence was measured following the addition of 150 mM KSCN. The resting [Cl⁻]ᵢ was obtained by calculating the ratio of the fluorescence measured in the absence of Cl⁻ (F₀: F_{Cl = 0} - F_{SCN}) to that measured at the resting [Cl⁻]ᵢ (F_{Rest}: F_{Cl = \text{rest}} - F_{SCN}) and fitting the values to the calibration curve, with a Kᵣ of 30.6 M⁻¹.

**Drugs**

The following drugs were used: tRNA from Roche (Mannheim, Germany), D(-)-2-amino-5-phosphono pentanoic acid (D-AP5) and 6-cyano-7-nitroquinoxalin-2,3-dione (CNQX) from Tocris Cookson (Ballwin, MO, USA), Denhardt’s solution from Nacalai tesque (Kyoto, Japan), NBT-2 emulsion and D-19 developer from Kodak (Rochester, NY, USA), fura-2 acetoxyl methyl and pluronic F127 from Dojindo (Kumamoto, Japan), dextran sulfate, tetrodotoxin (TTX), (-)-bicuculline methiodide, GABA, L-glutamate, nifedipine, methylsulfuric acid potassium salt and gramicidin from Sigma (St. Louis, MO, USA), MEQ from Molecular Probes (Eugene, OR, USA).

Through this report, “intact” is used to refer to neurons on the side contralateral to the facial-nerve section.

**RESULTS**

**Facial-nerve transection downregulates expression of KCC2 mRNA**
We performed unilateral transection of the facial nerve in adult rats. The number of axotomized neurons stained by thionin was comparable to those of intact neurons throughout the observation period. To assess the general expression patterns and the time course of the changes in the expression of KCC2 and NKCC1 mRNAs in the facial nucleus after axotomy, we evaluated in situ hybridization signals using film autoradiography.

Dark-field photomicrography revealed that the very intense KCC2 mRNA level was in decline as early as the first post-operative day and that it was almost abolished 3 days after the axotomy (Fig. 1A). This severe downregulation of KCC2 mRNA expression was sustained for 3 weeks before a gradual recovery. By 16 weeks after the axotomy, the KCC2 mRNA level had recovered to the control level (Fig. 1C). Bright-field photomicrography revealed that the KCC2 mRNA hybridization signals were positive in 95.1 ± 2.3% (mean ± S.D. throughout text) of large-sized (50 µm) cells (considered to be facial motoneurons) but not in the small-sized presumed glial cells (Fig. 1B). After the precipitate fall that immediately followed axotomy, recovery was evinced by 43.5 ± 8.5% of neurons being positive at 8 weeks and 81.8 ± 9.3% at 16 weeks (Fig. 1, B and C). There are reportedly no interneurons in the facial nucleus and only a minority of cells project via an extra-facial-nerve route from this nucleus (Røste 1989), so most neurons in the ipsilateral facial nucleus (i.e. facial motoneurons) were, not surprisingly, affected by the axotomy.

We used young rats, aged 10-12 days, for all the optical imaging and patch-clamp recordings. To compare the characteristics of facial motoneurons at this age with those of the adult, we studied changes in the expressions of KCC2 mRNA in the facial nucleus on P10 following unilateral facial-nerve transection 3 days earlier (n = 5). The expression of
KCC2 mRNA was comparable to that of the adult on intact side and it was clearly reduced on the axotomized side as in adult (Fig. 2A). There were no apparent signs of cell loss or severe deterioration such as swelling (Fig. 2A). At 8 weeks after transection, the KCC2 mRNA level showed a substantial recovery, the time course being comparable to that seen in the adult (not shown).

In P10 rats (n = 5), NKCC1 mRNA was expressed at levels comparable to those seen in the adult (n = 5). Bright-field observation showed that the hybridization signals for NKCC1 were localized both in neurons and in glial cells (Fig. 2B). Facial-motoneuron axotomy did not change the expression of NKCC1 mRNA either in motoneurons or in glial cells at any day after operation either in adult or young rats. Fig. 2B shows that there was no apparent difference in the expression of NKCC1 mRNA between intact and axotomized facial nuclei on P10 following transection 3 days earlier.

[FIG. 2 near here]

**Facial-nerve transection increased [Cl⁻]ᵢ in motoneurons**

To study the functional consequences of facial nerve transection on Cl⁻ homeostasis, we compared the resting [Cl⁻]ᵢ of axotomized neurons with that of intact neurons. First, we examined the $E_{\text{GABA}}$ in axotomized neurons by means of gramicidin-perforated patch-clamp recording. This technique allows measurement of $E_{\text{GABA}}$ with the intracellular Cl⁻ intact (Ebihara et al. 1995). In the current-clamp mode, GABA (100 µM) induced a hyperpolarization in intact neurons (n = 3) and a depolarization in axotomized neurons (n = 6) (Fig. 3A). Axotomized neurons had a resting membrane potential of $-65.8 \pm 5.2$ mV (n = 7) and an $E_{\text{GABA}}$ of $-52.1 \pm 5.7$ mV, while the corresponding values in intact neurons were
–65.6 ± 1.5 mV (n = 5) and –70.9 ± 5.3 mV (Fig. 3A). We calculated [Cl\textsubscript{i}] for each neuron from the Nernst equation, using measured $E_{\text{GABA}}$ and a [Cl\textsubscript{o}] of 132.5 mM. The [Cl\textsubscript{i}] in axotomized neurons was 17.8 ± 4.6 mM (n = 7), a value significantly higher than that obtained for intact neurons 8.5 ± 4.6 mM (n = 5; Mann-Whitney U test, p < 0.001).

We also compared the resting [Cl\textsubscript{i}] by optical imaging using MEQ. To this end, we changed the perfusion medium from standard ACSF to calibration solution containing 0 mM Cl\textsuperscript{-} together with tributyltin and nigericin. This allowed us to obtain a ratio of MEQ fluorescence values (resting [Cl\textsubscript{i}] over 0 mM [Cl\textsubscript{o}]). We estimated the resting [Cl\textsubscript{i}] from Stern-Volmer plots with $K_q = 30.6$ M\textsuperscript{-1}. The resting [Cl\textsubscript{i}] of axotomized neurons was 24.4 ± 17.3 mM (n = 10; 3 slices), significantly higher than that in intact neurons (11.1 ± 8.5 mM, n = 9; 3 slices; Mann-Whitney U test, p < 0.05) (Fig. 3B). Although absolute [Cl\textsubscript{i}] values measured by this method may not be strictly reliable (Fukuda et al. 1998b; Wöll et al. 1996), collectively, the above results suggest, in terms of comparison, that the depolarizing action of GABA in axotomized neurons is due to an elevated [Cl\textsubscript{i}] and a depolarized Cl\textsuperscript{-} equilibrium potential.

[FIG. 3 near here]

**GABA-mediated rise in intracellular Ca\textsuperscript{2+} induced by facial-nerve transection**

We loaded facial motoneurons with fura-2, measured the ratio of the fluorescence intensities excited at 340 nm and 380 nm ($R_{F340/F380}$) and used it to calculate [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 4A). Intact neurons had a resting [Ca\textsuperscript{2+}]\textsubscript{i} of 43.5 ± 7.3 nM (n = 15; 4 slices), and this was altered neither by 100 µM GABA nor 20 µM bicuculline (n = 15; 4 slices; Fig. 4B). In axotomized facial motoneurons, resting [Ca\textsuperscript{2+}]\textsubscript{i} was 57.6 ± 8.6 nM (n = 24; 5 slices), significantly higher
than in intact cells (p < 0.005, Mann-Whitney U test). In two thirds of cells tested after axotomy (16/24; 5 slices), GABA (100 µM) evoked marked [Ca\textsuperscript{2+}]i increases (by 46.5 ± 8.1 nM). Bicuculline not only blocked this increase but also decreased the baseline Ca\textsuperscript{2+} level, by 12.1 ± 2.6 nM (n = 8, p < 0.005; 3 slices) to a level comparable to that seen in intact cells (Fig. 4C). The increases in [Ca\textsuperscript{2+}]i evoked by GABA were completely blocked by 50 µM nifedipine, an L-type Ca\textsuperscript{2+}-channel blocker (n = 10; 3 slices; Fig. 4D) and by 1 µM tetrodotoxin (TTX), a sodium-channel blocker (n = 12; 3 slices: not shown), while the resting [Ca\textsuperscript{2+}]i was altered by neither of these agents. In contrast, D-AP5 did not block GABA-evoked [Ca\textsuperscript{2+}]i transient but reduce the baseline Ca\textsuperscript{2+} level by 7.5 ± 3.1 nM (n = 14, p < 0.05; 3 slices; Fig. 4E).

**[FIG. 4 near here]**

**[Ca\textsuperscript{2+}]i changes in response to glutamate in facial motoneurons**

To help us evaluate functional alterations in local neural circuits, we studied changes in the [Ca\textsuperscript{2+}]i responses to high-dose (100 µM) and low-dose (5 and 10 µM) glutamate. In intact neurons, bath-application of glutamate (100 µM) for 8 min increased the peak [Ca\textsuperscript{2+}]i by 131.9 ± 11.2 nM (n = 18; 4 slices) with a return to baseline 15.0 ± 3.6 min after the end of the glutamate application (Fig. 5A). In axotomized neurons, glutamate evoked larger and more prolonged (> 40 min, not returned to baseline during observation period) increases in the [Ca\textsuperscript{2+}]i (by 216.3 ± 12.6 nM, n = 16; 4 slices; Fig. 5C). In intact neurons, addition of bicuculline prolonged (27.8 ± 5.4 min) and enlarged the glutamate-evoked [Ca\textsuperscript{2+}]i increases (after bicuculline, by 169.1 ± 12.4 nM, n = 9; 3 slices; Fig. 5A). By contrast, in axotomized neurons the glutamate-evoked [Ca\textsuperscript{2+}]i increases were reduced by...
bicuculline in both amplitude (after bicuculline, by 181.4 ± 15.6 nM, n = 19; 5 slices) and
duration (35.7 ± 6.4 min; Fig. 5C).

We also studied changes in the [Ca^{2+}]_i responses to high-dose glutamate in the
presence of TTX (1 μM) to clarify the alternative network-based effects of GABA and
 glutamate. In intact neurons, addition of TTX prolonged the glutamate-evoked [Ca^{2+}]_i
increases both duration (29.3 ± 5.0 min) and amplitude (by 159.6 ± 25.7 nM, n = 19; 5
slices; Fig. 5B), whereas in axotomized neurons the glutamate-evoked [Ca^{2+}]_i increases were
reduced by TTX in duration (32.2 ± 4.7 min) and amplitude (by 182.4 ± 33.1 nM, n = 12; 3
slices; Fig. 5D). In the presence of TTX, additions of bicuculline affected neither duration
nor amplitude of glutamate-evoked [Ca^{2+}]_i increases in intact (30.4 ± 5.7 min, by 171.1 ±
14.0 nM, n = 8; 3 slices; Fig. 5B) and in axotomized (31.4 ± 5.9 min, by 176.4 ± 18.3 nM, n
= 11; 3 slices; Fig. 5D) neurons.

【FIG. 5 near here】

Bath-applications of low-dose glutamate (5 and 10 μM) for 2-min failed to evoke an
[Ca^{2+}]_i response in intact neurons (n = 9; 3 slices; Fig. 6A) even in the presence of
bicuculline (n = 10; 3 slices; Fig. 6B), indicating these dose were not sufficient to evoke any
Ca^{2+} transients. However, in axotomized neurons low-dose glutamate raised the [Ca^{2+}]_i
level (by 25.6 ± 8.1 nM in response to 5 μM glutamate; by 49.9 ± 8.7 nM in response to 10
μM) (n = 9; 3 slices; Fig. 6C). The α-amino-3-hydroxy-5-methylisoxazole-4-propionate
(AMPA)-receptor antagonist CNQX (10 μM) caused only marginal reduction (n = 12; 3
slices; Fig. 6D), while the NMDA-receptor antagonist D-AP5 (50 μM) blocked this effect of
low-dose glutamate (n = 10; 3 slices; Fig. 6E). Addition of TTX (n = 13; 4 slices; Fig. 6F)
and bicuculline (n = 9; 3 slices; Fig. 6G) abolished these low-dose glutamate-induced [Ca^{2+}]_i,
increases. These results indicate that endogenous GABA exerts on excitatory action in cooperation with glutamate in axotomized facial motoneurons to facilitate Ca\(^{2+}\) influx through NMDA receptor.

**FIG. 6 near here**

**Spontaneous [Ca\(^{2+}\)]\(_i\) oscillations induced in axotomized facial motoneurons**

We used low-Mg\(^{2+}\) (1 mM or 0.5 mM) ACSF in order to reduce Mg\(^{2+}\) block of NMDA receptors and/or enhance presynaptic release of transmitters. Oscillation-like spontaneous Ca\(^{2+}\) transients hardly occurred at all in intact neurons even in 0.5 mM extracellular Mg\(^{2+}\) concentration ([Mg\(^{2+}\)]\(_o\)) (Fig. 7A). In contrast, in axotomized neurons spontaneous [Ca\(^{2+}\)]\(_i\) oscillations occurred even in normal ACSF (20/28; 3 slices) and their amplitude and frequency, as well as the resting [Ca\(^{2+}\)]\(_i\), increased as the [Mg\(^{2+}\)]\(_o\) was reduced (Fig. 7B). These effects of low-Mg\(^{2+}\) ACSF, on resting [Ca\(^{2+}\)]\(_i\) and [Ca\(^{2+}\)]\(_i\) oscillations, were reduced by the addition of D-AP5 (50 µM; Fig. 7C), while CNQX (10 µM) was ineffective (Fig. 7D). The [Ca\(^{2+}\)]\(_i\) oscillations were also reversibly diminished by TTX (n = 10; 3 slices; Fig. 7E). In some axotomized neurons, there were synchronous spontaneous [Ca\(^{2+}\)]\(_i\) oscillations during perfusion with low-Mg\(^{2+}\) ACSF (3 of 10 slices; Fig. 7F). The synchronous or non-synchronous spontaneous [Ca\(^{2+}\)]\(_i\) oscillations and the increase in resting [Ca\(^{2+}\)]\(_i\) were reversibly blocked by 20 µM bicuculline (Fig. 7F). This suggests that they are mediated by endogenous GABA\(_A\)-receptor activation.

**FIG. 7 near here**
DISCUSSION

Depolarizing action of GABA following change in Cl⁻ homeostasis

Of [Cl⁻], regulators, KCC2, which normally carries Cl⁻ out of the cell along with K⁺, is largely responsible for keeping [Cl⁻], low in mature neurons (Jarolimek et al. 1999; Kakazu et al. 1999; Rivera et al. 1999), endowing them with hyperpolarizing responses to GABA (Ganguly et al. 2001; Lu et al. 1999; Rivera et al. 1999). NKCC1, which carries Cl⁻ into the cell using Na⁺-driving forces, helps to maintain a high [Cl⁻], in immature neurons, with the result that GABA acts in an excitatory manner (Kakazu et al. 1999; Plotkin et al. 1999; but see DeFazio et al. 2000). In our study, intact facial motoneurons displayed hyperpolarizing responses to GABA, however, after axotomy GABA caused depolarization (Fig. 3A), and the resting [Cl⁻] of axotomized neurons was significantly higher than that of intact neurons as demonstrated both by gramicidin-perforated patch-clamp recordings and by optical imaging using MEQ. The observed downregulation of KCC2 mRNA without changes in NKCC1 is consistent with an increase in [Cl⁻]. Thus, the depolarizing action of GABA in axotomized neurons may be caused by a positive shift in the equilibrium potential for Cl⁻ consequent upon the [Cl⁻] increase that follows KCC2 downregulation as reported previously (Nabekura et al. 2002).

Interaction of GABAergic excitation and Ca²⁺ signaling

In the present study, bicuculline blocked the Ca²⁺ increases evoked by GABA in axotomized neurons, suggesting that this response is mediated by the GABAₐ-receptor Cl⁻ channel. Nifedipine also blocked the [Ca²⁺], increases, indicating that the Ca²⁺ influx
occurred through L-type voltage-dependent Ca\textsuperscript{2+} channels (VDCa\textsuperscript{2+}s; Ganguly et al. 2001; Nabekura et al. 2002; Obrietan and van den Pol 1995; van den Pol et al. 1996; Yuste and Katz 1991). Since TTX also blocked this Ca\textsuperscript{2+} influx, our data suggest that the GABA-evoked [Ca\textsuperscript{2+}]\textsubscript{i} rise in axotomized neurons is primarily mediated by an initial depolarization due to a Cl\textsuperscript{-} efflux, with the ensuing action potential causing opening of VDCa\textsuperscript{2+}s. These results are comparable to previous reports (Nabekura et al. 2002; van den Pol et al. 1996).

We evaluated alterations in the local neural circuitry by studying changes in the [Ca\textsuperscript{2+}]\textsubscript{i} responses to glutamate. Both the amplitude and duration of glutamate-evoked [Ca\textsuperscript{2+}]\textsubscript{i} increases were greater in axotomized neurons than in intact neurons. Bicuculline decreased the glutamate response in axotomized neurons but enhanced it in intact neurons. In axotomized neurons, detectable [Ca\textsuperscript{2+}]\textsubscript{i} increases could be evoked by glutamate concentrations too low to induce a response in intact neurons. The addition of bicuculline abolished these responses. Since the addition of bicuculline had no further effects in the presence of TTX, the endogenous GABA\textsubscript{A} receptor-mediated actions induced by axotomy might be network-mediated. These results suggest that feed-forward GABAergic inhibition in intact neurons is changed to feed-forward GABAergic excitation in axotomized neurons, with the consequence that the threshold for glutamate-induced [Ca\textsuperscript{2+}]\textsubscript{i} increases was reduced (see Fig. 8).

In immature hippocampal neurons (Ben-Ari et al. 1988) and immature vagal motoneurons (Furukawa et al. 2000), the voltage-dependent Mg\textsuperscript{2+} block of NMDA channels are reduced. In addition to this, the depolarizing action of GABA, achieved via GABA\textsubscript{A}-receptors in immature neurons also tends to remove Mg\textsuperscript{2+} block (Ben-Ari et al. 1997; Ben-Ari 2002; Khazipov et al. 1997; Leinekugel et al. 1997). In adult vagal motoneurons,
axonal injury leads to the reaquisition of the immature characteristics of NMDA receptor (Furukawa et al. 2000) and GABAergic action (Nabekura et al. 2002). If this also occurred in the axotomized facial motoneurons, GABAergic depolarization might further reduce the voltage-dependent Mg\(^{2+}\)-block of NMDA channels, thus facilitating Ca\(^{2+}\) influx through them. This hypothesis is compatible with the present results that bicuculline and d-AP5 each but not CNQX blocked the [Ca\(^{2+}\)]\(_i\) increase induced by low-dose glutamate. This could provide for a synergy between GABA and glutamate, thus making GABA an excitatory transmitter as shown previously in immature hippocampal neurons (Ben-Ari et al. 1989, 1997; Ben-Ari 2002; Khazipov et al. 1997; Leinekugel et al. 1997). The implication is that neural injury may cause neurons to reacquire greater plasticity, with some immature characteristics. Indeed, a GABA-induced Ca\(^{2+}\) increase after an injury may allow the neuron to modulate gene expression (Bading et al. 1993; Berninger et al. 1995), influence growth-cone guidance (Obrietan and van den Pol 1996) and possibly reduce cell death resulting from the presence of a sub-optimal cytosolic Ca\(^{2+}\) (Franklin and Johnson 1992). Thus, a GABA-induced elevation in [Ca\(^{2+}\)]\(_i\) is likely to promote neuronal recovery.

**Mechanisms underlying GABA\(_A\)-receptor-mediated increase in resting [Ca\(^{2+}\)]\(_i\) and Ca\(^{2+}\) oscillations**

In axotomized facial motoneurons, in which the resting [Ca\(^{2+}\)]\(_i\) was significantly higher than in intact cells, bicuculline not only blocked the GABA-evoked [Ca\(^{2+}\)]\(_i\) increase but also decreased the baseline Ca\(^{2+}\) level (Fig. 4). In whole-cell patch-clamp recording, spontaneous postsynaptic currents (sPSCs) in axotomized neurons were blocked by bicuculline, whereas not in intact neurons (not shown). These results suggest that
endogenous GABA<sub>A</sub>-receptor activation (Flint et al. 1998; LoTurco et al. 1995; Owens et al. 1996) may help to raise basal Ca<sup>2+</sup> levels in such damaged neurons. Since d-AP5 had comparable effect with bicuculline on resting [Ca<sup>2+</sup>], activation of NMDA-receptor may also be involved in increases in resting [Ca<sup>2+</sup>]. Although effects of NMDA and GABA<sub>A</sub> receptors on resting [Ca<sup>2+</sup>], may imply depolarization of axotomized neurons by tonic activation of these receptors, resting membrane potential were comparable between intact and axotomized neurons in the present and in the previous (Nabekura et al. 2002) studies. A TTX-insensitive background activation of these receptors could increase resting [Ca<sup>2+</sup>], since [Ca<sup>2+</sup>], transients last longer than the accompanying membrane potential transients.

We demonstrated here that spontaneous [Ca<sup>2+</sup>], oscillations were present in axotomized neurons, a phenomenon reversibly blocked by bicuculline, suggesting GABA<sub>A</sub> receptor involvement, though a possibility of another mediator than GABA<sub>A</sub> to participate in the axotomy-induced [Ca<sup>2+</sup>], oscillations cannot be ruled out. In these neurons, the amplitude and frequency of the spontaneous [Ca<sup>2+</sup>], oscillations were increased as [Mg<sup>2+</sup>]<sub>o</sub> was lowered and were completely abolished by d-AP5 but not by CNQX. Thus, reduced Mg<sup>2+</sup>-dependent block of NMDA receptor-channels may be further facilitated by endogenous GABA<sub>A</sub>-receptor-mediated depolarization in axotomized neurons, so that this could induce spontaneous [Ca<sup>2+</sup>], oscillations by elevating [Ca<sup>2+</sup>], set-point via NMDA-receptor activation (Leinekugel et al. 1997). These phenomena might be network-driven, since [Ca<sup>2+</sup>], oscillation was TTX sensitive (see Fig. 8).

**Functional significance of the GABA<sub>A</sub>-mediated [Ca<sup>2+</sup>], oscillations**

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GABA$_A$-mediated spontaneous synaptic potentials can occur early in postnatal development, which can precede evoked GABAergic synaptic potentials in the neocortex (Luhmann and Prince 1991). The early maturation of GABA-release mechanisms and the early development of GABA$_A$-mediated spontaneous synaptic events suggest that GABA has trophic effects on developing neurons and a functional role in synaptogenesis (Barbin et al. 1993; Behar et al. 1996; Spoerri 1988). Since deafferentation, excepting GABAergic terminal (Vaughan 1994), occurs in the axotomized facial motor nucleus (Blinzinger and Kreutzberg 1968; Søreide 1981), GABA$_A$-receptor-mediated depolarization might be related to regeneration of these synapses. Previous morphological and physiological studies indicate the regeneration of neural circuitry in the facial nucleus with transient upregulation of GABAergic afferent to motoneurons (Vaughan 1994) and increases in neural excitability (Nishimura et al. 1992). Therefore, the presence of the TTX-sensitive Ca$^{2+}$ oscillations in axotomized neurons would be the result of an increase in the amount of activity within the facial nucleus caused by alterations in the intrinsic properties such as Cl$^-$ homeostasis.

In conclusion, Ca$^{2+}$ oscillation induced by the switch to GABAergic excitation that occurs in axotomized neurons, and which is induced by a change in the Cl$^-$ homeostasis following KCC2 downregulation, could play an important role in neural survival and regeneration in the facial nucleus. Although the present findings were obtained in young animals, based on the similarities in the KCC2 mRNA level and its reduction by axotomy, a potential for induction of a GABA-mediated excitatory events might be maintained in adulthood.
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FIGURE LEGENDS

Fig. 1. Facial-nerve transection downregulates KCC2 mRNA expression in the facial nucleus. (A) Dark-field photomicrographs showing rat facial nucleus expressing KCC2 mRNA hybridization signals 1 day, 3 days, 4 weeks, 8 weeks and 16 weeks after facial-nerve transection. Note marked reduction in KCC2 mRNA in the axotomized facial nucleus in the period from 3 days to 4 weeks, with recovery by 16 weeks. The areas surrounded by dashed lines indicate the regions of facial nuclei. Bar = 1 mm. (B) Bright-field photomicrographs of counter-stained section shows that KCC2 mRNA hybridization signals localized in facial motoneurons (M) were severely downregulated in axotomized neurons 3 days after transection. Note recovery of signals at 16 weeks after facial-nerve transection. Signal-positive cells were large in size and considered to be motoneurons (M), whereas small-sized presumed glial cells (G) were lacking in signals. Bar = 50 μm. (C) Time course of recovery in the percentage of facial motoneurons expressing KCC2 mRNA on the axotomized side. Error bars = S.D. (n = 5).

Fig. 2. Expressions of KCC2 mRNA and NKCC1 mRNA in 10-day-old rat facial nucleus after axotomy. (A) Expression of KCC2 mRNA in 10-day-old rat facial nucleus: on the intact side it was comparable to that of the adult, but it was clearly reduced on the axotomized side at 3 days after axotomy. Bright-field photomicrographs show that KCC2 mRNA hybridization signals in facial motoneurons (M) were clearly reduced on the axotomized side. Bars = 1 mm (dark-field photomicrograph) and 50 μm (bright-field photomicrograph). (B) Dark- and bright-field photomicrographs show lack of difference in expression of NKCC1 mRNA between intact and axotomized facial nucleus at 3 days after
axotomy. The areas surrounded by dashed lines indicate the regions of facial nuclei. Note that NKCC1 mRNA signals were detected both in motoneurons (M) and in presumed glial cells (G).

**Fig. 3. Increases in resting [Cl\(^-\)]\(_i\) after axotomy.** (A) By gramicidin-perforated patch-clamp recordings in the current-clamp mode, GABA (100 µM) hyperpolarized intact neurons whereas depolarized axotomized neurons. Reversal potential for GABA-evoked currents in voltaged-clamp mode shifted to the positive direction after axotomy. GABA was applied using pressure-pulses of 35-100 KPa for 10-50 msec. I-V curves for GABA-evoked currents in intact and axotomized neurons are shown at right. (B) Estimation of resting [Cl\(^-\)]\(_i\) by means of Cl\(^-\) imaging. Values for maximum and total quenchable MEQ fluorescence were obtained in 0 mM Cl\(^-\) and 150 mM SCN\(^-\), respectively. Resting [Cl\(^-\)]\(_i\) in individual neurons was estimated from the Stern-Volmer relationship. Resting [Cl\(^-\)]\(_i\) was significantly higher in axotomized neurons than in intact neurons (*graph at right*).

**Fig. 4. [Ca\(^{2+}\)]\(_i\) transients elicited by GABA application in facial motoneurons.** (A) Facial motoneurons loaded with fura-2 were clearly identified by fluorescence excited at 380 nm. Note that there are no apparent differences in size or shape between axotomized and intact facial motoneurons. Circles in the loaded neurons correspond the area of the ratio measurements. Bar = 50 µm. (B) Intact neurons responded neither to GABA (100 µM) nor to bicuculline (20 µM). (C) Axotomized facial motoneurons showed an [Ca\(^{2+}\)]\(_i\) rise in response to GABA (100 µM) that was completely blocked by bicuculline (20 µM). Note that bicuculline also decreased the baseline Ca\(^{2+}\) level, suggesting that endogenous GABA\(_A\)-
receptor activation might be partly responsible for the basal $[Ca^{2+}]_i$.  
(D) $[Ca^{2+}]_i$ increases evoked by GABA were also completely blocked by nifedipine (50 µM) but this agent did not alter the baseline $[Ca^{2+}]_i$ level.  (E) D-AP5 (50 µM) did not block GABA-evoked $[Ca^{2+}]_i$ transient but reduced the baseline $[Ca^{2+}]_i$ level.  Note that in axotomized neurons (C-E), basal $[Ca^{2+}]_i$ is higher than that in intact neurons (B).

**Fig. 5.** $[Ca^{2+}]_i$ changes in response to high-dose (100 µM) glutamate.  (A) In intact neurons, glutamate (100 µM, closed bar) evoked $[Ca^{2+}]_i$ increases that had returned to baseline 15 min after termination of glutamate application (left panel).  In the presence of bicuculline, glutamate-evoked $[Ca^{2+}]_i$ increases were larger and more prolonged, the level not returning to baseline until 30 min after glutamate application (right panel).  (B) In the presence of TTX, the glutamate-evoked $[Ca^{2+}]_i$ increases were enhanced in both duration and amplitude (left panel).  Addition of bicuculline did not change the responses apparently (right panel).  (C) In axotomized facial motoneurons, glutamate evoked increases in $[Ca^{2+}]_i$ that were larger and more prolonged than those seen in intact neurons.  Note that $[Ca^{2+}]_i$ did not return to baseline level within the time-frame of the experiment, and that the baseline level was higher than in intact neurons (left panel).  Addition of bicuculline to axotomized facial motoneurons reduced baseline $[Ca^{2+}]_i$ and shortened the duration of the glutamate-evoked $[Ca^{2+}]_i$ increases.  Note that in the presence of bicuculline, both the size and duration of glutamate-induced $[Ca^{2+}]_i$ increases, as well as the baseline $[Ca^{2+}]_i$ level, were comparable between intact and axotomized neurons (right panel).  (D) The glutamate-evoked $[Ca^{2+}]_i$ increases were reduced by TTX in duration and amplitude in axotomized neurons (left panel).  Further addition of bicuculline did not affect the responses
significantly (*right panel*). Bicuculline and TTX were continuously perfused before, during, and after bath application of glutamate.

**Fig. 6.** **[Ca^{2+}]_i** changes elicited by low-dose (5 and 10 µM) glutamate. Intact neurons showed no response to low-dose glutamate (5 µM, closed bar; 10 µM, opened bar) in the absence (*A*) or presence (*B*) of bicuculline. In axotomized neurons, application of low-dose glutamate evoked transient [Ca^{2+}]_i increases (*C*). CNQX did not (*D*) but D-AP5 (*E*), TTX (*F*), and bicuculline (*G*) abolished these responses. CNQX, D-AP5, TTX, and bicuculline were continuously perfused before, during, and after bath application of glutamate.

**Fig. 7.** Spontaneous [Ca^{2+}]_i oscillations occurred in axotomized facial motoneurons. (*A*) Spontaneous [Ca^{2+}]_i oscillations hardly occur at all in intact neurons even in low (1 or 0.5 mM) [Mg^{2+}]_o. (*B*) In axotomized neurons, spontaneous [Ca^{2+}]_i oscillations occur, their amplitude and frequency increasing as [Mg^{2+}]_o is progressively reduced. (*C*) These effects of low-Mg^{2+} ACSF, on resting [Ca^{2+}]_i, and [Ca^{2+}]_i oscillations, were blocked by the addition of D-AP5. (*D*) Application of CNQX did not block these [Ca^{2+}]_i oscillations. (*E*) Addition of TTX blocked these [Ca^{2+}]_i oscillations reversibly. (*F*) Among axotomized neurons, synchronous spontaneous [Ca^{2+}]_i oscillations were occasionally seen during superfusion with medium containing 0.5 mM [Mg^{2+}]_o. These [Ca^{2+}]_i oscillations were reversibly abolished by bicuculline. Results illustrated in (*A*)-(*F*) were from six different slices. Note that baseline [Ca^{2+}]_i rose progressively as [Mg^{2+}]_o was lowered in axotomized neurons but not in intact neurons.
Fig. 8. Schematic illustration of possible mechanisms underlying the elevation in resting $[\text{Ca}^{2+}]_i$ and generation of $[\text{Ca}^{2+}]_i$ oscillations in axotomized facial motoneurons.

Left: under normal conditions, GABA hyperpolarizes facial motoneurons by causing an influx of Cl$^-$ through GABA$\text{A}$ receptor-channels. Such GABAergic hyperpolarization may inhibit opening of voltage-dependent Na$^+$ channels (VDNa$^+$s), voltage-dependent Ca$^{2+}$ channels (VDCa$^{2+}$s) and NMDA receptor-channels; hence, high-dose glutamate-induced increases in $[\text{Ca}^{2+}]_i$ are attenuated by a presumed feed-forward GABAergic inhibition.

Right: in axotomized neurons, in which Cl$^-$ extrusion is inhibited by KCC2 downregulation, an accumulation of Cl$^-$ occurs via unaltered NKCC1. GABA thus depolarizes axotomized facial motoneurons by causing an efflux of Cl$^-$ through GABA$\text{A}$ receptor-channels, and hence opening the VDNa$^+$, with the ensuring action potential causing opening of VDCa$^{2+}$ and inducing a transient Ca$^{2+}$ influx. The endogenous GABA$\text{A}$-receptor-mediated depolarization may contribute to removal of the Mg$^{2+}$-block, decreasing threshold for glutamate (NMDA)-induced $[\text{Ca}^{2+}]_i$ increases and enhancing even a weak effect of glutamate. When glutamate is released from presynaptic neurons within the axotomized facial nucleus, the existing collapse of the Cl$^-$ gradient could thus convert feed-forward GABAergic inhibition to excitation, provoking GABA$\text{A}$ receptor- and NMDA receptor-mediated spontaneous $[\text{Ca}^{2+}]_i$ oscillations.
Fig. 1

A. Axotomy and Intact tissue over time:
- 1d
- 3d
- 4w
- 8w
- 16w

B. Comparison of Axotomy and Intact tissue:
- 3d
- 16w

C. Graph showing KCC2-positive neurons (%) over days after operation.
Fig. 2

A  P10 KCC2
Axotomy 3d  Intact

Axotomy 3d  Intact

B  P10 NKCC1
Axotomy 3d  Intact

Axotomy 3d  Intact
Fig. 3

A  
Intact  
Axotomy 3d  

GABA 100 µM  
-65 mV  
-61 mV  
GABA 10 µM  
-50  
-70  
-90  
100 pA  
200 ms  

GABA 100 µM  
-80  
-50  
-20  
-100  
-150  
150  

B  
Intact  
Axotomy 3d  

Fluorescence (Arbitrary unit)  
0  
600  
800  
200  
400  
600  
800  
0 10 20 30 40  
Time (min)  

Tributyltin (20 µM) + Nigericin (14 µM)  

Resting [Cl] (mM)  
0  
5  
10  
15  
20  
25  
30  
35  
P < 0.05  
Intact  
Axotomy 3d  

- mM

- 5

- 10

- 15

- 20

- 25

- 30

- 35

- P < 0.05

- Intact

- Axotomy 3d

- 150 mM KSCN

- 0 mM Cl

- 150 mM KSCN

- 0 mM Cl
Fig. 4

A  Fura-2 Fluorescence
    Axotomy 3d     Intact

B  Intact
    GABA      Bicuculline
    [Ca^{2+}] (nM)  
    100        50         0
    5 min

C  Axotomy 3d
    GABA      Bicuculline
    [Ca^{2+}] (nM)  
    150        100       50
    5 min

D  GABA      Nifedipine
    [Ca^{2+}] (nM)  
    150        100       50
    5 min

E  GABA      D-AP5
    [Ca^{2+}] (nM)  
    150        100       50
    5 min
Fig. 5

A  Intact

B  Intact/ +TTX (1 μM)

C  Axotomy 3d

D  Axotomy 3d/ +TTX (1 μM)

[Graphs showing changes in [Ca\(^{2+}\)] over time with and without Bicuculline and 100 μM glutamate]
Fig. 6

A. Intact
B. Intact + Bicuculline (20 μM)
C. Axotomy 3d
D. Axotomy 3d + CNQX (10 μM)
E. Axotomy 3d + D-AP5 (50 μM)
F. Axotomy 3d + TTX (1 μM)
G. Axotomy 3d + Bicuculline (20 μM)

5 μM glutamate
10 μM glutamate
Fig. 7

A  Intact

B  Axotomy 3d

C  Axotomy 3d (0.5 mM Mg\(^{2+}\))

D  Axotomy 3d (0.5 mM Mg\(^{2+}\))

E  Axotomy 3d (0.5 mM Mg\(^{2+}\))

F  Axotomy 3d (0.5 mM Mg\(^{2+}\))
Fig. 8

Intact

Axotomized

Hyperpolarization by GABA

Facial muscles

Depolarization by GABA

Facial muscles

GABAergic hyperpolarization

$[Cl^-]_i = 9 - 11$ mM

GABAergic depolarization

$[Cl^-]_i = 18 - 24$ mM

Endogenous GABA receptor activation