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GABA Itself Promotes the Developmental Switch of Neuronal GABAergic Responses from Excitation to Inhibition

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Summary

GABA is the main inhibitory neurotransmitter in the adult brain. Early in development, however, GABAergic synaptic transmission is excitatory and can exert widespread trophic effects. During the postnatal period, GABAergic responses undergo a switch from being excitatory to inhibitory. Here, we show that the switch is delayed by chronic blockade of $GABA_A$ receptors, and accelerated by increased $GABA_A$ receptor activation. In contrast, blockade of glutamatergic transmission or action potentials has no effect. Furthermore, GABAergic activity modulated the mRNA levels of KCC2, a K^+-Cl^- cotransporter whose expression correlates with the switch. Finally, we report that GABA can alter the properties of depolarization-induced Ca^{2+} influx. Thus, GABA acts as a self-limiting trophic factor during neural development.

In the adult central nervous system, γ-amino-butyric acid (GABA) is the primary inhibitory neurotransmitter. It regulates a neuron's ability to fire action potentials either through hyperpolarization of the membrane potential or through shunting of excitatory inputs. During early postnatal development, however, GABAergic synaptic transmission is excitatory, able to elevate the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and even capable of triggering action potentials (Mueller et al., 1984; Luhmann and Prince, 1991; Yuste and Katz, 1991; Reichling et al., 1994; Wang et al., 1994; Leinekugel et al., 1995; Obrietan and van den Pol, 1995; Chen et al., 1996; Owens et al., 1996; Khazipov et al., 1997). Over a limited postnatal period, in the hippocampus, neocortex, and hypothalamus, as well as other regions of the brain, there is a switch of the electrophysiological (depolarization to hyperpolarization) and biochemical (Ca^{2+}-mediated signaling) properties of GABAergic transmission (Mueller et al., 1984; Ben-Ari et al., 1989; Cherubini et al., 1991; Luhmann and Prince, 1991; Owens et al., 1996).

The $GABA_A$ receptor channel predominantly conducts Cl^- ions. Consequently, the nature of GABAergic transmission, excitatory versus inhibitory, is determined primarily by the electrochemical gradient for Cl^-, which depends on the intra- and extracellular concentrations of Cl^-. This electrochemical gradient sets the reversal potential for GABAergic currents (E_{GABA}; the membrane voltage at which GABAergic currents change their direction). Recent work has shown that the developmental transformation of GABAergic synaptic transmission from depolarizing to hyperpolarizing is due to a shift in E_{GABA} toward a more hyperpolarized potential, which is likely the result of an ontogenetic decrease in the intracellular Cl^- concentration ($[Cl^-]_i$; Cherubini et al., 1990; Luhmann and Prince, 1991; Chen et al., 1996; Owens et al., 1996). Indeed, changes in the mRNA level for the K^+-coupled Cl^- transporter KCC2 have been shown to correlate with the modification of GABAergic transmission (Lu et al., 1999; Rivera et al., 1999; Vu et al., 2000). KCC2 increases the rate of Cl^- extrusion, thus leading to a reduction in $[Cl^-]_i$ and a consequent shift in E_{GABA} toward more hyperpolarized potentials (Jarolimek et al., 1999; Kakazu et al., 1999; Rivera et al., 1999).

This conversion of GABAergic transmission from depolarizing to hyperpolarizing is also accompanied by a change in GABA-mediated biochemical signaling. Only during this early developmental period, depolarizing GABAergic potentials activate voltage-dependent Ca^{2+} channels (VDCCs) and elevate $[Ca^{2+}]_i$ (Connor et al., 1987; Yuste and Katz, 1991; Wang et al., 1994). Such GABA-induced elevation of $[Ca^{2+}]_i$ is likely to play a critical role in the maturation of the nervous system. For instance, GABA-mediated increases in $[Ca^{2+}]_i$ can induce BDNF expression (Berninger et al., 1995) and promote neuronal survival and differentiation (LoTurco et al., 1995; Marty et al., 1996; Ikeda et al., 1997). GABA-induced elevation of $[Ca^{2+}]_i$ may also be required to form, stabilize, and strengthen synaptic connections (Kirsch and Betz, 1998; Caillard et al., 1999; Kneussel and Betz, 2000).

While the developmental transformation of GABAergic transmission is well documented, little is known about signals that induce this transformation. Since neuronal activity is known to increase during development, we examined in the present study whether synaptic activity can regulate the switch of GABAergic transmission. We found that the change in GABA signaling was largely prevented by chronic blockade of $GABA_A$ receptors, and was accelerated by increased GABA receptor activation. Changes in the level of KCC2 mRNA tightly correlated with the observed changes in GABA signaling. In addition, we found that spontaneous GABAergic activity regulated the activation of voltage-dependent Ca^{2+} currents. These findings point to GABA as a critical maturation factor for the switch of the physiological and biochemical properties of GABA signaling.

Results

Switch of GABAergic Transmission from Depolarizing to Hyperpolarizing

To study the change in GABA signaling, we first monitored GABA-induced elevations of $[Ca^{2+}]_i$ over development. GABA-mediated depolarization was reflected by an increase in $[Ca^{2+}]_i$. Cultures of hippocampal neurons were loaded with the Ca^{2+}-sensitive dye Fluo-4 AM and changes in fluorescence were measured using confocal

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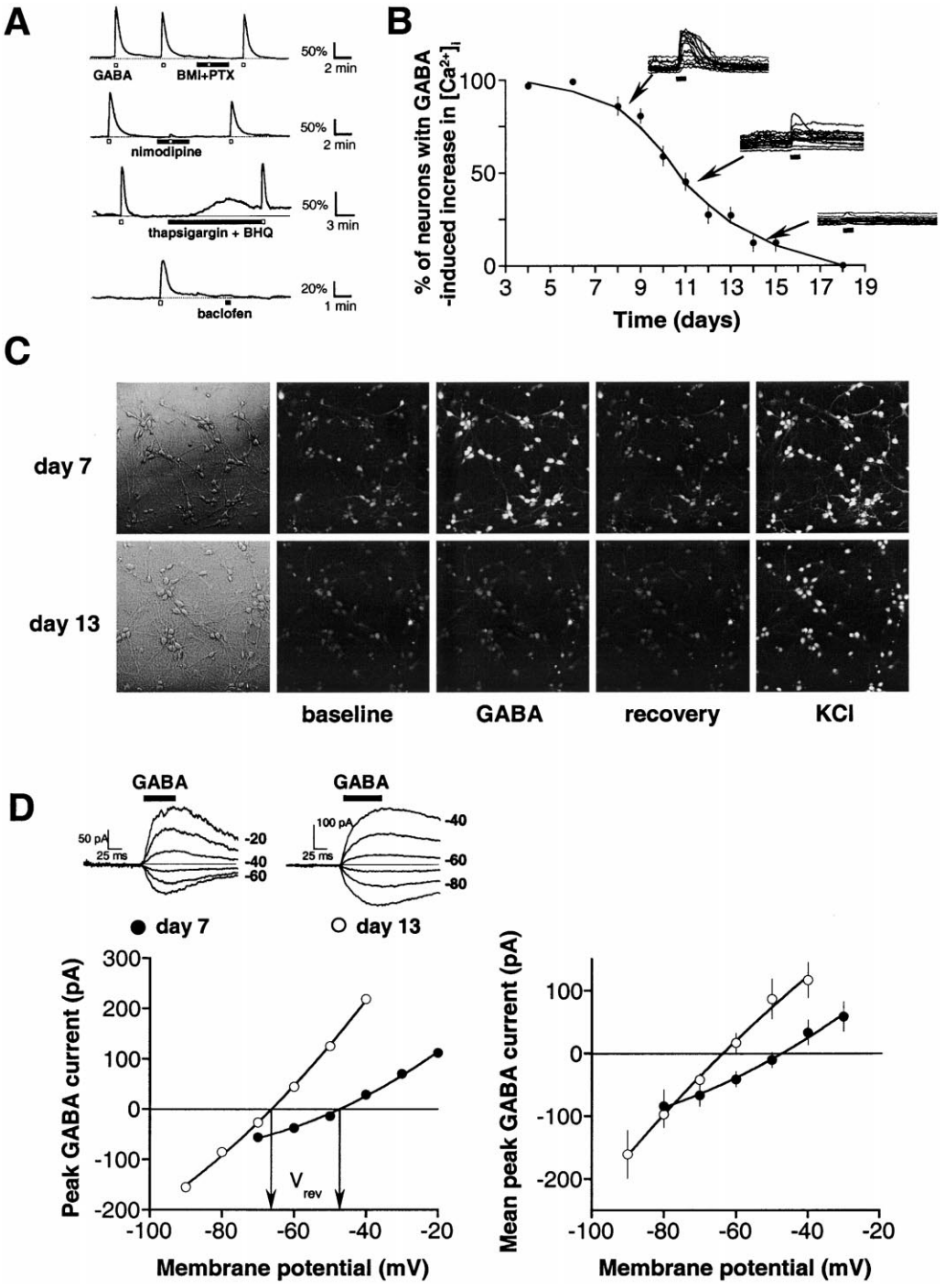

Figure 1. Developmental Changes in GABA-Induced Responses

(A) Pharmacological profile of GABA-induced elevations of $[Ca^{2+}]_i$ (BMI + PTX, 10 and 50 μM; nimodipine, 10 μM; thapsigargin + BHQ, 2 and 10 μM; and baclofen, 10 μM). Time course of changes in $[Ca^{2+}]_i$, assessed by Fluo-4 fluorescence intensity. Traces are average fluorescence intensity (% change shown by the scale) recorded from 30–40 randomly sampled neurons in response to pulses of GABA (open bar, 10 μM, 15 s). The elevation of $[Ca^{2+}]_i$ during application of thapsigargin + BHQ was attributed to emptying of intracellular Ca^{2+} stores.

(B) Time course of the GABA switch. The percentage of neurons exhibiting Ca^{2+} elevation in response to GABA is shown for different days after cell plating. Each point represents mean ± sem (n = 3 to 21 experiments, 121 in total). Each experiment involved recording from 30–40 neurons. Insets: Sample recordings from 20 randomly selected neurons at the time points indicated by the arrows.

(C) Representative recordings of Ca^{2+} imaging of young (7 day) and old (13 day) neurons. First panels show bright-field images. The fluorescence images on the right represent typical $[Ca^{2+}]_i$ in neurons before ("baseline"), during ("GABA"), and after ("recovery") application of a pulse of GABA (higher intensity represents higher $[Ca^{2+}]_i$). Image field = 604 × 604 μm.

(D) Left: Representative examples of peak GABA-induced currents versus membrane voltages (I-V relationship). The cells were voltage clamped at −70 mV and stepped to different potentials (−90 to +40 mV, 10 mV steps). E_{GABA} was calculated by fitting the I-V curve to a second-order polynomial function. Right: Averaged I-V curves (n = 11 in both cases).

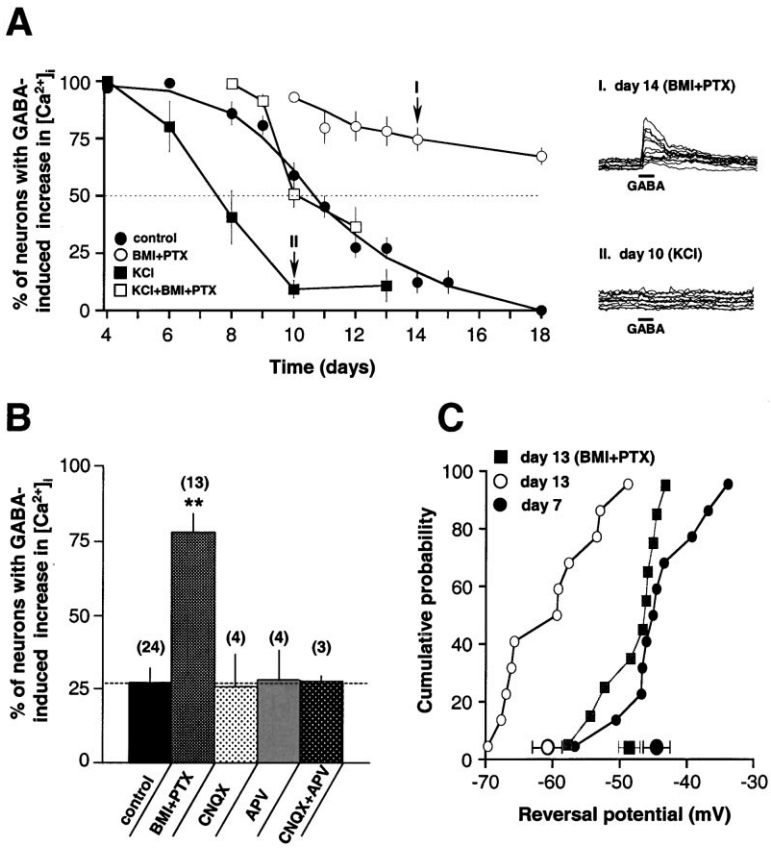

Figure 2. Role of Endogenous GABAergic Transmission in the Developmental Switch

(A) Time course of the switch in GABA signaling for neurons treated chronically with BMI + PTX (10 and 50 μM; n = 31, 1085 neurons), KCl (10 mM; n = 26, 910 neurons), and KCl + BMI + PTX (n = 14, 490 neurons), compared to control neurons (n = 121, 4235 neurons). In all experiments involving KCl, TTX and D-APV were included in the medium. Statistical comparisons were carried out for data collected for day 8, 10, and 12 neurons. BMI + PTX and KCl were significantly different from the untreated control and KCl + BMI + PTX ($p < 0.005$, single-factor ANOVA followed by a Bonferroni t test corrected for multiple comparisons). Insets: I, Representative recordings of day 14 neurons grown in the presence of BMI + PTX. II, Representative results on day 10 neurons grown in the presence of KCl (10 mM).

(B) Comparison of the effects of chronic blockade of $GABA_A$ and glutamate receptors at day 13 neurons. Numbers of experiments are indicated in parentheses. "**" denotes $p < 0.001$ (ANOVA).

(C) Cumulative probability histograms of E_{GABA} for day 7 and day 13 neurons (with or without chronic treatment with BMI + PTX). Each point represents E_{GABA} measurement from one cell. Data points with error bars below denote mean ± sem (n = 10 to 11). Data from both control day 7 and BMI + PTX day 13 were significantly different from the control day 13 ($p < 0.001$, Kolmogorov-Smirnov test).

microscopy. Neurons cultured for 4 to 9 days responded to exogenous applications of GABA (100 μM, 15 s pulse) with a rapid and reversible increase in $[Ca^{2+}]_i$. As shown in Figure 1A, these responses were largely blocked by a combination of $GABA_A$ receptor antagonists (bicuculline methoiodide, "BMI", 10 μM; picrotoxin, "PTX", 50 μM) or by the L-type Ca^{2+} channel antagonist nimodipine (10 μM), indicating that elevation of $[Ca^{2+}]_i$ is due to Ca^{2+} influx through L-type Ca^{2+} channels activated by $GABA_A$-receptor-mediated depolarization. In addition, application of baclofen, a $GABA_B$ agonist, had no effect on $[Ca^{2+}]_i$. Furthermore, depleting intracellular Ca^{2+} stores with thapsigargin (2 μM) and 2,5-di-t-butyl-1,4-benzohydroquinone (BHQ, 10 μM) did not alter the GABA-mediated Ca^{2+} transients, indicating that the elevation of $[Ca^{2+}]_i$ is a result of Ca^{2+} influx through VDCCs.

The percentage of neurons that responded to GABA with an elevation of $[Ca^{2+}]_i$ decreased with the age of the culture (Figures 1B and 1C). All neurons displayed GABA-mediated depolarization at day 4 to 6, while most neurons exhibited no detectable response by day 13. Regardless of whether a neuron responded to GABA, depolarization with a high concentration of extracellular KCl (100 mM) always induced robust elevations of $[Ca^{2+}]_i$ (Figure 1C), suggesting that the lack of responsiveness to GABA was due to a decrease in the extent of depolarization. These observations are consistent with previous findings from brain slices and cultured neurons (Luhmann and Prince, 1991; Chen et al., 1996; Owens et al., 1996; Wang et al., 1994), that GABA-induced elevations of $[Ca^{2+}]_i$ are developmentally regulated and that cultures of hippocampal neurons provide a useful model for studying the mechanism of the developmental switch in GABA signaling.

To directly assess the developmental shift in E_{GABA}, whole-cell perforated patch recording was used to measure the reversal potential of currents elicited by local application of GABA (10 μM, 50 ms pulse) in young (day 6–7) and mature (day 13–14) neurons. As shown in Figure 1D, E_{GABA} was significantly more hyperpolarized in mature neurons (−60.7 ± 2.2 mV; mean ± sem, n = 11) than in young neurons (−44.5 ± 2.0 mV; n = 11, $p < 0.001$), indicating that the developmental decrease of E_{GABA} contributes to the reduction of GABA-induced elevations of $[Ca^{2+}]_i$.

GABA Itself Promotes the Developmental Switch

Based on the hypothesis that the transformation of GABA signaling depends on neuronal activity, we examined the effects of chronic blockade of ionotropic transmitter receptors on the GABA-induced Ca^{2+} transients. Interestingly, GABAergic, but not glutamatergic activity was essential for the developmental change (Figures 2A–2C). Chronic blockade of $GABA_A$ receptors with BMI (10 μM) and PTX (50 μM) prevented the transformation in most neurons (note that similar effects were found in the presence of BMI alone; Figure 6E). In contrast, blockade of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors with D-APV (25 μM), the non-NMDA receptors with CNQX (15 μM), or both (CNQX+APV) did not affect the developmental time course (Figure 2B). In addition, chronic activation of the

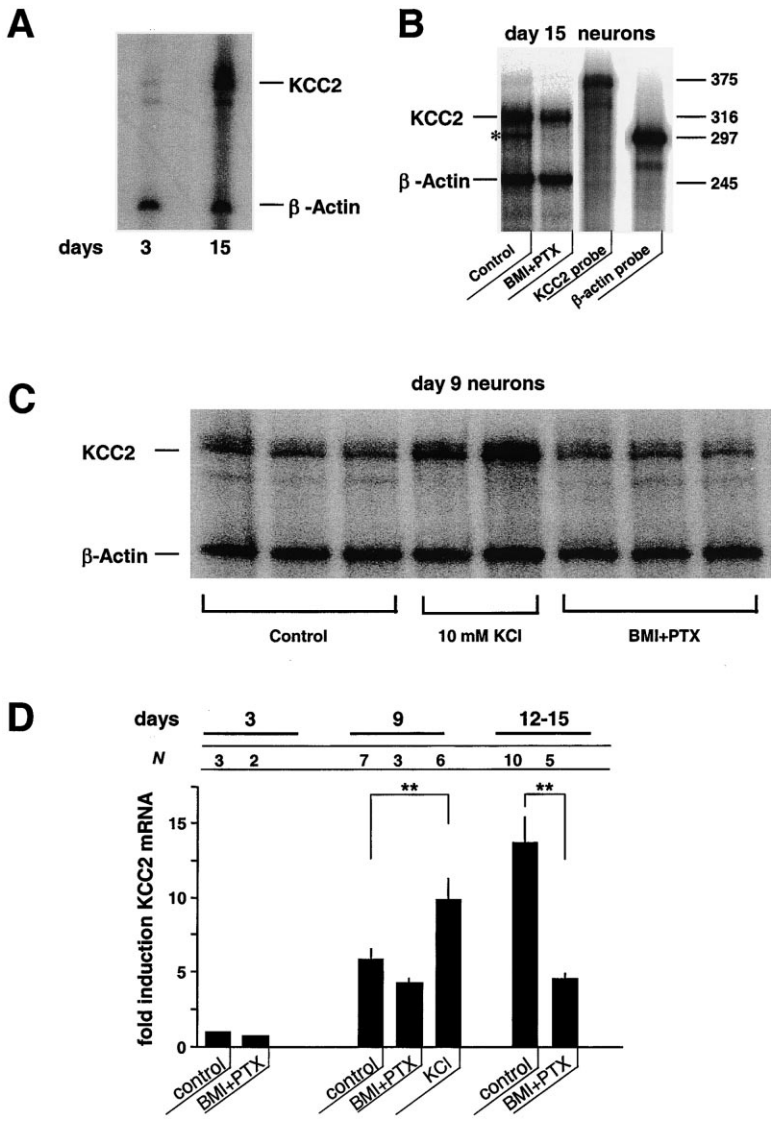

Figure 3. Modulation of KCC2 mRNA Expression by GABAergic Activity

Levels of KCC2 mRNA assessed by the RNase protection assay.

(A) Representative experiment displaying a higher level of KCC2 mRNA in day 15 neurons as compared to day 3 neurons.

(B) Representative experiment showing a decrease in KCC2 mRNA expression in day 15 neurons chronically treated with BMI + PTX, as compared to untreated controls. The thin band marked with "*" was due to residual undigested β-actin probe, as shown by the β-actin probe lane alone.

(C) The expression of KCC2 mRNA was increased by chronic depolarization with KCl (10 mM), and decreased in day 9 neurons following the BMI + PTX treatment. Replicate samples of results are shown.

(D) Summary of all results on KCC2 mRNA expression, assayed at day 3, 9, and 12–15. For each condition, KCC2 mRNA levels were normalized to that of β-actin and expressed as fold induction over the band intensity observed at day 3 in control neurons. "N" = number of experiments. Error bars = sem and "**" denotes $p < 0.005$ (t test).

metabotropic glutamate receptor mGluR3 with L-AP4 did not modify the switch (d11; 0.49 ± 0.07, n = 5, p = 0.57, t test). Thus, the developmental switch of GABA-mediated Ca^{2+} signaling is triggered by endogenous GABAergic transmission.

The maturational change in GABA-mediated Ca^{2+} signaling may result directly from a shift in the reversal potential of GABAergic currents. We therefore examined whether the ontogenetic modification of E_{GABA} was also regulated by GABAergic transmission. As shown in Figure 2C, chronic blockade of $GABA_A$ receptors prevented the shift of E_{GABA} toward hyperpolarized potentials in day 13 neurons. E_{GABA} remained at −48.4 ± 1.6 mV (n = 10), a value that was not significantly different from that of young (day 7) neurons under control conditions (p = 0.13, t test). These observations indicate that the developmental increase in GABAergic activity promotes the transformation of GABA signaling.

GABA Activates the Expression of the Cl^- Transporter KCC2

The hyperpolarizing shift in E_{GABA} has been correlated with an increase in KCC2 expression in hippocampal slices (Rivera et al., 1999). Since GABAergic activity drives this shift in E_{GABA}, we examined whether the ontogenetic increase in the levels of KCC2 mRNA is similarly modulated. KCC2 mRNA levels were quantified in day 3 and day 12–15 neurons using an RNase protection assay and normalized to the levels of β-actin (Lee and Costlow, 1987). Consistent with previous findings (Rivera et al., 1999), we observed an ~14-fold increase in the level of KCC2 mRNA in mature neurons (Figures 3A and 3D). At day 9 (during the onset of the switch), an ~6 fold increase in KCC2 expression was observed, although only ~20% of neurons failed to respond to GABA at this time (Figure 1B). This suggests a nonlinear relationship between the mRNA level of KCC2 and its functional effects (see Discussion). Furthermore, we explored whether chronic blockade of GABAergic activity with BMI + PTX altered the level of KCC2 mRNA. As shown in Figures 3B and 3D, KCC2 expression was decreased by 68 ± 4% (n = 5, $p < 0.001$) in comparison to age-matched control cultures (n = 10). To elevate GABAergic activity, we chronically depolarized the cultures with 10 mM KCl (see Figures 6C and 6D). We found that KCC2 message levels in day 9 neurons were

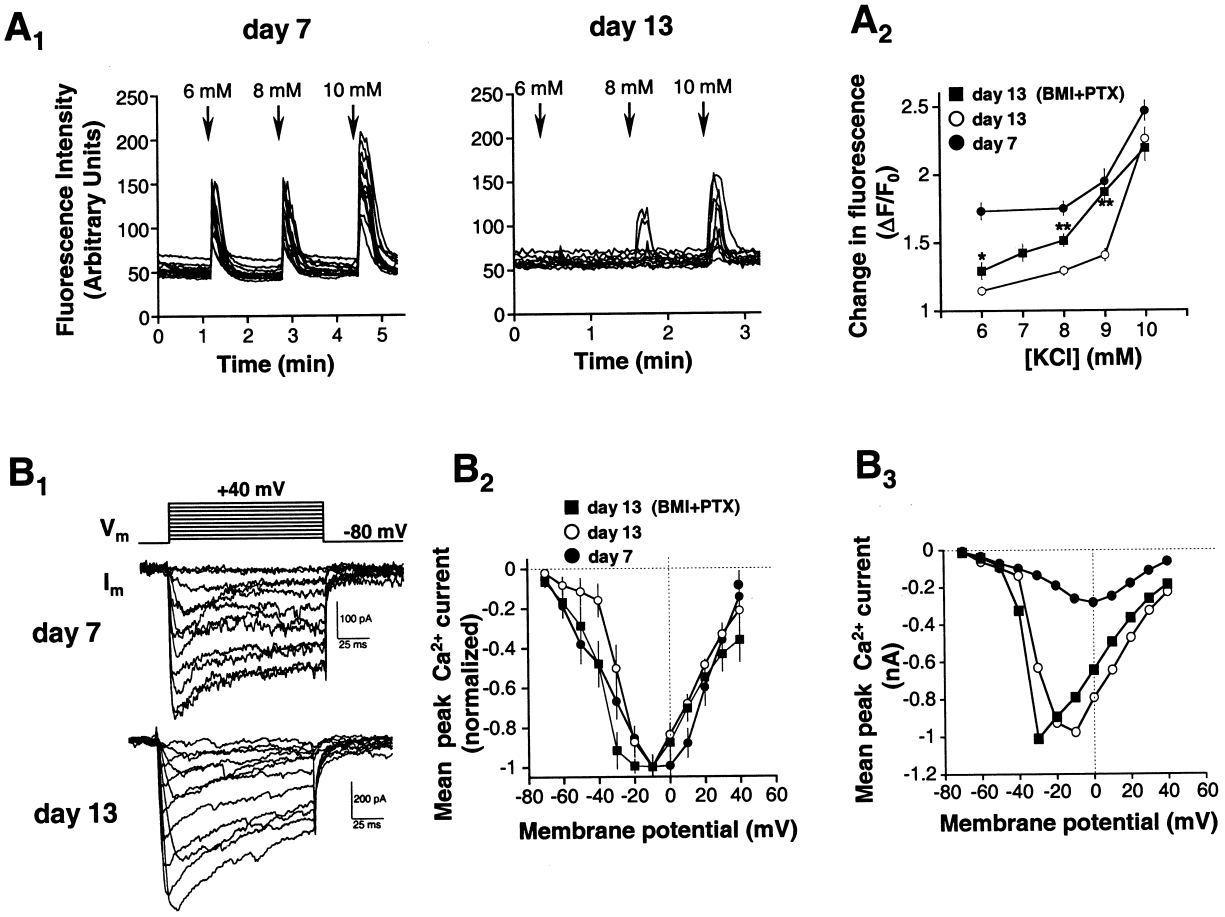

Figure 4. Regulation of Ca^{2+} Channel Properties during Development

(A_1) Representative recordings of changes in $[Ca^{2+}]_i$ induced by depolarization with 6, 8, or 10 mM KCl in day 7 and day 13 neurons. (A_2) Summary of normalized changes in Ca^{2+} fluorescence induced by KCl depolarization in young (day 7; n = 240 neurons), old (day 13; n = 260) untreated neurons, and old neurons chronically treated with BMI + PTX (n = 111). ("*" and "**" denote $p < 0.05$ and $p < 0.001$, respectively, when compared to the control day 13 treatment, single-factor ANOVA followed by a t test).

(B) GABA receptor blockade prevents the developmental shift in the activation of VDCCs. (B_1) Representative voltage-clamp recordings of Ca^{2+} currents. Inward Ca^{2+} currents (downward deflections) were elicited by step depolarizations (holding potential = −80 mV, step = 10 mV). (B_2) Normalized peak current versus voltage for day 7 (n = 12), day 13 (n = 9), and day 13 BMI + PTX (n = 11) neurons. On average, Ca^{2+} currents measured between −60 and −40 mV are 1- to 2-fold larger in younger neurons than in mature neurons (with $p < 0.05$ for −50 and −40 mV; n = 9 for 7 day, and n = 12 for 13 day). (B_3) Mean I-V curves for absolute peak current values (same data as in B_2). As expected, error bars were larger in these nonnormalized curves and were omitted for clarity.

enhanced by 69 ± 6% in comparison to control conditions (Figures 3C and 3D), and most neurons in the KCl-treated day 9 cultures had lost their responsiveness to GABA (Figure 2A). Taken together, these results support the notion that the expression of KCC2 can be regulated by GABA-mediated depolarization.

Developmental Regulation of Depolarization-Induced Elevations of $[Ca^{2+}]_i$

GABA-induced Ca^{2+} transients depend both on the extent of membrane depolarization as well as the properties of voltage-dependent Ca^{2+} influx. We thus examined whether depolarization-induced Ca^{2+} transients are modified during neuronal development. We measured changes in fluorescence intensity in response to increasing depolarization (induced by KCl) in young (day 7) and mature (day 13) neurons. While young cells displayed a robust increase in $[Ca^{2+}]_i$ at all levels of depolarization, mature neurons responded only to strong depolarization (Figure 4A). These measurements of KCl-induced changes in fluorescence intensity most accurately reflect depolarization-induced changes in "free" $[Ca^{2+}]_i$. Therefore, these results indicate that while a mild depolarization can robustly increase the free $[Ca^{2+}]_i$ in young neurons, moderate depolarization is required to appreciably increase the levels of $[Ca^{2+}]_i$ in mature neurons. We thus conclude that the switch in GABA-induced Ca^{2+} signaling involves two independent ontogenetic modifications: a hyperpolarizing shift in E_{GABA} and a reduction in the depolarization-induced elevation of free $[Ca^{2+}]_i$.

Changes in free $[Ca^{2+}]_i$ are likely to depend on two factors: (1) the activation profile of voltage-dependent Ca^{2+} currents (I_{Ca}), and (2) the Ca^{2+} buffering properties of a neuron. We conducted voltage-clamp recordings of I_{Ca} from young and mature neurons. Step depolarization evoked fast inward currents that displayed slow and partial inactivation (Figure $4B_1$). For young neurons, inward currents activated typically between −60 and −50

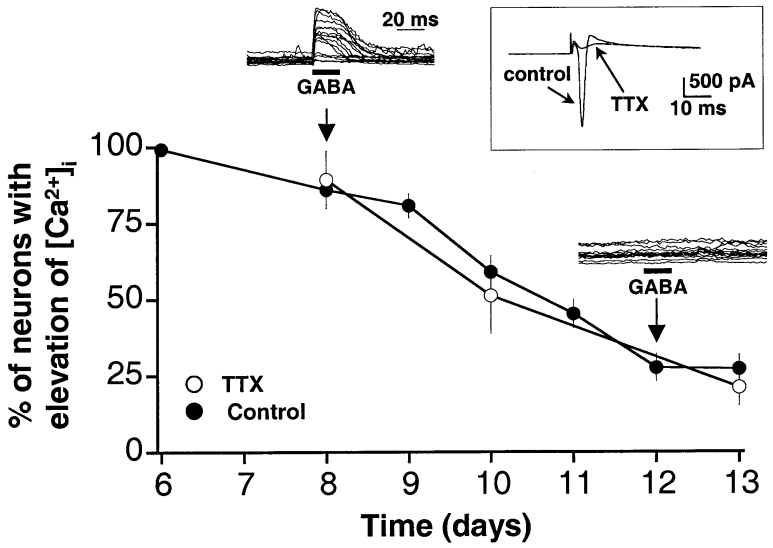

Figure 5. Spiking Is Not Required for the Developmental Transformation

Chronic blockade of action potentials with TTX (2 μM) did not affect the time course of the GABA signaling switch. I and II: Typical recordings of Ca^{2+} levels in neurons chronically treated with TTX, at the indicated time points. Inset: Membrane currents recorded from a day 6 neuron before and after application of 2 μM TTX.

mV, reaching a peak at about −10 mV. In contrast, mature neurons displayed a shift in the activation profile toward more hyperpolarized potentials, suggesting that the kinetics of I_{Ca} are developmentally regulated (Figure $4B_2$). However, the absolute amplitudes of the whole-cell currents for young and mature neurons at the hyperpolarized potentials (−70 to −50 mV) were similar (Figure $4B_3$). These results suggest that additional factors underlie the observation that mild depolarization induces large increases in free $[Ca^{2+}]_i$ only in young neurons (Figure 4A). One possible factor is that the cell surface of mature neurons is typically ~2-fold larger than that of young neurons. Thus, the current density at hyperpolarized potentials is likely to be higher in young cells. Developmental changes in the Ca^{2+}-buffering properties of neurons are also likely to contribute (Schierle et al., 1997; Rosenstein et al., 1998; Boukhaddaoui et al., 2000).

GABA Can Promote Developmental Changes in Depolarization-Induced Ca^{2+} Transients

Since GABA itself promoted the shift in E_{GABA}, we examined whether the properties of depolarization-induced Ca^{2+} transients are similarly regulated. As shown in Figure $4A_2$, chronic blockade of GABAergic transmission markedly reduced this developmental modification. In mature neurons chronically treated with BMI + PTX (day 13–14), KCl triggered a robust elevation in the free $[Ca^{2+}]_i$ that was significantly larger than those in the untreated parallel cultures. Although the ontogenetic change in the activation profile for I_{Ca} was reduced, the absolute levels of calcium influx were similar to that seen under control conditions (Figures $4B_{2-3}$). Taken together, these findings suggest that GABA promotes the developmental decrease in depolarization-induced Ca^{2+} transients, but this decrease is not fully accounted for by changes in voltage-dependent Ca^{2+} influx.

The Developmental Switch Does Not Require Neuronal Spiking

To test whether spiking is required for the switch of GABAergic signaling, neurons were cultured in the presence of tetrodotoxin (TTX, 2 μM), which blocks Na^+-dependent action potentials in these neurons (Figure 5). Interestingly, blocking Na^+ spikes did not affect the time course of the transformation, suggesting that spontaneous miniature depolarizing GABAergic synaptic currents (mGSCs) are sufficient to drive the developmental switch (see Figure 6B). Spontaneous mGSCs were first detected (at a low frequency) at day 7 (Figure 6A), and the frequency increased steadily to reach a plateau of about 0.5 Hz between days 11 and 12. Remarkably, the time course in which spontaneous GABAergic activity arises closely parallels that of the switch in GABA signaling (compare Figures 5 and 6A). It is noteworthy that although substantial glutamatergic activity was also present during this period (Figures 6A and 6B*b*), this excitatory activity did not contribute to the developmental switch in GABA signaling (Figure 2B).

It has been reported that chronic blockade of $GABA_A$ receptors may lead to increased activity even when GABA is depolarizing (Lamsa et al., 2000). Thus, it is possible that the effects of chronic blockade of $GABA_A$ receptors on the switch are the result of an increase in overall neuronal activity. To test this possibility, we compared the effects of chronic blockade of $GABA_A$ receptors alone (BMI) with those observed in the presence of BMI + TTX or BMI + CNQX in day 13 neurons (Figure 6E). As no significant differences were found under these conditions ($p > 0.2$, ANOVA), the effects of chronic BMI treatment were not a consequence of increased neuronal activity.

The notion that spontaneous GABAergic synaptic activity drives the developmental switch was further tested with KCl-induced depolarization (in the presence of TTX), which enhances the probability of transmitter release, thus increasing the frequency of mGSCs. As shown in Figures 6C and 6D, KCl (10 mM) induced a rapid increase in the mGSC frequency (190 ± 28%, $n = 4$, $p < 0.05$). Furthermore, chronic treatment with KCl (10 mM) markedly accelerated the time course of the transformation of GABA signaling, as more than 90% of all neurons failed to exhibit GABA-induced $[Ca^{2+}]_i$ elevation by day 10 (Figure 2A). We note that these same

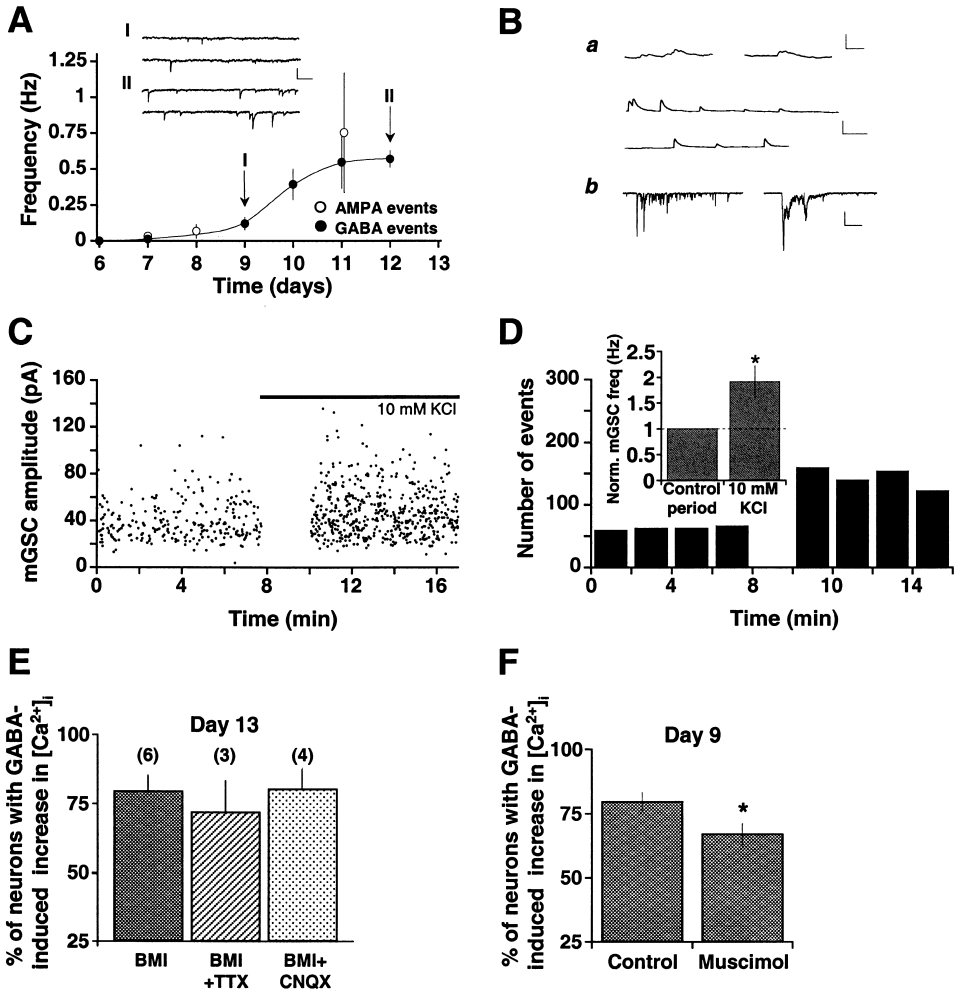

Figure 6. Developmental Changes in Spontaneous Activity

(A) Developmental increase in the frequency of spontaneous GABAergic and glutamatergic currents, which were recorded in the presence of TTX + CNQX/APV and TTX + BMI, respectively. Representative traces of mGSCs are shown (I and II). Scales: 10 pA, 0.5 s.

(B*a*) Spontaneous depolarizing GABAergic potentials recorded in current clamp using gramicidin perforated patch in the presence of CNQX. Resting potentials were approximately −60 to −70 mV. Scales: 10 mV, 0.3 s. (B*b*) Voltage clamp recordings (−70 mV) of spontaneous glutamatergic activity from cultures chronically treated with BMI + PTX. Scales: 100 pA, 0.3 s.

(C) An example of acute effects of 10 mM KCl on the frequency and amplitude of mGSCs. Each point represents a single mGSC.

(D) Changes in mGSC frequency after KCl treatment (the same experiment as shown in [C]). Inset: average mGSC frequency before and after KCl treatment ($n = 4$). ($p < 0.05$, paired t test).

(E) Summary of experiments involving chronic blockade of GABAergic activity only (BMI), GABAergic activity and spiking (BMI + TTX), and GABAergic and glutamatergic activities (BMI + CNQX).

(F) Comparison of chronic activation of $GABA_A$ receptors by muscimol (10–50 μM; $n = 8$) versus control conditions ($n = 21$). "*" denotes $p <$ 0.05 (t test).

neurons did respond to acute depolarization with KCl with a Ca^{2+} transient. Moreover, the acceleration of the switch by KCl was reversed in the presence of $GABA_A$ receptor antagonists (KCl + BMI + PTX treatment in Figure 2A). However, this time course was faster than the one observed for neurons treated only with BMI + PTX, suggesting that, in addition to increasing the mGSC frequency, KCl had additional effects (see Discussion). Taken together, these results indicate that the frequency of mGSCs can determine the kinetics of the developmental switch in GABA signaling. Consistent with this notion, chronic activation of $GABA_A$ receptors with muscimol (10–50 μM) produced a significant acceleration of the switch in day 9 neurons (Figure 6F).

Requirement for GABA-Mediated Ca^{2+} Influx

Early in development, GABA-induced depolarization evokes Ca^{2+} influx through voltage-dependent Ca^{2+} channels. Electrophysiological recordings from individual neurons showed that I_{Ca} was blocked by ~50% by the L-type Ca^{2+} channel antagonist nimodipine (10 μM) and ~90% by 10 μM $CdCl_2$ (commonly used as a high-threshold Ca^{2+} channel blocker; Figure 7A). As chronic blockade of all I_{Ca} was found to be deleterious for neuronal survival, a partial block of I_{Ca} was performed using moderate concentrations of nimodipine (0.5–1 μM). As shown in Figure 7B, chronic treatment with nimodipine significantly delayed the developmental switch. To assess whether this observation reflected a true shift in

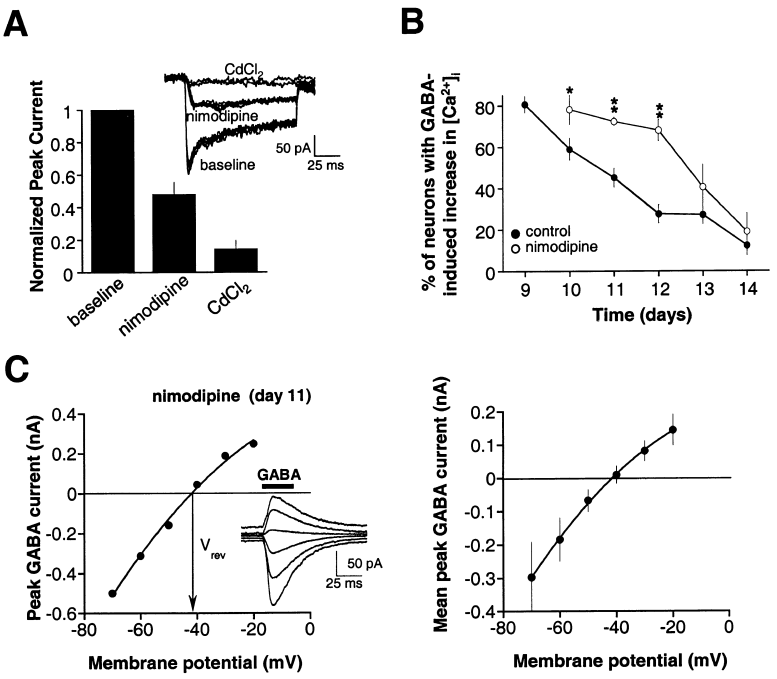

Figure 7. Role of Ca^{2+} Influx for the Developmental Switch of GABA Function

(A) Blockade of I_{Ca} by nimodipine and by $CdCl_2$ ($n = 6$). Inset: Representative traces of Ca^{2+} currents elicited by depolarizing pulses in a day 7 neuron, displaying the effects of nimodipine (10 μM) and $CdCl_2$ (10 μM).

(B) Effects of chronic blockade of L-type VDCCs on GABA-induced Ca^{2+} elevation in cultures of different ages. ("*" and "**" denote $p < 0.05$ and $p < 0.001$, respectively, t test).

(C) Effects of chronic blockade of L-type VDCCs on the shift in E_{GABA}. Left: Representative example of a GABA-induced I-V relationship. Cells were voltage clamped at −70 mV and stepped to different potentials (−70 to 0 mV, 10 mV steps). Right: Averaged I-V curve ($n = 9$). Note that E_{GABA} at day 11 in the presence of nimodipine is similar to that at day 7 in untreated cultures (Figure 1).

E_{GABA}, we measured E_{GABA} in cultures chronically treated with nimodipine (Figure 7C). We found that the shift in E_{GABA} was prevented at a time in which the effect of nimodipine on the GABA-induced elevations of $[Ca^{2+}]_i$ was most significant (day 11 neurons). Under these conditions, E_{GABA} was -42.5 ± 4.3 mV ($n = 9$), similar to that observed in untreated neurons at day 7 (-44.5 ± 2.0 mV; $n = 11$; $p > 0.2$). These findings support a model in which spontaneous GABAergic events elevate $[Ca^{2+}]_i$ and activate signaling pathways which can promote the developmental switch.

Discussion

The switch of GABA signaling from excitation to inhibition occurs during early postnatal brain development, when synaptic connections are being established, strengthened, and refined (Katz and Shatz, 1996). Using a model system of cultured hippocampal neurons, we tested the hypothesis that this switch is regulated by neuronal activity. We first characterized two aspects of GABAergic transmission—the electrophysiological (membrane depolarization) and the biochemical (Ca^{2+} signals)—which may serve different roles in the developing nervous system. We found that developmental changes in these two properties are related to a shift in E_{GABA} toward hyperpolarized potentials as well as a change in depolarization-induced elevations of $[Ca^{2+}]_i$. Our results also indicate that both of these changes can be triggered by GABAergic activity. The expression of the Cl^- transporter KCC2 was upregulated by increased spontaneous GABAergic activity, and downregulated following blockade of $GABA_A$ receptors, indicating that GABA-mediated depolarization is itself able to increase the expression of KCC2 and induce the maturation of inhibition (Figure 8). This suggests the notion that GABA-mediated excitation is self-limiting.

The Role of GABAergic Activity and Depolarization

We propose that the level of depolarizing GABAergic activity determines the rate of transformation of neuronal GABAergic responses. This is supported by the following three lines of evidence: (1) Blockade of $GABA_A$ receptors prevented the transformation; (2) elevating GABAergic activity or stimulating $GABA_A$ receptors accelerated it; and (3) the developmental increase in spontaneous GABA release tightly correlated with the time course of the transformation. To elevate synaptic activity, we chronically depolarized the neurons with KCl (10 mM) in the presence of TTX. That KCl-depolarization had indeed elevated GABAergic activity was shown by (1) the frequency of mGSCs was increased, and (2) the accelerated rate of transformation was significantly reduced when the KCl treatment was combined with blockade of $GABA_A$ receptors. However, the transformation still occurred under conditions of KCl-induced depolarization in the presence of $GABA_A$ receptor antagonists. As evident from Figure 4A, 10 mM KCl acutely induces a very robust elevation of $[Ca^{2+}]_i$ in immature neurons. Therefore, it is likely that chronic depolarization with 10 mM KCl induced a massive Ca^{2+} influx that bypassed the required specificity for GABA-mediated depolarization. This notion is supported by the fact that physiological sources of depolarization (namely glutamatergic activity and action potentials), although present at substantial levels, are unable to regulate the time course of the developmental switch (Figures 2B, 6A, and 6B).

Interestingly, in spinal cord neurons, the switch of GABA signaling is accelerated when neurons are plated on a higher density of astrocytes (Li et al., 1998). The mechanism underlying this process remains unknown.

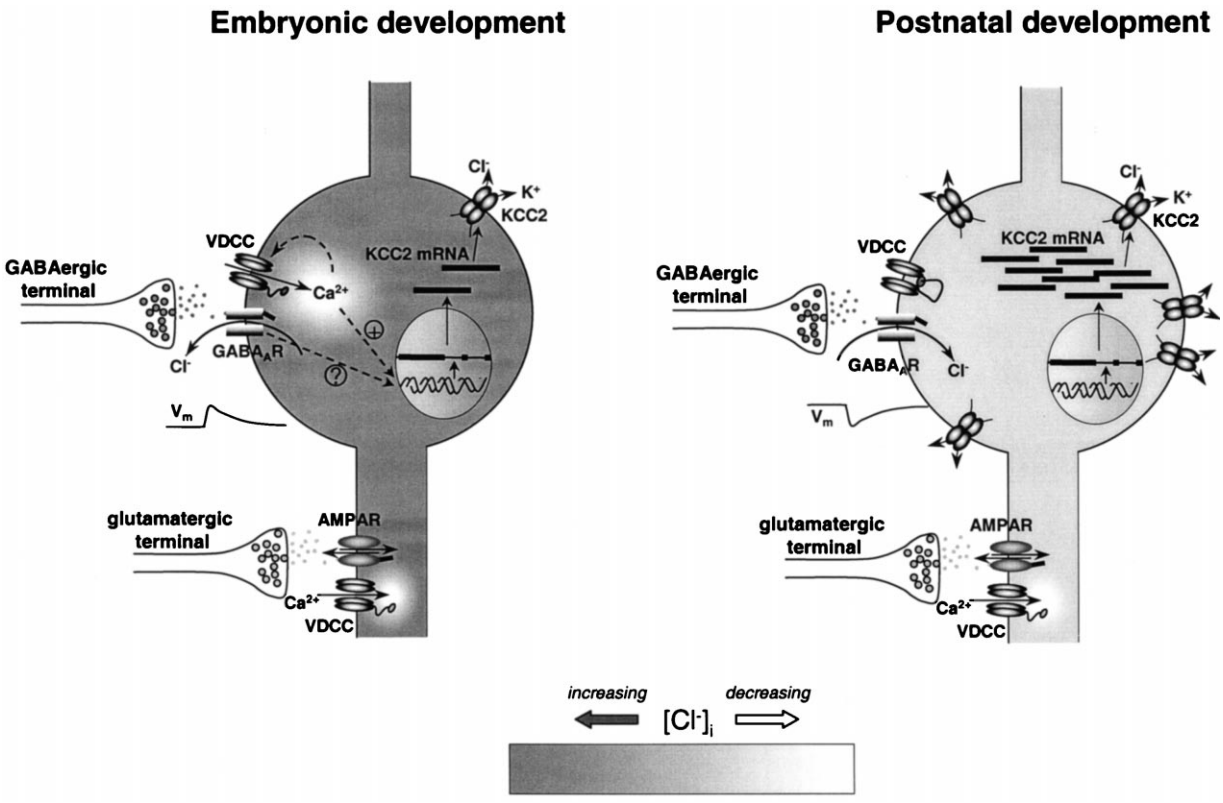

Figure 8. A Model for the Developmental Switch of GABAergic Transmission

During embryonic development, GABA acts as an excitatory neurotransmitter. GABA-mediated excitation can trigger Ca^{2+} influx through VDCCs, leading to an increase in the expression of the potassium-chloride cotransporter KCC2. This excitatory GABAergic activity can also regulate the activation profile of VDCCs. During early postnatal development, the increase in KCC2 activity lowers $[Cl^-]_i$ and reduces E_{GABA}, establishing GABA as an inhibitory neurotransmitter.

Recently, astrocytes have been shown to dramatically increase the number and maturity of functional synapses in cultured neurons (Ullian et al., 2001). Thus, it is possible that astrocytes might accelerate the maturation of GABAergic synaptic transmission, thereby accelerating the time course of the switch.

Specificity of GABA-Mediated Calcium Signaling

Spontaneous GABAergic and glutamatergic activity are both present during the time window of the developmental switch (Figures 6A and 6B; Hsia et al., 1998; van den Pol et al., 1998; Lamsa et al., 2000; Palva et al., 2000). GABAergic events are indeed depolarizing (3–20 mV), and the level of depolarization is enhanced by summation of unitary events. Such depolarizing responses are likely to trigger Ca^{2+} influx through VDCCs (Koester and Sakmann, 1998). Surprisingly, GABAergic but not glutamatergic activity promotes the transformation of GABAergic transmission (Figures 2A and 2B).

What is the signal that is specifically activated by GABA but not glutamate? Nimodipine delayed the switch (Figures 7B and 7C), indicating that Ca^{2+} influx through L-type VDCCs participates in the signaling cascade initiated by GABA. Since glutamate-mediated depolarization also activates L-type VDCCs (Yuste and Katz, 1991; Deisseroth et al., 1998), this finding suggests that either GABA-mediated elevations of $[Ca^{2+}]_i$ are spatially localized, or that additional Ca^{2+}-independent processes are required. Consistent with the notion of GABA-specific signaling is the growing evidence for an extensive protein network centered around gephyrin, a protein that may link $GABA_A$ receptors to other signaling proteins within the inhibitory postsynaptic specialization (Kneussel and Betz, 2000).

GABA-Dependent Regulation of KCC2 Expression

In hippocampal slices, the temporal expression pattern for KCC2 coincides with the postnatal switch of GABA-signaling (Rivera et al., 1999; Vu et al., 2000). In these cultures, there is a similar developmental increase in the level of KCC2 mRNA (Figure 3). We found that the level of KCC2 mRNA can be regulated by GABAergic activity. The level of KCC2 mRNA was reduced by chronic blockade of $GABA_A$ receptors and increased by chronic depolarization with KCl (which enhanced spontaneous neurotransmitter release, see Figures 6C and 6D). The observed increase in KCC2 mRNA levels may result from enhanced transcription or an increase in the stability of existing mRNAs. Given that the developmental increase in KCC2 mRNA levels will result in a decrease in the $[Cl^-]_i$ and a reduction of the GABA-mediated depolarization (Jarolimek et al., 1999; Kakazu et al., 1999; Rivera et

al., 1999), these results further support the notion that GABA-mediated excitation is self-limited.

While we have monitored the expression of KCC2, other genes may also contribute to the switch. The expression of NKCC1, a Na^+-K^+-2 Cl^- transporter, is also developmentally regulated (Plotkin et al., 1997; Kakazu et al., 1999; Lu et al., 1999). NKCC1 participates in Cl^- influx, and its expression is reduced during the same time window in which KCC2 expression is increased, suggesting a mechanism for reducing Cl^- influx as the Cl^- efflux is increased. It may be noted that at day 9, the expression of KCC2 was 6-fold higher than that at day 3, while only 20% of the cells had failed to respond to GABA with Ca^{2+} elevation (Figures 1B and 3D). This could be attributed to a high rate of Cl^- influx due to NKCC1 activity during this early period of transformation.

GABA-Induced Modifications of I_{Ca}

In addition to the developmental transformation of GABAergic transmission, we found that neuronal maturation involved a shift in the threshold for the activation of I_{Ca} (Figure 4). Pharmacological analysis of Ca^{2+} currents suggested that, in both young and mature neurons, I_{Ca} seems to reflect primarily the activation of "high-threshold" VDCCs, with ~50% of those channels corresponding to the L-type (Figure 7A). Our findings suggest that either the subunit composition of Ca^{2+} channels is modified or that the expression of low-threshold Ca^{2+} channels decreases with age. Consistent with this idea, the expression of Ca^{2+} channel genes appears to be developmentally regulated (Gruol et al., 1992; Hilaire et al., 1996; Desmadryl et al., 1998). In fact, the functional expression of low threshold T-type Ca^{2+} channels has been shown to decrease in cultured hippocampal neurons between day 4 and day 16, a time window similar to that studied here (Chameau et al., 1999). Since chronic blockade of $GABA_A$ receptors (Figure 4B) reduced the developmental shift in the threshold for the activation of I_{Ca}, GABAergic transmission can trigger the observed developmental changes in Ca^{2+} currents.

Two Faces of GABA Signaling: Membrane Depolarization and Ca^{2+} Elevation

GABA is able to induce electrophysiological (membrane depolarization) and biochemical (Ca^{2+}signaling) effects, which may serve different roles in the developing nervous system. While the extent of GABA-mediated depolarization depends primarily on E_{GABA}, Ca^{2+}-dependent signaling through GABA is dependent both on E_{GABA} and the properties of depolarization-induced elevation of $[Ca^{2+}]_i$; these two aspects may be independently regulated (Obrietan and van den Pol, 1997). Previous work has suggested that the GABA-induced depolarization may serve to either facilitate or inhibit neuronal activity under different circumstances in the developing brain (Ben-Ari et al., 1989; Chen et al., 1996; Garaschuk et al., 2000; Lamsa et al., 2000). A depolarizing GABAergic potential can result in a shunting inhibition or can facilitate excitation, depending on the relative timing and spatial distribution of GABAergic and glutamatergic inputs (Chen et al., 1996). In addition, GABA-mediated depolarization has been proposed to play a role in the activation of NMDA receptors and the maturation of glutamatergic transmission (Ben-Ari et al., 1997).

GABA-induced elevation of $[Ca^{2+}]_i$ is likely to promote neuronal maturation. In the initial stages of brain development, depolarizing GABAergic potentials can prevent DNA synthesis, leading to the differentiation of cortical progenitor neurons (LoTurco et al., 1995). Furthermore, GABA-mediated Ca^{2+} increases can induce BDNF expression (Berninger et al., 1995) and the differentiation into specific neuronal phenotypes (Marty et al., 1996). GABA-induced elevation of $[Ca^{2+}]_i$ may also be required to form, stabilize, and strengthen synaptic connections (Kirsch and Betz, 1998; Caillard et al., 1999; Kneussel and Betz, 2000). For glycinergic transmission, there is a similar developmental transformation from excitation to inhibition (Reichling et al., 1994; Wang et al., 1994). Interestingly, Ca^{2+} influx triggered by glycine-mediated depolarization is required for clustering of postsynaptic glycine receptors (Kirsch and Betz, 1998), an important step in synaptogenesis. Similarly, Ca^{2+} influx mediated by GABA may also participate in postsynaptic differentiation (Kneussel and Betz, 2000). Finally, there is increasing evidence that Ca^{2+} influx through synaptically activated L-type Ca^{2+} channels is important for activation of gene expression (Murphy et al., 1991; Berninger et al., 1995; Deisseroth et al., 1996, 1998).

During development, these two aspects of GABAergic synaptic transmission—depolarization and Ca^{2+} influx—are transformed. We have demonstrated that GABA itself promotes this transformation. Such a self-limiting trophic action of GABA allows for an activity-dependent transition of the nervous system from its early dependence on global excitation to the requirement of mature neural circuits, where inhibition plays a critical role in its development and function.

Experimental Procedures

Cell Culture and Chronic Treatments

Hippocampi from E18 rats were trypsinized (15 min, 37°C), washed, and gently triturated by passing the tissue through a Pasteur pipette with a fire-polished tip. Neurons were plated at 500,000 cells/ml on poly-L-lysine coated coverslips in 35 mm dishes. The plating medium was DMEM (BioWhittaker, Walkersville, MD) containing10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) and 10% Ham's F12 with glutamine. Twenty four hours after plating, the culture medium was completely replaced by Neurobasal medium without glutamine (Gibco, Life Technologies) with B-27 supplement (Gibco). These serum-free conditions supported the growth of neurons but not glial cells. Cultures were fed once a week by adding ~0.4 ml of neurobasal medium. Chronic treatments were started 2 days after plating. BMI, D-APV, TTX, GABA, baclofen, CNQX, Nimodipine, PTX (Sigma), thapsigargin, and BHQ (Calbiochem) were added daily from fresh stocks prepared at 1000-fold the final concentrations (indicated in the Results section), except for KCl, which was added only once. All chronic treatments involving KCl (10 mM) were done in the presence of TTX and D-APV (2 and 50 μM).

Calcium Imaging

Neurons were loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-4 for 20–40 min at room temperature in HEPES buffered saline solution (HBS, in mM: NaCl 150, KCl 3, $CaCl_2$ 3, $MgCl_2$ 2, HEPES 10, and glucose 5; pH 7.4; osmolarity 310 mOsm) containing 15 μM CNQX and 10 μM BMI in the presence of 5 μM Fluo-4AM dissolved in DMSO (Molecular Probes, Eugene, OR). HEPES-buffered solution (in the absence of HCO_3^-) was used throughout the experiments, thus consistent with the notion that changes in $[Cl^-]_i$ can account

for the switch in GABA signaling (Staley et al., 1995). All recordings were carried out at room temperature, using HBS containing 10 μM CNQX. Solutions were exchanged using a multibarreled perfusion system (at 1–2 ml/min). Recordings were performed using a confocal laser scanning microscope BioRad MRC1024MP (Biorad, Hemel Hempstead, England). The scanhead was connected to an Olympus BX50WI upright microscope and images were collected using a 20× water-immersion objective. A total of 30–40 neurons were studied in each experiment. Somatic regions of ~20–50 pixels were chosen for quantification of fluorescence intensity. Sampling of average pixel intensity was performed at 0.5 Hz. Transmitted and fluorescent images reflecting Ca^{2+} dynamics were acquired (picture size of 512 × 512 pixels) using the Biorad Lasersharp acquisition program. Data were analyzed using custom made LabView Software (National Instruments) on a PC. A neuron was considered responsive to GABA if the peak $\Delta F/F_0$ was larger than a threshold, defined as six standard deviations (SD) of the baseline noise. This "six SD" criterion was developed by repeatedly comparing visual analysis on blind experiments with threshold analysis, until the results matched.

Electrophysiology

All experiments were carried out using the perforated-patch whole cell recording technique. Neurons were visualized using phase-contrast on a Nikon inverted microscope. Recordings were carried out using patch clamp amplifiers (Axopatch 200B, Axon Instruments, Foster City, CA). Signals were filtered at 2 kHz, sampled at 10 kHz and analyzed using Pclamp 6.0 software (Axon Instruments). Series resistance was compensated at 80% (lag 30–60 μs). Micropipettes were made from borosilicate glass capillaries (KG-33, Garner Glass) with a resistance of ≈2 MΩ. For recordings of E_{GABA}, pipettes were tip filled with internal solution and then back filled with internal solution containing 20 μg/ml gramicidin A (dissolved in methanol, Sigma). The internal solution contained (in mM): K-gluconate 154, NaCl 9, $MgCl_2$ 1, HEPES 10, and EGTA 0.2; pH 7.4; osmolarity 300 mOsm. The bath solution contained HBS with 15 μM CNQX, and was constantly perfused at a rate of ~1 ml/min. GABA (100 μM, 50 ms, 5 psi) was locally applied every 10 s through a micropipette connected to a Picospritzer (General Valve Corporation). For recordings of Ca^{2+} currents, pipettes were back filled with a high $CsCl_2$ internal solution containing 200 μg/ml amphotericin B (dissolved in DMSO, ICN). The high $CsCl_2$ internal solution contained (in mM): $CsCl_2$ 154, NaCl 9, $MgCl_2$ 1, HEPES 10, and EGTA 0.2; pH 7.4; osmolarity 300 mOsm. The bath solution was a modified HBS containing (in mM): NaCl 150, KCl 3, $BaCl_2$ 10, $MgCl_2$ 2, HEPES 10, glucose 5, and tetraethylammonium 10; TTX 2 μM. Leak and capacitive currents were subtracted using the average of four hyperpolarizing pulses. Recordings of miniature synaptic currents were performed using amphotericin B and high $CsCl_2$ internal solution. The bath solution was HBS with CNQX (15 μM) for GABA-mediated currents, and with BMI (10 μM) for glutamate-mediated currents. Events were filtered at 2 kHz and detected online using the WCP software (kindly provided by J. Dempster, University of Strathclyde, Scotland). All experiments were performed at 22–24°C. Experiments were rejected if the leak current exceeded 100 pA.

Quantitation of KCC2 mRNA in Cultured Neurons

Total RNA was harvested from neurons treated with vehicle, BMI + PTX or 10 mM KCl. Cells were lysed with guanidine isothiocyanate (600 μl) and purified (RNeasy, Qiagen). An antisense RNA template was generated using 316 bp of noncoding sequence residing at the distal 3′ end of the mRNA sequence (Payne et al., 1996). An antisense β-actin probe (245 bp) was mixed with the KCC2 probe and used as an internal standard in all assays. ^{32}P-labeled riboprobes were generated using Maxiscript (Ambion), and RNase protection assays were performed using Hybspeed (Ambion). Briefly, 3.5 micrograms of total RNA were treated with a mixture of the KCC2 and β-actin antisense probes (5×10^4 cpm each) and annealed at 68°C for 10 min. The samples were then immediately treated with RNase T1 (200 U/ml) for 30 min at 37°C. The protected RNA species were separated over a 5% acrylamide gel containing 8 M urea, and then dried. Radioactive band intensities were then measured (Molecular Dynamics Phosphorimager) and quantified (NIH image v1.62, Bethesda MD).

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