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Development of GABA responsiveness in embryonic turtle cortical neurons

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The whole-cell patch-clamp method was used to study the development of functional GABA receptors in cortical neurons dissociated from embryonic turtles. GABA elicited an increase in membrane conductance, even from cells obtained from the earliest stages of corticogenesis. The GABA-mediated conductance had a mean value 7.4 times greater than membrane 'leak' conductance and increased with developmental age. In all stages studied, the response inverted polarity at a value approximating E_{Cl} and was blocked by applications of bicuculline, suggesting that it was mediated by $GABA_A$ receptors. GABA receptors are thus present and functional very early in corticogenesis, preceding electrogenesis, synaptogenesis, and full neuronal differentiation.

γ-Aminobutyric acid (GABA) is the principal inhibitory transmitter in cortex and plays an important role in modulating cortical excitability. Its role during corticogenesis, when it may have a trophic function [6], will be better defined when the timetable for the appearance of the GABA responsiveness in cortical neurons is determined.

We used embryonic cortical neurons of the turtle (*Pseudemys scripta elegans*) as our experimental preparation. Turtle cortical development shares patterns of neurogenesis, cellular differentiation, and synapse formation with mammalian corticogenesis [11]. However, turtle cortex follows an outside-in gradient of development and thus provides advantageous temporal and spatial features for studying developmental questions. In addition, the trilaminar cortex of adult cortex has been reported to share anatomical, biochemical, and physiological properties with mammalian cortex, to which it may be phylogenetically related [8, 15, 16]. It contains a single layer of pyramidal neurons, and ultrastructural and immunohistochemical evidence suggests that GABA-immunoreactive non-pyramidal neurons in all 3 layers make inhibitory synaptic contacts onto pyramidal cell dendrites and perikarya [3, 9].

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In this paper, we used the whole-cell patch-clamp technique to examine GABA responsiveness in acutely dissociated turtle cortical neurons starting at ages prior to cortical plate formation. This technique allowed us to look at the direct effects of GABA while circumventing the technical difficulties involved in obtaining conventional intracellular recordings from small developing neurons. Gravid turtles (Tangi Turtle Farms, Ponchatoula, LA) were rapidly decapitated and their eggs were removed and placed in glass bowls kept in a humidified 30°C incubator. Cortical slabs, which range from 100 to 400 μm in thickness, were dissected out, placed in cold saline containing (in mM): NaCl 130, KCl 5, $CaCl_2$ 1, $MgCl_2 \cdot 6H_2O$ 1, dextrose 25, HEPES 20, and cut into 1 mm^2 fragments. The pieces of tissue were then placed in saline containing 1.6 mg/ml papain type IV (Sigma) and 1 mM cysteine, and gently stirred at room temperature for 5–15 min. Tissue fragments were rinsed and triturated in 1 ml of fresh saline using fire-polished Pasteur pipettes of decreasing tip diameter. The cell suspension was transferred to a 35 mm tissue culture dish which was placed on the stage of an inverted microscope and superfused with saline containing a final concentration of 3 mM $CaCl_2$.

The whole-cell patch-clamp technique described by Hamill et al. [12] was used. Patch-clamp pipettes (1–10 MΩ) were filled with solution containing (in mM): KCl 120, HEPES 10, EGTA 5, $MgCl_2$ 1 (pH = 7.2), and 1–5 GΩ seals were obtained. An estimate of neuronal membrane surface areas was obtained from the membrane capacitance of the cells (1 $\mu F/cm^2$).

The criteria of Yntema [21] were used to determine the stage of the embryos. Corticogenesis begins at approximately stage 15. At stage 16, the earliest age at which cortical hemispheres can be readily identified and surgically isolated, neural precursor cells are in the process of undergoing mitosis in the ventricular zone. By stage 20, a loose cortical plate has appeared and synaptic profiles can be detected at the electron microscope level [11]. The cortical plate becomes better defined and neurons with more mature dendritic and synaptic features become more abundant by stage 22. The data reported here were obtained from 50 cells recorded from embryos of stages 17, 19, 20, 23, and 26 (newly hatched) turtles. Neurons could be distinguished from glial cells by their morphological features (phase-bright and three-dimensional, confirmed by absence of glial fibrillary acidic protein staining). In addition, since pyramidal neurons outnumber non-pyramidal neurons in adult cortex by a ratio of at least 17:1 [3], it is likely that most neurons from which we recorded were pyramidal. We observed no differences in the response characteristics of neurons with different morphologies.

We were able to record from cells as young as stage 17 and observed differences in the current–voltage (I–V) relationship with developmental age. Stage 17 cells had a steady outward current at higher voltage steps (Fig. 1A) but no inward current. At stages 19 and 20, 57% ($n = 16$) of cells had an early component of inward current preceding the outward current. All cells from stage 23 embryos and postnatal animals had both prominent early inward and steady outward currents (Fig. 1B). The possibility that a small early inward current in younger neurons might be masked by the large outward current was ruled out since the amplitude of the inward current

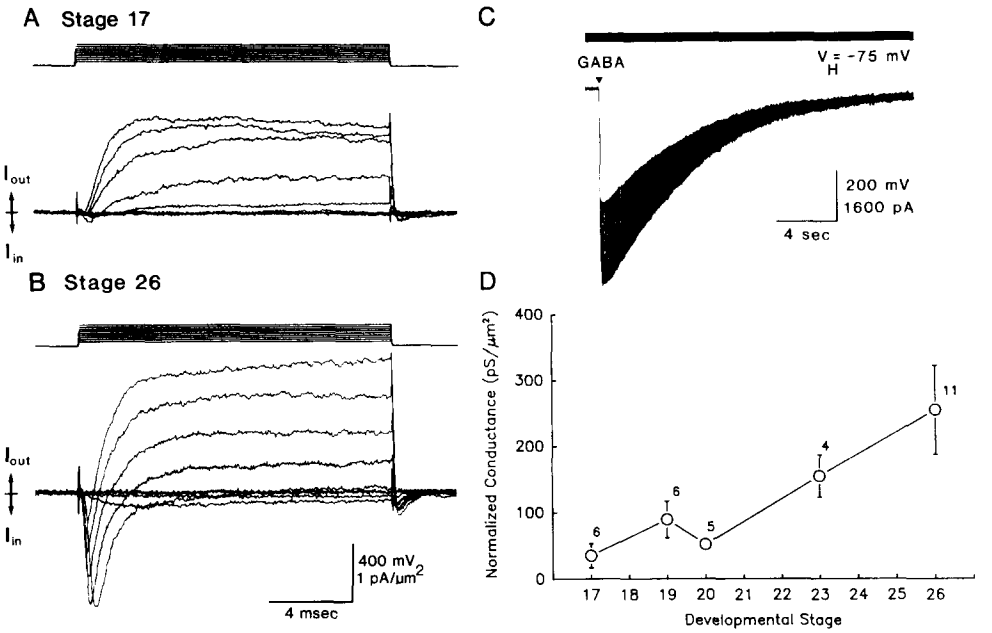

Fig. 1. A, B: *I V* properties of stage 17 and stage 26 cortical neurons under whole-cell clamp. Cells were held at −100 mV and stepped to increasing depolarizing voltages (first step to −70 mV, subsequent steps at 15 mV increments). Current amplitudes are normalized to membrane area to represent current per unit membrane area. Leak and capacitive currents have been substracted. Neuron from stage 17 embryo (A) shows very little, if any, inward (downward) current, although an outward (upward) current is apparent. Membrane capacitance = 8.0 pF, estimated membrane area = 800 μm^2. Stage 26 cortical cell (B) shows both inward and outward components of current. There may also be a small, sustained inward current which may be attributed to calcium. Membrane capacitance = 9.6 pF, estimated membrane area = 960 μm^2. C: GABA response of whole-cell-clamped cortical neurons from a stage 26 (hatchling) turtle. Top trace, voltage; bottom trace, current. Cell was held at −75 mV and 30 mV depolarizing voltage steps of 10 ms duration were applied every 40 ms to monitor the 'leak' (baseline) membrane conductance. GABA (0.5 mM) was pressure-ejected (Digital Valve) onto the cell body by applying N_2 gas (70–150 kPa) to the back of drug-filled pipettes broken to tip diameters of 1–2 μm. Pipettes were positioned, under visual guidance, less than 20 μm from the perikarya. A saturating pulse of GABA (arrowhead) produced an increase in membrane conductance of an inward (downward) current (outward flux of Cl^- ions). Recovery was complete in approximately 12 s. D: plot of GABA conductance (mean ± S.E.M.; normalized to membrane area) at different developing stages. Numbers indicate the number of cells to which GABA was applied. GABA conductance was measured by subtracting the leak conductance prior to GABA application from the conductance value obtained at the peak of the response using the protocol shown in C. For GABA conductance measurements, values for membrane potential were adjusted in each case to compensate for the voltage drop due to the series resistance of the patch pipette. Space clamp errors were negligible. (We estimate that during a 100-fold increase in membrane conductance, there would be, at most, a 5% steady state voltage error at the tip of a 60-μm-long, 1-μm diameter dendrite [14], which corresponds to the most extensive dendrite obtained in a dissociated neuron.)

recorded with CsF (100 mM) electrodes never exceeded that illustrated in Fig. 1A ($n = 6$). Therefore, while voltage-activated outward currents are present in all neurons even at the earliest stages of development, prominent inward currents are not universally present until later stages.

Although very young cells lacked voltage-activated inward currents, nearly all neurons tested (48 out of 50) across all stages responded to GABA application with a large inward current accompanied by an increase in conductance (e.g. Fig. 1C). The mean increase was 7.4-fold, from a mean leak conductance of 3.4 nS to a mean total conductance of 25.2 nS (range = 1.6- to 99-fold). The response desensitized during prolonged GABA application, and subsequent applications usually had less effect. Application of control saline solution produced no response.

The GABA-mediated conductance was determined for cells dissociated at different stages and was normalized to membrane area. As shown in Fig. 1D, GABA-mediated conductance increases during embryonic development. The developmental increase in GABA-mediated ionic conductance may be due to an increase either in the number of GABA channels or in single channel conductance; single-channel recordings would distinguish between these two possibilities. Preliminary autoradiographic studies using [^{3}H]flunitrazepam, a ligand specific to the benzodiazepine receptor, have revealed a developmental increase in the number of binding sites (Lee and Kriegstein, unpublished observations), providing indirect evidence that an increase in the actual number of GABA receptors is at least partially responsible for the greater GABA-mediated conductance in neurons from the later stages.

Activation of $GABA_A$ receptors causes an increase in Cl^- permeability [4], while $GABA_B$ receptors mediate an increase in membrane permeability to K^+ [10, 18]. For the cell illustrated in Fig. 2A, brief applications of GABA at different membrane potentials revealed a reversal potential of +3 mV. This value approximates the expected Nernstian equilibrium potential for Cl^- (3.3 mV). A mean of 0.8 mV (S.E.M. = 2.0 mV) was obtained for the reversal potential of the GABA response for 21 neurons tested. In addition, the GABA response was sensitive to the $GABA_A$ receptor antagonist, bicuculline. Bicuculline (10 μM) was effective in eliminating responsiveness to GABA in 9 out of 9 cells from embryonic stages 17 and 19 (Fig. $2B_1$). The GABA response of 6 of these 9 cells gradually recovered to their original levels, while the remaining 3 showed only partial recovery. The GABA responses of 3 out of 3 cells tested from stages 20 and 23 were sensitive to bicuculline, and all 3 showed almost complete recovery within 90 s (Fig. $2B_2$). However, in some cases such as the one shown in Fig. $3B_2$, it appeared that bicuculline did not completely eliminate the GABA response. This effect could have resulted from the presence of bicuculline-insensitive receptors, but since bicuculline is a competitive antagonist, our observation may also reflect the failure of bicuculline application to block all the $GABA_A$ receptors. Baclofen (0.1 mM), a $GABA_B$ receptor agonist, had no effect on cells ($n = 5$) obtained from stage 19 and 26 animals. This may be due to the truncation of dendritic processes since $GABA_B$ receptors may be located on apical dendrites. However, we also failed to find responses to baclofen when recording from pyramidal neurons in intact adult turtle cortex.

Our data indicate that the Cl^- channel-mediating $GABA_A$ receptor is present in embryonic cortical neurons. The absence of a depolarizing GABA response similar to that described in immature rabbit hippocampus [17] may also be due to the truncation of dendritic processes, since depolarizing GABA responses have been associated

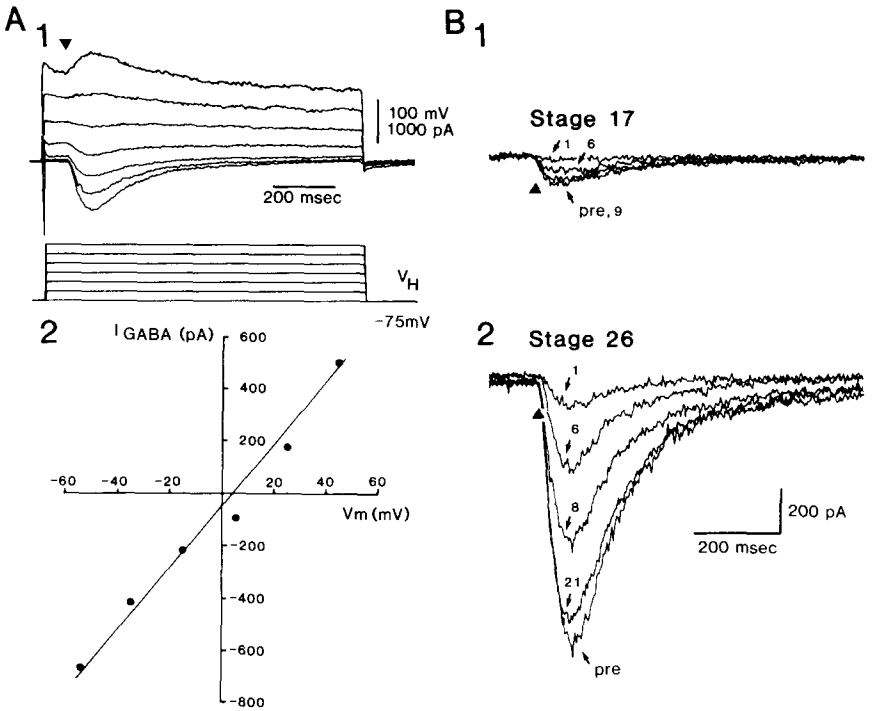

Fig. 2. A: the GABA response reverses at approximately E_{Cl}. (1) Small, short (4–10 ms) non-saturating pulses of GABA were applied while the cell was stepped to different membrane voltages from a holding potential of −75 mV (top). The resulting current traces show a reversal in polarity of the GABA current from an inward (downward) to an outward (upward) current. (2) Values for GABA current (I_{GABA}) were obtained by measuring the peak GABA response compared to the current amplitude immediately prior to GABA application. By plotting I_{GABA} as a function of membrane voltage (V_m), a GABA current reversal potential of 3 mV was determined for this cell. The Nernstian equilibrium potential for Cl^- is 3.3 mV (extracellular $[Cl^-] = 130$ mM, pipette $[Cl^-] = 122$ mM), suggesting that Cl^- is the primary ion responsible for the GABA current. B: GABA response is blocked by bicuculline in stage 17 (1) and 26 (2) cortical neurons. Pre traces are control responses to discrete pulses of GABA prior to bicuculline application. GABA was applied every 4 s and numbers indicate the number of 45-s epochs elapsed following application of bicuculline (e.g. trace 1 was obtained 4 s after bicuculline was applied).

with distal dendritic application [15]. Alternatively, the intracellular perfusion of Cl^- from our recording electrodes may have abolished regional differences in the Cl^- gradient that could affect the reversal potential of transmitter-evoked responses [13].

Immunohistochemical studies using antisera to GABA and its synthetic enzyme, glutamic acid decarboxylase (GAD) have shown that GABA-positive neurons are present by stage 16 [2]. Therefore, the substrate for GABAergic inhibition – GABA-containing cells and GABA-responsive cells – is present very early on in development, prior to synapse formation. The role of these precocious GABAergic elements has yet to be determined. In embryonic mammalian cortex, GAD is found in neurons prior to synaptogenesis [5, 20], and in developing rat cortex, GABA-accumulating neurons are present before the appearance of inhibitory synaptic profiles [1, 6, 7, 19].

Wolff [19] reported that synaptogenesis begins after GABA-accumulating cells appear and terminates with a selective increase in GABAergic synapses. These observations suggest that GABA may play an transient role in synaptogenesis, and it would be interesting to determine whether such a role might be disrupted by bicuculline. It would also be important to study the physiological responses of developing cortical neurons to other amino acid transmitters such as the excitatory amino acid, glutamate, to determine whether precocious expression of receptors is a general feature of synaptic development or is selective for the GABAergic system. Such studies may also provide additional insight into the interaction between excitatory and inhibitory systems during cortical development.

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