EXCITATORY ACTIONS OF GABA DURING DEVELOPMENT: THE NATURE OF THE NURTURE

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In the immature brain, GABA (γ-aminobutyric acid) is excitatory, and GABA-releasing synapses are formed before glutamatergic contacts in a wide range of species and structures. GABA becomes inhibitory by the delayed expression of a chloride exporter, leading to a negative shift in the reversal potential for choride ions. I propose that this mechanism provides a solution to the problem of how to excite developing neurons to promote growth and synapse formation while avoiding the potentially toxic effects of a mismatch between GABA-mediated inhibition and glutamatergic excitation. As key elements of this cascade are activity dependent, the formation of inhibition adds an element of nurture to the construction of cortical networks.

IONOTROPIC
A term that describes a receptor that exerts its effects through the modulation of ion channel activity.

In the adult brain, the equilibrium between excitation and inhibition is an essential feature that must be maintained to avoid pathological consequences. In the adult mammalian brain, GABA (γ-aminobutyric acid)-releasing synapses (the principal source of inhibition) and synapses that use glutamate (the principal excitatory transmitter) operate through IONOTROPIC receptor channels that are permeable to anions and cations, respectively. Agents that block GABA synapses generate seizures, whereas agents that enhance inhibition have sedative, anticonvulsant and anxiolytic actions. Conversely, excessive activation of glutamatergic synapses leads to devastating neurological conditions. A dilemma is therefore encountered in sculpting neuronal connections during brain development: a mismatch between the strength of GABA and glutamatergic synapses might either prevent growth and synapse formation if the former prevails, or cause toxicity if the latter becomes operative first.

Here, I discuss a sequence of events that seems to have evolved to provide a solution to this dilemma. This sequence consists of three independent but indispensable developmental features that were first described in neonatal hippocampal slices[1]. First, GABA is initially excitatory as a result of a high intracellular concentration of chloride ($[Cl^-]_i$). Second, GABA-releasing and glutamatergic synapses are formed sequentially. Third, there is a primitive network-driven pattern of electrical activity in all developing circuits — the giant depolarizing potentials (GDPs), which are generated in part by the excitatory actions of GABA. This pattern allows the generation of large oscillations of intracellular calcium, even in neurons that have few synapses, and an activity-dependent modulation of neuronal growth and synapse formation. Later on, once a sufficient density of glutamate and GABA synapses has been generated and inhibition becomes necessary, a chloride-extruding system becomes operative, an event that seems to be activity dependent. As a result, chloride is efficiently pumped from the intracellular milieu, GABA begins to exert its conventional inhibitory action, and the primitive pattern is replaced by more diverse and elaborate patterns of activity.

The principal elements of this sequence of events have been observed in many brain structures and animal species, which indicates that they have been maintained throughout vertebrate evolution. Interestingly, the main steps of this cascade, including the shift from excitatory to inhibitory actions of GABA, are modulated by neuronal activity, providing an element of nurture in the construction of the developing network. So, the molecular switch that controls $[Cl^-]_i$ is a pivotal point in the

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Box 1 | GABA is a highly conserved developmental signal

GABA (γ-aminobutyric acid) and glycine chloride channels have been described throughout the animal and vegetal kingdoms, and phylogenetic studies indicate that they belong to a ligand-gated ion channel family that is orthologous to the vertebrate glycine channels[111,112]. In plants, cytosolic glutamic acid decarboxylase (GAD) synthesizes GABA in response to environmental stress, and the secretion of GABA serves to buffer cytosolic pH changes in plant cells[113,114]. GABA is also a source of nitrogen in pollen and is present in sunflowers, where it is involved in ethylene production[115]. Moreover, it is involved in the ripening of tomatoes and wheat seeds[116,117]. The sponge Porifera, which belongs to the earliest evolutionary Metazoan phylum, has $GABA_B$ receptors that are similar to mammalian metabotropic receptors[118].

GABA operates as a signal in a wide range of species, including insects, at early developmental stages. For example, GABA is expressed during the post-embryonic development of beetle antennae[119], and GABA postsynaptic currents (PSCs) are observed at the mid-gastrula stage in *Drosophila*[120]. Blockers of GABA uptake in larval and adult *Drosophila* affect behaviour and increase the amplitude of endplate junction potentials[121]. Calcium oscillations are present in the mushroom bodies of the insect brain[122], and GABA has an important role in their generation and in olfactory cues[123,124]. Although information is lacking at present, it is likely that the mechanisms that regulate the differentiation of the GABA neurotransmitter system, and the associated enzymes and transporters, are evolutionarily conserved in insects and worms.

Therefore, GABA is an ancillary signal that participates in communication during early development. Further studies will be required to determine whether, as in the developing mammalian brain, the formation of GABA synapses precedes the formation of glutamatergic contacts in these non-mammalian organisms.

transition from a silent structure with silent neurons to one that has a highly diversified range of electrical currents and billions of excitatory and inhibitory synapses. As GABA is also used as a communication signal in plants and primitive organisms (BOX 1), this sequence is also discussed within an evolutionary framework.

GABA depolarizes immature neurons

An early study indicated that a developmental shift in the actions of GABA takes place in chick neurons in culture[2]. Using neonatal hippocampal slices from birth to two weeks of age, Y.B.-A. and co-workers[1] reported that the activation of GABA synapses in young neurons produces a depolarization instead of the characteristic hyperpolarization. In these neurons, unlike those of adults, the reversal potential for chloride (also referred to as the electrochemical equilibrium potential, E_{rev} or E_{Cl}; see BOX 2) was at a more depolarized level than the resting membrane potential (V_{rest}), indicating that $[Cl^-]_i$ was higher in neonatal neurons. In rodents, several observations indicate that the activation of $GABA_A$ receptors produces a depolarization and an increased concentration of intracellular calcium ($[Ca^{2+}]_i$) in immature but not adult neurons in a wide range of brain structures, including the hippocampus[3–10] and neocortex[11–15], the hypothalamus[16–18], the spinal cord[19–22], the ventral tegmental area[23] and the cerebellum[24,25]. Interestingly, glycine receptors, which operate through chloride-permeable channels and are also inhibitory in the adult, show a similar developmental shift in the rat brain stem[26,27] and spinal cord[28].

A developmental shift in the actions of GABA has been observed in a range of species, including embryonic *Xenopus* tadpoles[29], spinal neurons of *Xenopus* larvae[30], embryonic zebrafish[31], chick embryos[32] and turtle embryonic retina[33,34]. In fact, so far, no exception to this shift has been found, so it seems to be a general rule that has been conserved throughout evolution, at least in vertebrates. Interestingly, another example of the developmental chloride shift is observed in optic nerves, in which GABA depolarizes immature but not adult neurons[35,36]. Also, GABA and glutamic acid decarboxylase (GAD; the synthetic enzyme for GABA) are expressed in astrocytes of the developing but not the adult optic nerve[36], indicating that developmental alterations in the function of GABA are not restricted to neurons.

Perforated-patch-clamp recordings, which do not alter $[Cl^-]_i$, show an important developmental change in E_{Cl} (BOX 2). For example, in neurons of the lateral superior olive, E_{Cl} is shifted from –46 to –82 mV between birth and postnatal day (P) 10 (REF. 37). In spite of the permeability of GABA and glycine channels to bicarbonate, the developmental shift seems to be due primarily to a higher $[Cl^-]_i$ (REFS 7,30). $[Cl^-]_i$ has been measured using two chloride-imaging techniques[38,39], both of which revealed a shift in E_{Cl}. However, these imaging techniques are highly sensitive to pH alterations and might provide an inadequate estimate of $[Cl^-]_i$. Also, perforated-patch recordings might lead to an underestimate of V_{rest} in immature neurons, owing to their small size[40]. In spite of these caveats, it is clear that the concentration of $[Cl^-]_i$ is higher in immature than in adult neurons by 20–40 mM. This is sufficient to shift the actions of GABA from inhibition to excitation (see BOX 2 and below). So, in immature neurons, the net active chloride transport is inwards, so that anions flow out of the neuron when GABA channels are opened.

Developmental expression of KCC2

The intracellular accumulation of chloride in immature neurons could be generated either by the early expression of an importer or by the delayed expression of an exporter. Two families of transporters have been studied: the Na^+–K^+–$2Cl^-$ co-transporters (NKCCs), which typically raise $[Cl^-]_i$, and the K^+–Cl^- co-transporters (KCCs), which normally lower $[Cl^-]_i$ below its electrochemical equilibrium potential[41]. NKCC1, which is driven by sodium and potassium gradients, accumulates intracellular chloride, is expressed at early developmental stages, and is responsible for the high $[Cl^-]_i$ in dorsal root ganglia and in neocortical neurons[42,43].

However, several observations[7,43,44] indicate that the key player in the developmental switch from GABA-mediated excitation to inhibition is the K^+–Cl^--coupled co-transporter KCC2 (FIG. 1). First, changes in the levels of KCC2 messenger RNA in hippocampal cultures and slices correlate with the modification of GABA actions. Second, the transfection of KCC2 into hippocampal neurons converts the actions of GABA from excitatory to inhibitory, and GABA is excitatory in mice that lack KCC2. Third, the activation or blockade of $GABA_A$ receptors alters both the E_{rev} of GABA and the levels of KCC2. However, because of the intrinsic heterogeneity of immature neurons — some might have no functional synapses, whereas others already generate

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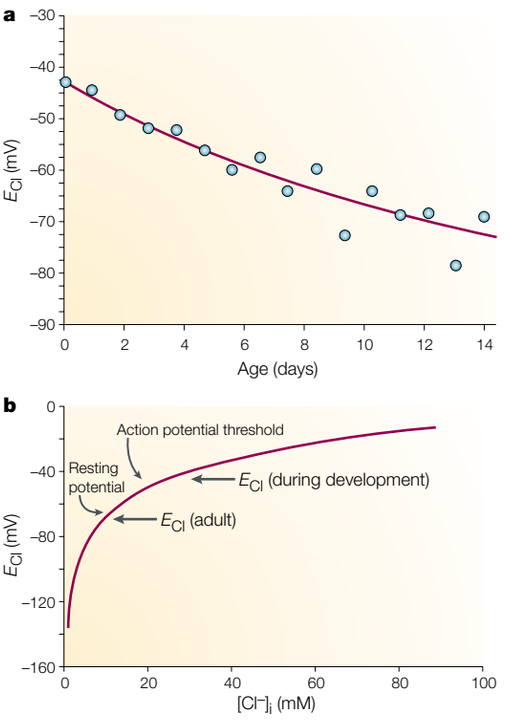
Box 2 | **Equilibrium potentials and the ins and outs of chloride**[125]



Anions are distributed differentially across the cell membrane. The main anions of the intracellular fluid are organic molecules, such as negatively charged amino acids, proteins and nucleic acids, whereas chloride is the principal anion in the extracellular fluid. Under physiological conditions, the concentration gradient for chloride — that is, the difference between the external and internal concentrations — is 140 mM – 7 mM, so there will be an influx of chloride when chloride-permeable channels, such as GABA (γ-aminobutyric acid) type A receptors, open. However, the direction and magnitude of ion diffusion will be determined by both the concentration gradient and the membrane potential (V_m), which forces ions to move in a particular direction according to their charge. The electrochemical equilibrium potential (E_m; also known as the reversal potential) for a given ion is the membrane potential at which the concentration-gradient force that tends to move a particular ion in one direction is exactly balanced by the electrical force that tends to move the same ion in the reverse direction. For cations, these values are 0 mV for sodium and 100 mV for potassium — far from a typical resting potential (V_{rest}) of –65 mV. By contrast, E_{Cl} in the adult is only a few mV more hyperpolarized than V_{rest} (that is, –75 mV), so the net driving force is small. In the rodent hippocampus, we have shown that E_{Cl} decreases with age during the postnatal period (see part a of the figure).

Developing neurons have a higher intracellular concentration of chloride ($[Cl^-]_i$) than adult neurons. To estimate $[Cl^-]_i$, recordings are made using the perforated-patch-clamp technique, in which a solution is used to makes perforations in the membrane that are not permeable to chloride and so do not change the genuine $[Cl^-]_i$. In spite of their limitations for small neurons, perforated-patch recordings indicate that $[Cl^-]_i$ is in the order of 20–25 mM in young hippocampal neurons (part b; adapted, with permission, from REF. 45 © 2001 Macmillan Magazines Ltd). An important feature of chloride gradients is that even small changes in $[Cl^-]_i$ can have profound consequences. Indeed, the curve that relates the reversal potential to the transmembrane chloride concentration (the Nernst equation) is steep at physiological concentrations of chloride[45]. So, small changes in $[Cl^-]_i$ are sufficient to cause the GABA reversal potential to be either below or above the resting membrane potential and the threshold for action potential generation (part b). During development, when a $[Cl^-]_i$ higher than 25 mM is sufficient to induce the shift, higher concentrations are not required. Therefore, when the channels are activated, there is an efflux of chloride, leading to a depolarization that can generate sodium and calcium action potentials and remove the voltage-dependent magnesium block from NMDA channels.

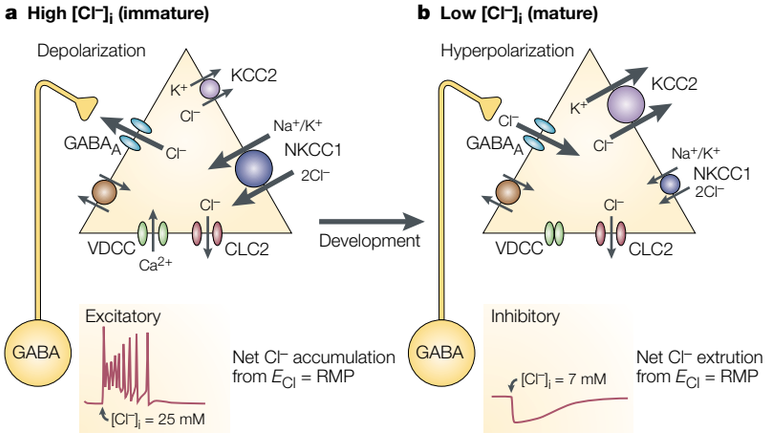


Figure 1 | **Early expression of NKCC1 and late expression of KCC2 determines developmental changes in $[Cl^-]_i$.** Schematic diagram depicting the Na^+–K^+–$2Cl^-$ co-transporter NKCC1, the K^+–Cl^- co-transporter KCC2 and voltage-gated calcium currents, as well as the gradients of chloride ions. **a** | NKCC1 expression predominates in immature neurons, in which the intracellular concentration of chloride ($[Cl^-]_i$) is relatively high. **b** | KCC2 expression predominates in mature neurons. Note that the activation of GABA (γ-aminobutyric acid) type A receptors generates an efflux of chloride and an excitation of immature neurons, and an influx of chloride and an inhibition of adult neurons. CLC2, voltage-gated chloride channel 2; E_{Cl}, chloride reversal potential; RMP, resting membrane potential (V_{rest}); VDCC, voltage-dependent calcium channel. Adapted, with permission, from REF. 42 © 1998 The American Physiological Society.

network-driven patterns (see below) — the expression of KCC2 at birth will vary even between adjacent neurons. Further studies will be required to determine the relationship between KCC2 expression and functional maturation.

Poo and colleagues used calcium imaging to measure the actions of GABA, perforated-patch recordings to measure E_{Cl}, and RNASE PROTECTION ASSAYS to estimate KCC2 activity in rodent hippocampal neurons in culture[7], and they showed a simultaneous change in all three parameters. Interestingly, blocking $GABA_A$ receptors with bicuculline and picrotoxin prevented the shift from taking place; that is, the KCC2 transporter was not expressed and GABA continued to exert a depolarizing action. By contrast, blocking glutamate receptors did not modify the outcome, and the shift took place at the right time. So, the switch seems to be mediated by the activation of GABA synapses themselves. Most intriguingly, blocking all ongoing activity by continuous applications of the sodium channel blocker tetrodotoxin did not prevent the shift from excitation to inhibition. This implies that the presence of miniature postsynaptic currents (PSCs), which are generated by the action-potential-independent quantal release of GABA, is sufficient to trigger the expression of KCC2 and a reduction in $[Cl^-]_i$. In other words, all that is needed to produce the shift is an ongoing release of GABA, even when all the network

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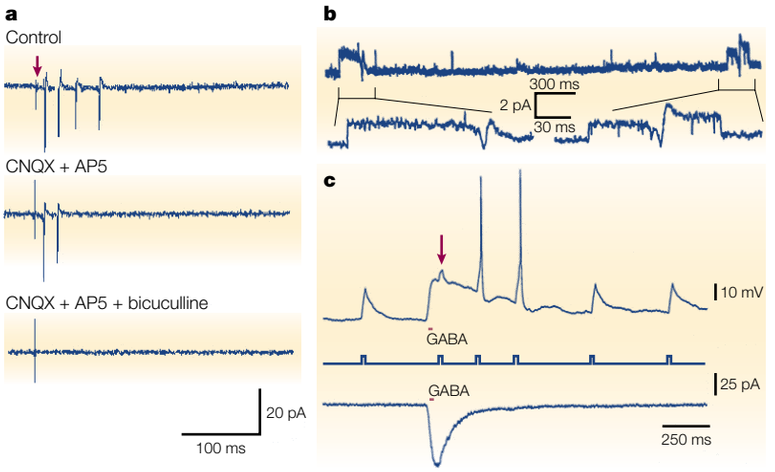

Figure 2 | **Dual excitatory/inhibitory effects of GABA in immature neurons.** **a** | Cell-attached recordings from neonatal pyramidal neurons in hippocampal slices. Electrical stimulation (red arrow) evokes four action currents. Two are blocked by the application of glutamate AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and NMDA (*N*-methyl-D-aspartate) receptor antagonists, and the remaining spikes are blocked by the GABA (γ-aminobutyric acid) type A receptor antagonist bicuculline, indicating that the synaptic release of GABA has generated action currents in this neuron[5]. AP5, D(-)-2-amino-5-phosphonovaleric acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione. **b** | Activation of a single GABA channel generates action currents in embryonic spinal cord neurons. Adapted, with permission, from REF. 20 © 1995 The Physiological Society. **c** | Shunting and excitatory actions of GABA on a hypothalamic neuron. Repeated current pulses produced a depolarization. Applications of a pulse of GABA (red bars) either reduced the amplitude of the depolarizations (red arrow) or augmented them to generate an action potential. The lower trace shows an example of the inward current generated by a pulse of GABA[53].

activity is blocked and the terminals are disconnected from their parent soma.

The observation that miniature PSCs can mediate such an important change is consistent with several recent indications that these currents are essential for the expression of receptors and the establishment of functional synapses. So, the KCC2 signal provides a "new form of feedback of $GABA_A$ receptors", in which GABA itself promotes the shift from excitation to inhibition through $GABA_A$-receptor-mediated PSCs[45]. This effect is mediated by calcium signals that ultimately lead to the expression of a transporter and a shift in the actions of GABA from excitatory to inhibitory through a reduction in $[Cl^-]_i$. However, it will be important to determine whether a similar sequence takes place *in vivo*, and whether its time course parallels the formation of the circuit and, in particular, the maturation of interneurons and pyramidal neurons. Moreover, applications of a GABA antagonist prevented the shift in the actions of GABA but not the expression of the KCC2 protein, indicating that some elements in the transition remain to be discovered.

These observations provide direct evidence that electrical activity controls the establishment of the main type of transmitter-mediated inhibition, and that GABA itself has a central role in this sequence. GABA has also been shown to positively stimulate several essential developmental functions, including neuronal migration, cell division and neuritic growth[46]. It is particularly intriguing that in spite of their central role in plasticity, NMDA (*N*-methyl-D-aspartate)-type glutamate receptors do not seem to control this central step, and they are not involved in the shift from GABA excitation to inhibition. Also, the blockade of NMDA receptors produces an extensive increase in neuritic arborization, indicating that the activation of these receptors exerts an effect that is opposite to that of GABA[47,48].

GABA excitation: an poco ma non troppo

If GABA is an excitatory transmitter, the threshold of action potential generation must be below the E_{Cl}, and the amplitude of the depolarization will depend on the net driving force for chloride ions. Cell-attached recordings have indeed shown that the activation of GABA receptors[20] and GABA synapses[3–5,10,16–18,49,50] leads to the generation of sodium action potentials (FIG. 2a). Because of the high INPUT RESISTANCE of immature neurons, the opening of single GABA channels can generate action potentials[20] (FIG. 2b). Extracellular recordings of neurons also reveal a developmental shift, such that electrical activity is increased by GABA receptor agonists in neonatal neurons and is reduced at later stages[50]. In recent studies, single NMDA or potassium channels have been recorded to determine V_{rest} in immature neurons[51]. With this approach, $[Cl^-]_i$ is not affected and important elements are not washed out from the intracellular milieu. These studies indicate that V_{rest} is similar in immature and adult neurons: about –75 mV (REF. 51, and R. Tyzio, Y.B.-A. and R. Khazipov, unpublished observations). So, in neonatal neurons, depolarizing GABA currents are an important driving force (BOX 2).

The activation of GABA receptors also generates calcium currents by directly activating voltage-dependent calcium channels[4,14,24–25,42,52,53]. The depolarization produced by the activation of GABA receptors is sufficient to remove the voltage-dependent magnesium block from NMDA channels by shifting the affinity of NMDA channels for magnesium and inducing an increase in $[Ca^{2+}]_i$ (REF. 52). So, GABA operates in synergy with NMDA channels in immature neurons[54] (FIG. 3). This feature is important, as NMDA-induced currents provide a more substantial component of the overall activity in the developing circuit than in the adult[55]. This is due to the differential expression of NMDA receptor subunits in immature and adult neurons, leading to currents with a longer duration[56]. Furthermore, GABA-induced currents have a relatively slow time course of desensitization in immature neurons compared with adult neurons[57]. As GABA-induced depolarizing currents constitute the principal, if not the sole, source of depolarization at this stage, their synergistic action on NMDA receptors is a key factor that will enhance neuronal activity in the network and facilitate the generation of synchronized patterns of activity that are a hallmark of developing networks (see below).

However, things are not quite as simple as is implied above. Although synaptic currents with a reversal potential that is negative to the action potential threshold are always inhibitory, depolarizing PSCs are not necessarily excitatory. GABA-induced depolarizing currents can inhibit neuronal activity if they concomitantly decrease the effectiveness of glutamatergic excitatory postsynaptic

RNASE PROTECTION ASSAY
A technique that is used to measure the quantity of messenger RNA that corresponds to a given gene in an RNA sample. A labelled RNA probe that is complementary to the relevant sequence is hybridized with the RNA sample; any RNA that does not hybridize with the probe is then digested away using ribonuclease. The undigested mRNA can then be quantified on an electrophoresis gel.

INPUT RESISTANCE
An estimate of the cell Ohmic resistance ($V = IR$).

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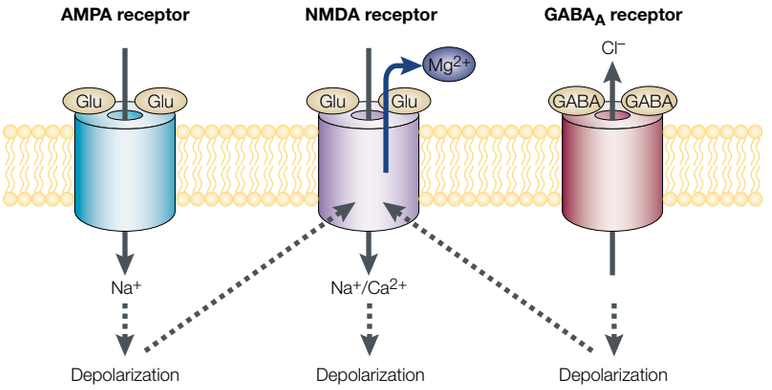

Figure 3 | **Synergistic actions of GABA, NMDA and AMPA receptors in developing neurons.** The activation of GABA (γ-aminobutyric acid) type A receptors in hippocampal neurons recorded in neonatal slices generated a depolarization that was sufficient to remove the voltage-dependent magnesium block of NMDA (*N*-methyl-D-aspartate) receptors. Therefore, in neonatal neurons, GABA can activate NMDA receptors and increase the intracellular concentration of calcium. The data were obtained by both cell-attached determination of the affinity of magnesium for NMDA receptors and by confocal microscope analysis of the increase in intracellular calcium that was obtained with GABA receptor and NMDA receptor agonists. In immature cells, as in mature neurons, the depolarization produced by the activation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors also involves the magnesium block of NMDA channels. Glu, glutamate.

currents (EPSCs), without themselves generating an excitation by clamping the membrane potential to E_{Cl} (REF. 58). The depolarization that is produced by GABA can also lead to the inactivation of sodium conductance through a shunting mechanism that raises the spike threshold[50,59,60,61,62]. So, GABA can exert dual excitatory/inhibitory actions in immature neurons. In keeping with this, the $GABA_A$ receptor agonist isoguvacine and the ALLOSTERIC modulator diazepam induce biphasic changes in the electrical activity of neonatal neurons[50]. Similarly, applications of glutamate generate an action potential at certain phases of the GABA-mediated PSCs and block it at others[53] (FIG. 2c).

So, GABA synapses initially excite their targets, but they can also shunt and reduce the excitation by glutamatergic EPSCs, the net effect depending on the levels of ongoing activity, the density of GABA receptors and the excitability of the network. The dependence of the shunting actions of GABA on the density of glutamatergic synapses might provide a developmentally regulated loop that provides a progressive reduction in the excitatory actions of GABA as a function of the degree of maturation of the neuron. The initial dual excitatory and shunting action of GABA is a general rule that is respected in many structures and species.

The GABA–glutamate sequence

In their initial study, Y.B.-A. *et al.*[1] reported that the application of a $GABA_A$ receptor antagonist blocked ongoing activity in immature hippocampal slices, in contrast to antagonists of ionotropic glutamate receptors, which often had no effect. This indicated that GABA synapses are formed before glutamatergic ones. Similar effects have now been observed in a wide range of structures in the rodent brain (see above). However, the interpretation of observations that rely on bath-applied drugs is particularly difficult in the neonatal brain, as developing neurons, even from the same population, are very heterogeneous — some might only just have divided, whereas others already have functional synapses (see below).

To show directly that functional synapses mature sequentially, it is essential to examine in parallel the synaptic currents that are generated in a neuron and to determine its developmental stage by measuring the volume of its arborization. In a recent study[63], a large sample of CA1 hippocampal pyramidal neurons was recorded at birth (P0), their PSCs identified and the neurons reconstructed *post hoc* to identify their degree of maturation (FIG. 4a). We found three populations of pyramidal neurons at birth. First, 'silent' neurons, which show no spontaneous or evoked PSCs, even in the presence of toxins that elicit transmitter release. These neurons have a small soma and an axon, but no apical dendrites. However, they have functional $GABA_A$ and glutamate receptors. Second, 'GABA-only neurons', which show GABA-mediated but not glutamatergic PSCs. These are more differentiated than the first group, with a bigger soma and a small apical (but no basal) dendrite. Third, 'GABA-and-glutamate neurons', which show both GABA- and glutamate-mediated PSCs. These are more highly developed than the GABA-only group, and they have an apical dendrite that reaches the distal part of the molecular layer, and a basal dendrite. From the capacitance of the neuron, which correlates with the degree of arborization, it is possible to predict whether the neuron will be silent, will have only GABA synapses, or will have glutamate EPSCs as well. So, axons develop before apical dendrites, and basal dendrites are formed last. The formation of GABA synapses requires that the principal neurons have an apical dendrite. This sequence probably reflects the time that has elapsed since these neurons became postmitotic. At birth, the youngest neurons are still silent, whereas cells that became postmitotic at an earlier stage already have GABA and glutamate synapses.

Similar observations have now been made in primate neurons *in utero*[64] (FIGS 4c,5). Hippocampal neurons were recorded from macaque monkey embryos (embryonic day (E) 85 to E154; birth date is E165). At E85, CA1 pyramidal neurons are silent, and at E105 they have GABA but not glutamate synapses. At the latter but not the former stage, they also have dendrites that penetrate into the STRATUM RADIATUM. Neurons that have both GABA and glutamate synapses also have more elaborate dendrites. Moreover, there is a parallel formation of spines. Axons are formed before dendrites, and at E120, the peak dendritic growth rate is attained (400 μm day^{-1}). GABA synapses are formed first, and the expression of glutamatergic EPSCs coincides with the formation of spines, starting around E105 and establishing a peak rate of 200 synapses per day on a pyramidal cell at E125. Around birth, it is estimated that pyramidal neurons have as many as 7,000 spines. Initially, the $GABA_A$ receptor antagonist bicuculline blocks ongoing activity (in keeping with an excitatory action of GABA), and from

ALLOSTERIC
A term used to describe proteins that have two or more binding sites, in which the occupancy of each site affects the affinities of the others.

STRATUM RADIATUM
A region adjacent to the pyramidal cell layer of hippocampal area CA1. It contains few cell bodies, but is rich in dendrites that project from the pyramidal cells.

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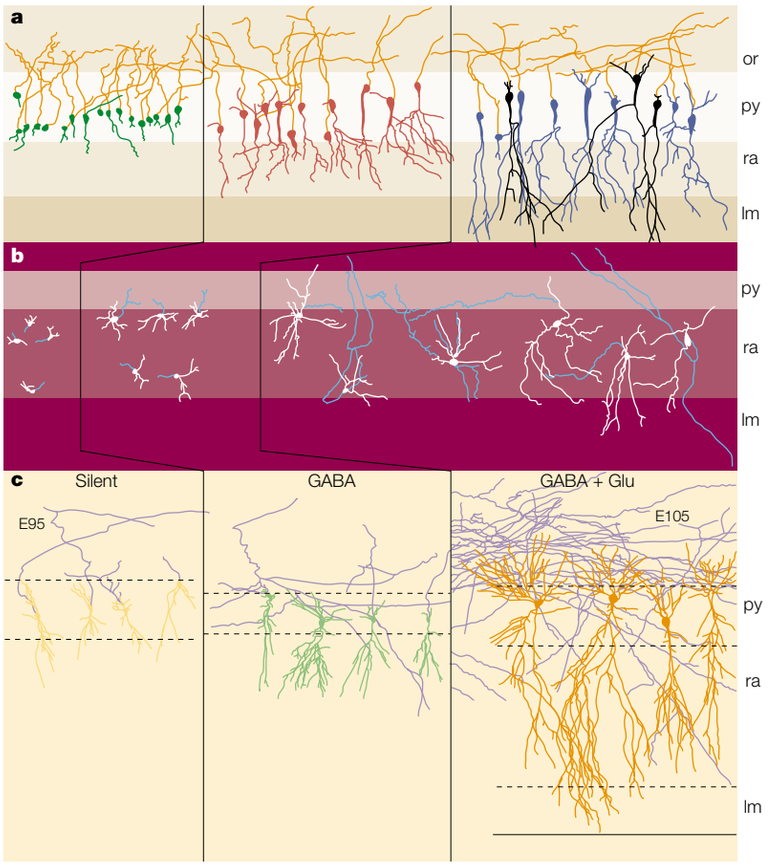

Figure 4 | **Sequential formation of GABA and glutamate synapses in the developing primate and rodent hippocampus. a**,**b** | Rat pyramidal neurons (**a**) and interneurons (**b**) were patch-clamp recorded from postnatal-day-0 hippocampal slices, the spontaneous and evoked excitatory postsynaptic currents (PSCs) determined, and the neurons filled with dyes for *post hoc* morphological reconstruction. Note that 80% of pyramidal neurons are silent with no functional PSCs, 10% have only GABA (γ-aminobutyric acid)-mediated PSCs, and the remaining 10% have both GABA and glutamate (Glu) PSCs. This correlates with the degree of dendritic and axonal arborization. Interneurons follow a similar developmental gradient, with GABA synapses being established before glutamate synapses, but at an earlier stage. So, at birth, only 3% of the interneurons are silent, and most have both GABA and glutamate PSCs. Part **a** adapted, with permission, from REF. 63 © 1999 Society for Neuroscience; part **b** adapted, with permission, from REF. 71 © 2002 Federation of European Neuroscience Societies. **c** | Similar results were obtained in the primate hippocampus *in utero*. At the mid-embryonic stage, most CA1 pyramidal neurons are silent. Note the difference in the development of pyramidal neurons with no functional synapses, with GABA-only synapses, and with GABA and glutamate synapses. Adapted, with permission, from REF. 64 © 2001 Society for Neuroscience. E, embryonic day; lm, stratum lacunosum moleculare; or, stratum oriens; py, stratum pyramidale; ra, stratum radiatum.

E105 onwards, seizures are generated with an increasing severity that parallels the formation of glutamate synapses. So, within a few weeks, pyramidal neurons shift from a largely silent structure to one that can generate integrative signals shortly before birth. The general template is similar to that of the rodent hippocampus, although it takes shape at an earlier stage. This provides a unique opportunity to compare precisely the key elements of the formation of a network in the two species, although several quantitative elements are now available for the primate but not the rodent brain. So, the GABA–glutamate sequence seems to have been retained throughout evolution, and its phase shift might provide an accurate estimate of the equivalence of structures in different species during development.

If GABA synapses are formed first, then synaptic markers should enable us to determine their exact location. Immunocytochemical observations using anti-synaptophysin or anti-GAD antibodies indicate that the first synapses of the principal neurons in the hippocampus are formed on the apical dendrites[65,66]. At an early developmental stage, there are no synapses on the somata of the principal cells. As glutamate fibres are already present *in utero*[67–69], the delayed formation of glutamatergic synapses is not determined by the late arrival of the inputs, but by the maturity of the postsynaptic target. So, the conditions for the formation of GABA and glutamate synapses differ: GABA synapses are formed on contact between the axons of GABA neurons and the dendrites of pyramidal neurons, whereas glutamatergic synapses require a more developed target. Studies using electron microscopy will be required to determine the density of GABA terminals on the soma and dendrites of developing neurons.

If GABA synapses become functional before glutamatergic ones, then GABA-synthesizing interneurons would be expected to mature at an early stage. Indeed, interneurons become postmitotic at an earlier stage than pyramidal neurons, originate from a different source and follow a different migration pathway (tangential, as opposed to the radial migration of principal neurons[70]). In a recent study, Gozlan and collaborators[71] carried out a systematic morphological and functional study of embryonic and postnatal hippocampal interneurons, and they reported a similar GABA–glutamate sequence, albeit at an earlier stage (FIG. 4b). At birth, only 5% of interneurons are silent, in contrast to 80% of the principal neurons[71]. Moreover, most interneurons (80%) have both GABA and glutamate synapses, in contrast to 10% of pyramidal neurons. Among E18–E20 neurons, virtually all pyramidal neurons are silent, whereas most interneurons already have GABA and glutamate synapses. So, all the activity in the rodent hippocampus *in utero* is provided by GABA synapses that are formed between interneurons, and even glutamatergic synapses are formed on interneurons before they are established on the principal cells. Therefore, interneurons are both the source and the targets of the first synapses to be established in the hippocampus, and probably also in other brain structures.

Interestingly, even within the population of interneurons, those that innervate the apical dendrites of pyramidal neurons mature before those that innervate the cell body, implying a dendrite–soma gradient for GABA synapse formation[71]. A recent study also showed that GABA transporters become operative after glutamate transporters, indicating that, in immature networks, GABA will not be efficiently removed from the extracellular space, allowing a more efficient action on target neurons (M. Demarque *et al.*, unpublished observations).

In summary, the sequential expression of GABA and glutamate is not restricted to one type of neuron, and the events that condition this general sequence are programmed accordingly. Clearly, the earlier formation of

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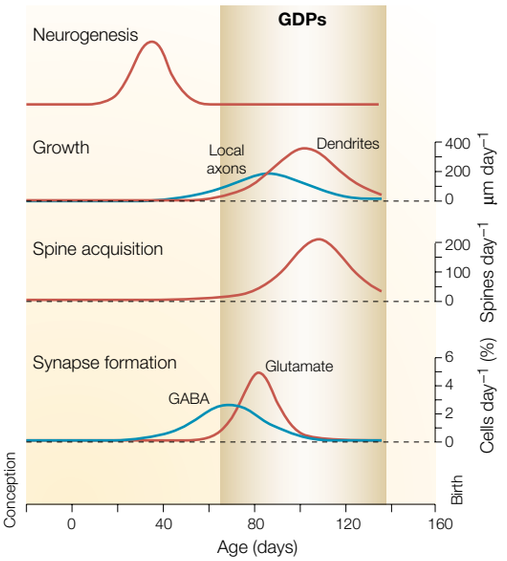

Figure 5 | **Quantitative description of the sequence of events during the maturation of GABA and glutamate synapses in primate neurons *in utero*.** The embryos were removed by caesarean surgery from pregnant macaque females (embryonic day (E) 85 to E154; birth date is E165), and the hippocampi dissected and slices prepared. The physiological properties were determined first, and neurons reconstructed *post hoc*. The curves depict the speed of the various parameters (d*v*/d*t*). Neurogenesis takes place before E80 (data for pyramidal neurons and interneurons are shown, granule cells are not included). Note that axons develop before dendrites, GABA (γ-aminobutyric acid) synapses before glutamate synapses, and that giant depolarizing potentials (GDPs) provide all the activity during most of the embryonic phase. Shortly before birth, GDPs disappear and are replaced by more diversified patterns of activity. Adapted, with permission, from REF. 64 © 2001 Society for Neuroscience.

GABA synapses seems to be a general rule, and for a short period of time, which depends on both the structure and the species studied, GABA synapses offer the only transmitter-gated communication between neurons.

Early-formed GABA synapses are plastic

As noted above, a central element of the shift from excitatory to inhibitory actions of GABA is modulated by activity-dependent mechanisms. Recent studies indicate that other events that are related to this shift are also activity dependent. Gubellini *et al.*[72] recorded from silent neurons in neonatal hippocampal slices; these cells express receptors for both GABA and glutamate, but have no functional synapses, so no PSCs are observed. These authors showed that repetitive intracellular depolarizing current pulses led to the expression of the first functional GABA synapses. A silent neuron became active 30 min after a series of pulses (FIG. 6a). The neuron showed spontaneous and evoked GABA-mediated PSCs, but no glutamatergic EPSCs. So, a rise in $[Ca^{2+}]_i$ that is produced by intracellular postsynaptic stimulation is sufficient to activate the machinery that is required for the operation of GABA synapses.

Another factor that might affect the development of neuronal circuits is the long-term plasticity of early GABA synapses. If GABA is an excitatory transmitter that interacts positively with NMDA receptors and calcium channels, the activation of GABA receptors might cause long-lasting alterations in synaptic transmission, as has been shown extensively for glutamatergic synapses. Studies of GABA synaptic plasticity during development have provided strong evidence for changes in synaptic efficacy. The activation of NMDA receptors by GABA-induced depolarization generates a long-term depression of GABA-mediated PSCs, whereas the activation of calcium channels by GABA-mediated PSCs leads to the long-term potentiation of depolarizing GABA-mediated potentials[73,74] (FIG. 6b). These alterations in synaptic efficacy are mediated by a persistent change in the release of GABA. So, depending on the source of the rise in $[Ca^{2+}]_i$, increasing the activity can either enhance or depress the release of GABA. These alterations in synaptic efficacy are observed only when the activation of GABA receptors generates a depolarization, and they are not observed in more adult neurons when the chloride gradient is outward. So, GABA excites immature neurons, and this excitation leads to long-term alterations in synaptic efficacy, much like the long-term changes in synaptic strength that are mediated by excitatory glutamate receptors. As the network-driven activity of immature neurons can trigger synaptic plasticity, activity might modulate the efficacy of the principal transmitter during early development. The stimulation that is required to generate these forms of plasticity is provided by the largely GABA-mediated GDPs, which contribute all the activity at the initial stages of development.

GDPs: a signature of developing networks

A unique pattern formed by GDPs seems to dominate ongoing neuronal activity at early developmental stages. Initially described in the hippocampus through intracellular recordings[1], GDPs are long-lasting (~300 ms), recurrent (0.1 Hz) depolarizing potentials that provide most of the activity in the rodent hippocampus during the first two weeks of postnatal life. They have been observed in cultures and in slices in all the populations of hippocampal neurons, including CA3 and CA1 pyramidal neurons, granule cells and interneurons[5–6,9,52,64,75–78]. They have also been observed *in vivo*[75], and in the intact hippocampus *in vitro*, where they propagate from the rostral to the caudal pole along developmental gradients, and from one side of the hippocampus to the other by the time of birth[77]. Although they are referred to by different names, GDPs and similar network-driven patterns are present in all brain structures, including the rabbit and primate hippocampus, the rodent cerebellum and neocortex[79,80], and the rodent, chick and *Xenopus* spinal cord[81–85]. Retinal waves, which are present in rats, turtles and chicks[86], also have similar features to GDPs, although their generating mechanisms might differ. So, GDPs constitute another basic property of developing networks that "play a similar melody"[54].

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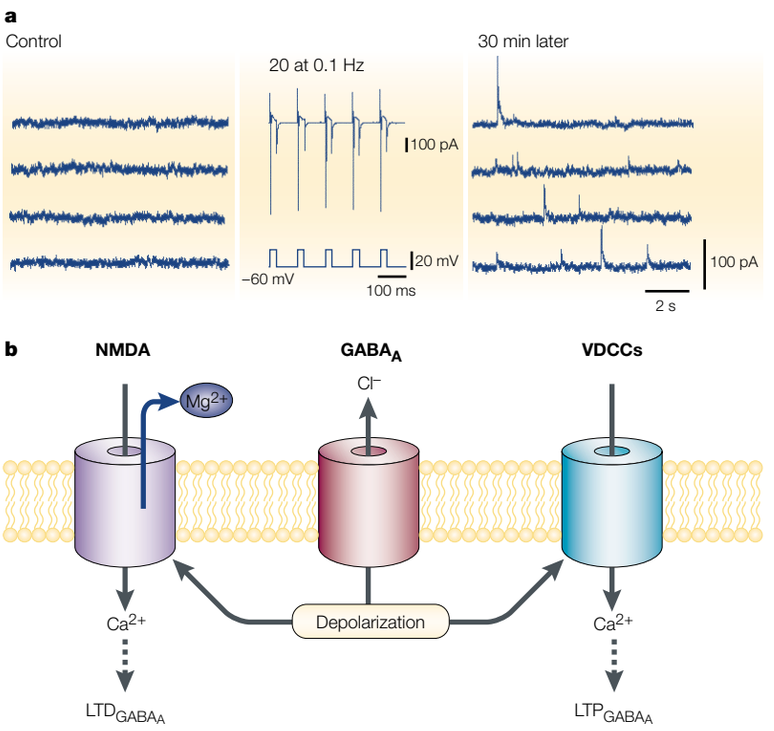

Figure 6 | **Activity-dependent mechanisms modulate early GABA synapses.** **a** | Establishment of the first GABA (γ-aminobutyric acid) synapses is activity dependent. A silent neuron was recorded in a neonatal slice. Repeated current pulses, which generate calcium currents, induced the expression of spontaneous GABA-mediated postsynaptic currents (PSCs) within 30 min. Adapted, with permission, from REF. 72 © 2001 Federation of European Neuroscience Societies. **b** | Long-term potentiation (LTP) and long-term depression (LTD) of GABA synapses in developing hippocampal neurons. Brief tetani trigger long-term alterations in the synaptic efficacy of $GABA_A$-receptor-mediated PSCs. When NMDA (*N*-methyl-D-aspartate) receptors are activated, LTD of GABA-mediated PSCs is observed; when these are blocked and voltage-dependent calcium channels (VDCCs) are activated, robust LTP results. Both are generated by a rise in the concentration of intracellular calcium, probably associated with different second-messenger cascades. The maintenance of depression or potentiation is likely to be mediated by presynaptic mechanisms; that is, a persistent alteration in transmitter release[73,74].

DECAY TIME CONSTANT
The initial decay of an excitatory postsynaptic potential (EPSP) can usually be fit by a single exponential function. The time constant derived from this fit describes how quickly an EPSP decays.

THETA ACTIVITY
Rhythmic neural activity with a frequency of 4–8 Hz.

GAMMA ACTIVITY
Rhythmic neural activity with a frequency of 25–70 Hz.

CONDITIONAL POINT MUTATION
A mutation that is expressed at a given developmental stage and/or in a given brain region or neuronal population.

The mechanisms that underlie the generation of GDPs have also been extensively studied, and the excitatory actions of GABA have a central role in their generation. In fact, GDPs are present as long as GABA exerts excitatory actions on a proportion of neurons in the circuit, and pure GABA GDPs have been recorded in neurons that have only GABA synapses[63]. However, glutamatergic synapses also contribute to their generation in neurons that have both GABA and glutamate synapses[6]. This was shown by experiments in which the intracellular poisoning of GABA receptors revealed underlying glutamatergic EPSCs that were mediated primarily by NMDA receptors[6]. As immature NMDA receptor subunits have a long DECAY TIME CONSTANT[55], their activation will generate prolonged synaptic currents. GDPs are associated with large calcium oscillations (FIG. 7), due to the activation of voltage-gated calcium currents and the synergistic action of GABA and NMDA receptors. The duration of the GDPs is also controlled by pre-synaptic G-protein-coupled $GABA_B$ inhibition, which operates at an early developmental stage[87–89]. This ensemble of mechanisms allows large calcium oscillations to be generated in developing networks.

GDPs also correlate with certain behaviours, as shown by a recent *in vivo* study in baby rats using patch-clamp and field recordings from the hippocampus[75]. In these animals, bursts occurred mainly during periods of immobility, sleep and feeding. The immature hippocampus showed only this single primitive pattern of bursts generated by GDPs, which propagated throughout the entire limbic system[77]. The wide repertoire of behaviourally relevant patterns observed in adults, including THETA ACTIVITY and GAMMA ACTIVITY, was not present before the end of the second postnatal week.

In the primate hippocampus, GDPs dominate until a few days before birth[64]. At this stage, GDPs disappear and the network is sufficiently developed to generate more elaborate patterns of activity (FIG. 5). Although the presence of GDPs in the human brain remains to be shown, the *tracé alternant* that was described four decades ago in preterm babies (around 25 weeks of age) is strikingly similar to GDPs[90,91]. So, it is likely that this primitive pattern, which has a poor informational content, provides a general growth signal by means of large calcium oscillations, and is replaced by more elaborate patterns when the circuit has reached a sufficient density of functional connections and the animal can achieve more integrative functions.

GDPs are key players in the electrical modulation of various functions that are essential for the developing network, including neuronal migration and growth, synapse formation and plasticity of developing GABA synapses[46]. GABA actions are mediated by a positive loop with brain-derived neurotrophic factor (BDNF)[11]. The excitation by GABA activates calcium currents in young (but not adult) hippocampal cultures, leading to the activation of c-*fos*, and BDNF mRNA and KCC2 protein expression, which leads to the molecular shift in GABA actions (S. Aguado *et al.*, unpublished observations). So, a scenario emerges in which GABA might promote growth and the construction of the network. By contrast, the activation of glutamate (particularly NMDA) receptors seems to have the opposite effect; that is, an arrest of growth[48] and a reduction in the number of receptors clusters[92] that provide a negative loop by which the target neuron might control its inputs.

So, these studies indicate that the activation of GABA synapses positively modulates the formation of the network. It was hoped that this could be confirmed by knocking out the genes for enzymes that control GABA functions. However, these experiments have provided limited information, most probably because of the wide range of functions in which GABA is involved. Mice that are completely deficient in the neuronal KCC2 co-transporter die at birth owing to respiratory failure, and have excitatory GABA and glycine responses[93]. Partial mutations with 5% of the transporter still present lead to early death associated with seizures, most probably due to failure of inhibition[94]. It might be necessary to generate CONDITIONAL POINT MUTATIONS that are expressed only at specific developmental stages to exclude adaptive alterations that take place in knockout mice. An alternative

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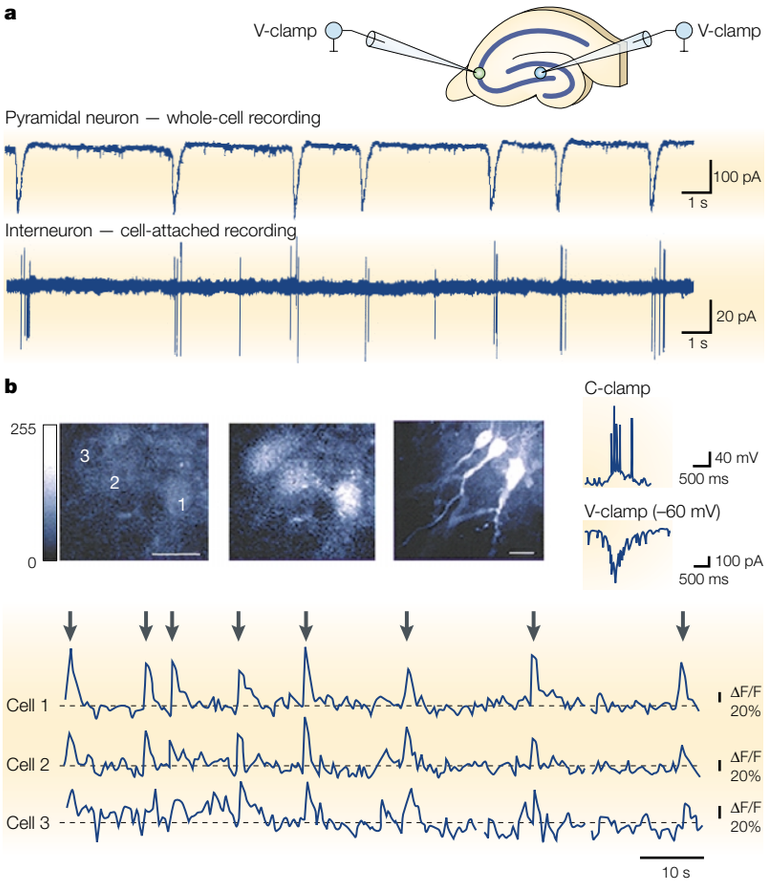

Figure 7 | **Giant depolarizing potentials in developing hippocampal neurons. a** | Giant depolarizing potentials (GDPs) recorded concomitantly in a neonatal slice from a pyramidal neuron and an interneuron. With the exception of a few spikes recorded from the interneuron, all the discharge occurs synchronously in these cells. V-clamp, voltage clamp. Adapted, with permission, from REF. 4 © 1997 The Physiological Society. **b** | Confocal microscope calcium imaging of pyramidal neurons in a neonatal hippocampal slice. Three neurons were filled with the calcium indicator Fluo3-AM by focal applications, and a fourth was patch-clamp recorded to visualize calcium changes. Note the recurrent, synchronized increase in the concentration of intracellular calcium ($[Ca^{2+}]_i$) and the parallel GDPs that were recorded from the fourth neuron. C-clamp, current clamp. Adapted, with permission, from REF. 5 © 1997 Elsevier Science.

approach might be to use mice that are deficient in factor(s) that are essential for the correct migration of interneurons[95,96], resulting in brain structures that are largely devoid of GABA synapses[97,98].

What are the advantages of this model?

As stressed above, constructing the brain with either inhibitory GABA or excitatory glutamate dominating at an early stage raises serious problems. An alternative to the developmental sequence that I have discussed would be a parallel formation of GABA and glutamate synapses, with a predetermined sensor that ensures a strict equilibrium at all developmental stages. However, this solution has several complicating factors. These include the need for synchronized migration, differentiation and growth of the various types of glutamatergic and GABA interneurons (there are over 40 types in the hippocampus[99]); the need for simultaneous expression of the cohort of neuronal and glial transporters, uptake and exchangers that control the concentration of glutamate and GABA; and several forms of activity-dependent synaptic plasticity that are generated by electrical activity or by hormonal and environmental factors. This solution would clearly require a predetermined sequence with no possibility of activity-dependent modulations of the developing network.

By contrast, the excitatory and shunting actions of GABA that I have described offer several advantages to the developing network. The proximity of E_{Cl} to the resting potential, even when it is depolarizing and excitatory, precludes possible excitotoxic actions, in contrast to glutamate synapses. In addition, even when GABA synapses are overactive, the shunting action prevents the generation of seizures in developing neurons. This problem is particularly acute in immature neurons, in which the high input resistance facilitates the generation of large currents. In addition, the relatively slow kinetics of GABA-mediated PSCs (at least three- to fivefold lower than that of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-receptor-mediated PSCs) will increase the probability that low-frequency consecutive events will summate in neurons that have few synapses. With few synapses that make use of AMPA receptors, this probability is small. The summation of consecutive GABA-induced currents, and the slow currents generated by the activation of NMDA receptor subunits that have slow kinetics, are key factors in the generation of the unique primitive synchronized patterns that are a basic feature of developing networks. Another point to consider is that, in GABA-only neurons, the shunting action of GABA will be limited because of the lack of glutamate synapses, and it is likely that the generation of action potentials by GABA currents will participate in the maturation of the neuron (see above). Further studies will determine the relationship between the activity of GABA synapses and the functionality of voltage-gated channels[18].

From an energetic point of view, it is less expensive for a growing process to pump small amounts of chloride than to extrude large amounts of it (to allow an early hyperpolarizing response to GABA) and to simultaneously export a large number of sodium ions (to allow a depolarizing response to glutamate). Although there are no quantitative data available, it is likely that immature neurons do not have their full complement of anionic proteins, hence the osmotic need for high levels of intracellular anions and notably high $[Cl^-]_i$. So, a rapidly growing process might express few, if any, protein pumps on its membrane if the $[Cl^-]_i$ is as high as the extracellular chloride concentration. Over time, growing processes will have the energy and the capacity to synthesize the proteins needed for signal transduction, so the sodium and chloride levels can be lowered for GABA and glutamate signalling. So far, a systematic survey of alterations in $[Cl^-]_i$ during development in peripheral (neuronal and non-neuronal) structures has not been carried out. By and large, peripheral neurons, such as those in ganglia and the myenteric system, have a higher concentration of chloride and chloride channels,

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and co-transporters are present in cultured heart cells, in which E_{Cl} is depolarizing[100–102].

A fascinating possibility is that the developmental sequence for chloride is valid only in neurons, whereas other cell types of the developing muscular, cardiac or digestive systems maintain a high $[Cl^-]_i$. Viewed from this perspective, the developing nervous system might have taken advantage of a general property (higher $[Cl^-]_i$ in developing cells), which might serve a purpose other than GABA excitation, perhaps being an evolutionarily conserved feature[103]. Interestingly, rat lactotrophs have depolarizing GABA-operated chloride channels[104], and inhibitors of oxidative phosphorylation affect $[Cl^-]_i$, indicating that it is regulated by mitochondria and is responsive to cell metabolism[105]. It should be stressed that immature neurons are highly resistant to anoxia[106] and that variations in $[Cl^-]_i$ lead to altered cell functions. Clearly, it will be important to determine the mechanisms that are responsible for the accumulation of $[Cl^-]_i$ in immature neurons, and its implications for the metabolism and general operation of the developing neuron.

Concluding remarks and remaining questions

This story leaves us with more questions than answers. First, these studies reflect the extent to which development is a dynamic process in which heterogeneity prevails. Adjacent pyramidal neurons can be very different at birth — some will be completely silent, whereas others will have a full repertoire of synapses and an inhibitory GABA-mediated system with an efficient KCC2 exporter and low $[Cl^-]_i$ (REFS 63,64,71) (FIG. 4). This heterogeneity is probably due to the time window during which neurons divide — in the rat, hippocampal pyramidal neurons can become postmitotic from 24 hours to 5 days before birth. These differences are even more important in species that have a more extended developmental period; for example, in primates, an interval of a few weeks separates the earliest- and latest-formed pyramidal neurons. As a consequence, physiological and pathological changes in neuronal activity will exert different effects on developing neurons, depending on their developmental stage. So, the effects of a given procedure on neuronal development must be examined in relation to the age of the neuron, not the embryonic or postnatal age.

Most importantly, the various steps in the sequence — that is, the shift from depolarization to hyperpolarization, the expression of functional glutamatergic synapses and the growth of processes and the clustering of receptors — are modulated by electrical activity and by the environment in general. So, activity-dependent mechanisms will have direct access to a crucial stage in the development of the network; that is, the shift from immature cells with few synaptic communications to a network in which the balance between excitation and inhibition allows the generation of a rich repertoire of behaviourally relevant patterns. A recent study, in which hippocampal neurons were patch-clamp recorded *in vivo* in neonatal rats, indicated that theta, gamma and other hippocampal patterns of network activity are first observed at the end of the second postnatal week[75]. It has been suggested that GDPs, which provide most of the initial activity, are a primitive signal with little informational content that propagates to all brain structures before the formation of functional entities and the generation of less stereotyped and behaviourally more relevant patterns[107]. Its main function is to increase $[Ca^{2+}]_i$. So, this shift might correspond to the stage at which functional units and circuits become able to elaborate coordinated actions, and when integrative functions become possible.

It is important to note that GABA interneurons, which are extremely heterogeneous[99,108], control the generation of behaviourally relevant network oscillations and patterns of activity in adults by means of multiple inhibitory modes[109]. They have also developed unique patterns of migration and routes of regulation [69–71,95–98], so they control the formation of a network right from the beginning. Brain assembly, including the formation of layered structures and fibre pathways, does not require transmitter release, and depends on a genetically determined programme[110]. However, the subsequent steps, and in particular, the formation of excitatory GABA synapses and the GABA–glutamate developmental sequence, might constitute a fundamental step in the construction of a functional cortical network and its reinforcement by neuronal activity. It is therefore reasonable to suggest that the shift of GABA actions is a developmentally regulated function that signals the shift from a genetically determined programme to one that takes neuronal and environmental factors into account. The proposed scenario is validated, at least in part, by the observation that the three features that I have discussed seem to have been conserved throughout evolution. Any exceptions to these rules would provide a better understanding of the mechanisms that are used to avoid the toxic effects of a mismatch between GABA and glutamate. These issues are of the utmost importance in the nature–nurture debate, and will no doubt facilitate the convergence of various disciplines to the benefit of developmental neurobiology.

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