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Trophic actions of GABA on neuronal development

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During brain development, transmitter-gated receptors are operative before synapse formation, suggesting that their action is not restricted to synaptic transmission. GABA, which is the principal excitatory transmitter in the developing brain, acts as an epigenetic factor to control processes including cell proliferation, neuroblast migration and dendritic maturation. These effects appear to be mediated through a paracrine, diffuse, non-synaptic mode of action that precedes the more focused, rapid mode of operation characteristic of synaptic connections. This sequential operation implies that GABA is used as an informative agent but in a unique context at an early developmental stage. This sequence also implies that by altering these effects, drugs acting on the GABA system could be pathogenic during pregnancy.

Introduction

Neurotransmitters have central roles in synaptic communication and convey most of the information required for operation of the brain and its networks. However, as is often the case in nature, this device is used more than once. Thus, it is now clear that GABA and glutamate operate before synapse formation (Box 1) and that, in addition to their roles in synapse communication, they have a trophic role in neuronal maturation. Recent studies also suggest that GABA is the first neurotransmitter to become functional in developing networks and provides most of the initial excitatory drive. GABA-mediated mechanisms thus have a central role both in early stages, when networks are non-existent and neurons are an ensemble of immature cells that have little communication, and later, when GABAergic synapses operate and the emerging network generates a coherent pattern of activity. In this respect, GABA provides an excellent example of the multiple forms and actions that a molecule can exert at different developmental stages. This review will examine the roles of GABA, particularly in relation to proliferation, neuronal migration, synapse formation and activity-dependent mechanisms that are essential for network construction.

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When GABA modulates progenitor proliferation and survival

In several preparations, GABA agonists exert important but contrasting effects on cell proliferation depending on the type of precursor investigated and the type of animal assayed (e.g. rats versus mice). Thus, GABA inhibits cell-cycle progression of precursors in neurospheres and organotypic striatal slices [1], shortens the cell cycle in cortical slices [2] and decreases DNA synthesis and the number of cells that incorporate bromodeoxyuridine (BrdU) in acute slices [3]. By contrast, GABA increases the proliferation of cerebellar granule cell precursors [4]. For these actions, GABA functions in association with various trophic factors. Thus, GABA or the GABA receptor agonist muscimol inhibits the proliferative effects of basic fibroblast growth factor (bFGF) on cortical progenitors, leading to an increased number of differentiated neurons [5]. Trophic factors, including epidermal growth factor (EGF) and FGF2, decrease GABA production [1] and $GABA_A$ receptor expression [5], providing a feedback signal to control cell division (Box 2).

Does GABA modulate cell death or cell survival? In general, even when excitatory, GABA does not promote cell death or cell survival of neuronal cultures (e.g. Ref. [4]). However, high doses of muscimol (10 mM) induce death of GABAergic neurons in 3D cultures [6]. Furthermore, treatment of pregnant rats with bicuculline during the post-proliferative phase [from embryonic day (E)18 to E21] reduces significantly the number of parvalbumin-immunoreactive neurons in the neostriatum [7]. However, these effects are difficult to interpret considering the complex actions of seizures generated in pregnant rats by bicuculline. This is also exemplified by the complex literature concerning the effects of antiepileptic drugs, many of which act on GABA receptors. These can cause apoptotic neurodegeneration in the developing rat brain [8] but whether this is mediated by concentrations that are obtained in humans is controversial.

When GABA modulates neuronal migration

Once immature neuroblasts generated in the germinal layers become postmitotic, they start to migrate into the cerebral tissue to reach their targets. In the cerebral cortex there are two different modes of migration – a radial mode for the principal pyramidal cells, and a tangential mode for the interneurons [9]. GABA, acting on several receptor subtypes (of both $GABA_A$ and $GABA_B$

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Box 1. GABA as a diffusible factor that acts before synapse formation

Cell precursors and maturing neurons express functional GABA receptors long before formation of functional synaptic contacts [40,44,45] (Figure Ia–c). These receptors might act as sensors to detect extracellular GABA, which is released from pioneer and migrating neurons [43], growth cones [46,47] and eventually glia [48]. In maturing brain, GABA and glutamate are released via soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent and SNARE-independent mechanisms (i.e. vesicular and non-vesicular modes, respectively) [45]. The latter could involve exchangers, gap junction hemichannels or volume-sensitive Cl^- channels [45]. Diffusion of GABA in the extracellular space is facilitated by delayed maturation of GABA transporters that, although present by the end of gestation [49,50], remain ineffective in the brains of perinatal rats and mice [45]. In maturing brain, GABA combined with glutamate activates very immature neurons by acting mainly on $GABA_A$ and NMDA receptors. Thus, local stimulation close to the recorded cells induces a current that is characterized by long kinetics. This type of response was observed at prenatal stages and in the first postnatal week but not in more mature rats (Figure Id). This response – referred to as 'early slow current' – is mediated by the activation of $GABA_A$, and to a lesser extent NMDA, receptors. Spontaneous events of slow kinetics were recorded even in immature munc 18-1 knockout mice [45], in which vesicular release of transmitters has been eliminated [51]. Tonic SNARE-independent release of GABA also occurs in immature neurons, because $GABA_A$ receptor antagonists generated a current (Figure Ic). Therefore, GABA is a paracrine factor during maturation that contributes to stabilize the resting membrane potential of immature neurons.

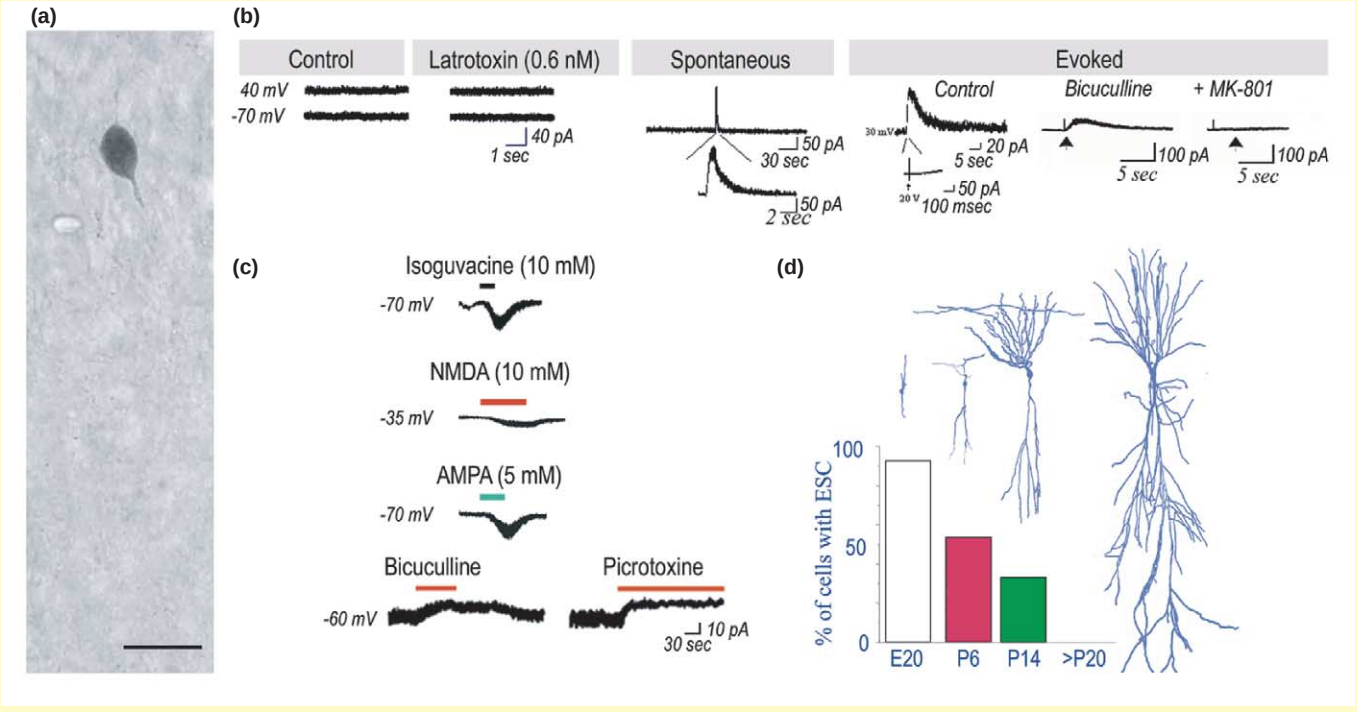

Figure I. GABA as a paracrine factor [45]. **(a,b)** Patch clamp recordings (b) of an immature pyramidal neuron injected with biocytine (a). This cell did not have functional synaptic contacts, as revealed by bath-application of latrotoxin, which stimulates vesicular exocytosis. However the cell displays spontaneous events of long kinetics. A single stimulation of the stratum radiatum evokes in this cell a current of long kinetics that is sensitive to bicuculline and the NMDA receptor antagonist MK-801, thus revealing a paracrine activity of GABA and glutamate although the activation of $GABA_A$ and NMDA receptors; AMPA receptors did not contribute to this slow-activated current. **(c)** Synaptically silent cells express functional $GABA_A$, NMDA and AMPA receptors as depicted by the currents evoked by specific agonists (isoguvacine in the case of $GABA_A$ receptors). In addition, application of $GABA_A$ receptor antagonists (e.g. bicuculline and picrotoxin) unmasks a constitutive release of GABA, which tonically activates $GABA_A$ receptors, thus contributing to maintain the resting membrane potential. **(d)** Early slow currents (ESCs) induced by the stimulation of the stratum radiatum are evoked in almost all maturing neurons from E20–P0 pups, but they are not evoked after P20. Representative biocytin-reconstructed pyramidal cells from animals sacrificed at E20, P6, P14 and P30 are illustrated. Scale bar in (a), 20 μm.

subclasses), modulates migrating neuroblasts as a motility-promoting, an acceleratory or a stop signal [2,10–13]. This has been observed in a diverse brain structures (cerebellar and cerebral cortices, and olfactory epithelium) and neuronal subtypes (e.g. granule cells, pyramidal neuroblasts and tangentially migrating interneurons).

Unfortunately, studies performed so far have used only *in vitro* preparations; there is to the best of our knowledge no *in vivo* description of neuronal migration in the offspring of animals treated with GABA receptor agonists or antagonists during pregnancy. From a clinical perspective, it is important to stress that $GABA_A$ agonists are frequently used as sedatives, tranquillizers, antiepileptic drugs or anaesthetics. They are used widely in paediatric and obstetric medicine. It is thus possible that such treatments during pregnancy influence migration in maturing foetuses, and result in cortical lamination defects or even formation of cortical ectopias. Teratogenic agents (e.g. alcohol or cocaine), physical agents (e.g. irradiation) and biological agents (e.g. viral infection) acting during the period of cell migration [14,15] might indeed result in cortical malformation. The best-documented examples are the alterations induced by alcohol consumption during pregnancy (foetal alcohol syndrome), which can include facial dimorphism, intrauterine growth retardation, brain lesions, cerebral dysfunction and mental retardation. Among the most characteristic lesions in this syndrome are disturbances of cortical lamination, neuronal ectopias

Box 2. Growth factors are important for early GABA signalling

The actions of neurotrophins and GABA are heavily interconnected, providing a major feedback mechanism. Thus, activity enhances expression of brain-derived neurotrophic factor (BDNF) and promotes its release; through the activation of trkB receptors, this promotes postnatal maturation of GABA-mediated inhibition, increasing the frequency of GABA-mediated postsynaptic currents (PSCs) in the cerebral and cerebellar cortices [52] and in cultured hippocampal neurons [53–55]. Antibodies against BDNF and NT-4 reduce the mean synapse-to-cell ratio (as determined by electron-microscopy) in P0 organotypic cerebellar cultures [56]. Conditional deletion of *trkB* in cerebellar precursors is associated with a dramatic reduction in numbers of GABAergic boutons and synaptic specializations, although the final number of GABAergic cells was unaffected [57]. However, several reports propose an opposite role for BDNF. Analysis of the superior colliculus of $BDNF^{-/-}$ mice proved that the chronic absence of BDNF was associated with a significant increase in the amplitudes of GABA-evoked spontaneous and miniature inhibitory PSCs, with no change to the frequency of miniature inhibitory PSCs or the degree of paired-pulse facilitation [58]. Most importantly, these effects were mimicked in normal mice by the tyrosine-kinase inhibitor K-252a, which blocks BDNF receptor signaling [58]. In addition, acute treatment of primary hippocampal cultures with BDNF reduces significantly the amplitudes of miniature inhibitory postsynaptic potentials, an effect that requires functional trkB receptors [59].

As proposed by Zafra *et al.* in 1991 [60], the interplay between excitatory and inhibitory activity determines the levels of *BDNF* expression, so that in immature brain GABA would cooperate with glutamate to enhance expression of this neurotrophin. In immature cultured hippocampal neurons, GABA and muscimol augment levels of intracellular Ca^{2+}, c-Fos mRNA and BDNF mRNA, in a GABA-receptor-dependent and Ca^{2+}-channel-dependent manner [61]. This effect is valid in immature neurons only – when GABA excites neurons – because $GABA_A$ agonists have no effects on c-Fos and BDNF mRNA levels in mature neurons [61,62]. It is thus plausible that the trophic effects of GABA in maturing brain are mediated in part via BDNF and trkB activation. Accordingly, application of exogenous BDNF to hippocampal organotypic explants mimicked the stimulatory effect of bicuculline on GAD65-immunoreactive terminals, and antibodies to BDNF decreased the density of such terminals in bicuculline-treated slices [33]. BDNF is also required for the effect of GABA on the expression of neuropeptide Y, because this effect was suppressed in cultures from BDNF-knockout embryos [63]. Interestingly, BDNF is produced by cortical and hippocampal pyramidal glutamatergic neurons but not by GABAergic interneurons, despite its dramatic action on neurochemical and morphological maturation of inhibitory neurons in the cerebellum, neostriatum, neocortex and hippocampus [52,54,63–67]. This paracrine mode of action of trophic factors, combined with the regulation of the action of GABA in principal cells, illustrates how GABA can in parallel modulate its own actions in principal cells and, via the same trophic factor, control maturation of the neurons from which it is released.

and reduced thickness of the cortical mantle. Interestingly, ethanol inhibits the proliferation of neuronal precursors and impairs their migration, and it augments neuronal death [16,17]. These effects are likely to be related to its action on $GABA_A$ and NMDA receptors [15], although the interference with other factors can also account for its adverse effects. Data from humans on the possible teratogenic actions of antiepileptic drugs (e.g. Ref. [18]) are also controversial. Clearly, more data are required to estimate the relevance of this important clinical issue and, notably, to determine the contribution of GABA to the teratogenic actions of antiepileptic drugs and seizures.

When GABA modulates neuronal arbour elaboration and differentiation

Spoerri [19] proposed GABA as a trophic or regulatory factor having observed that treatment of dissociated embryonic chick cortical and retinal cells using GABA (1 μM) increased the length and branching of the neurites and augmented the density of synapses. This was extended to mammalian neurons by Barbin and co-workers [20], who showed that $GABA_A$ receptor antagonists reduced the dendritic outgrowth of cultured rat hippocampal neurons (Figure 1). Subsequent studies in diverse brain structures, including cerebellar granule cells [21], cortical plate and subplate interneurons [22], spinal cord cells [23] and raphe nuclei 5-hydroxytryptamine (serotonin)-producing neurons [24], showed similar results. The trophic effects of GABA have been reproduced by agents acting on GABA synthesis, receptor activation or blockade, intracellular Cl^- homeostasis, or L-type Ca^{2+} channels. Furthermore, blockers of Ca^{2+}/calmodulin kinase II (CaMKII) or mitogen-activated protein kinase kinase reduce the trophic effects of GABA [21], suggesting that GABA exerts his neuritogenic role through Ca^{2+} influx and the subsequent activation of Ca^{2+}-dependent kinases. Interestingly, postsynaptic CaMKII also generates structural synaptic rearrangements between cultured cortical neurons [25], supporting the notion that CaMKII might be involved in the consolidation of specific synaptic inputs.

The contribution of GABAergic synapses to the formation of GABAergic and glutamatergic synapses is less well documented than the contribution of glutamate synapses to this process. The sequential expression of GABA and glutamate raises the possibility of sequential actions of the two transmitters. Because the paracrine (Box 1) and synaptic actions of GABA are excitatory and precede those of glutamate [26], it is possible that these actions also preferentially modulate earlier processes. Unfortunately, these issues have not been taken into account in earlier studies. Based on neuronal or slice cultures, the prevailing concept is currently one of a homeostatic plasticity mechanism that dynamically adjusts synaptic strengths in the correct direction to promote stability [27]. In this scheme, average neuronal activity levels are maintained by a set of homeostatic mechanisms: reducing excitation will lead to enhancement of excitatory synapses and conversely reducing inhibition will lead to enhancement of inhibitory synapses. In keeping with this model, chronic blockade of NMDA receptors in hippocampal slice cultures leads to a substantial increase in the frequency of miniature excitatory postsynaptic currents (mEPSCs) [28]. Furthermore, in cultured hippocampal neurons, removing inhibition leads to reduction of excitatory and enhancement of inhibitory synaptic strengths, whereas the opposite effects were observed after reduction of excitatory inputs [29]. However, the simple concept of homeostatic mechanisms would be less than adequate to explain transmitter-mediated regulation of synapse formation by activity during brain maturation. Indeed, there are multiple sequences during brain development that are not centred on the issue of equilibrium between excitation and

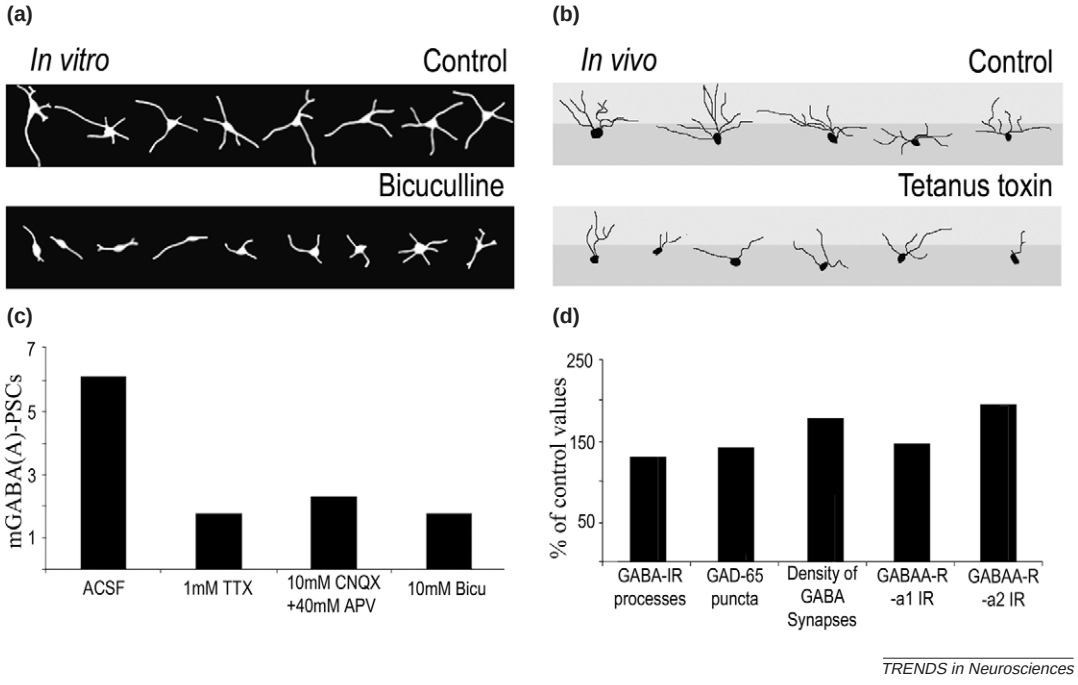

Figure. 1. Effects of GABA on dendrite outgrowth and synaptogenesis. **(a)** *In vitro* analyses have shown that treatment of neuronal cultures (here E18 rat hippocampal neurons) with $GABA_A$ receptor antagonists (e.g. bicuculline) results in a significant reduction of dendrite outgrowth [20]. **(b)** *In vivo* analysis confirmed that synaptic activity has an important effect on dendrite outgrowth, at least for basilar dendrites of hippocampal CA1 pyramidal cells [68]. P1 rat pups received a single injection of tetanus toxin into the hippocampus and the consequences on dendrite maturation were measured after 5 d [68]. Here, only cell bodies and basilar dendrites are shown. Grey colours outline the borders of the oriens (pale) and pyramidal cell (dark) layers. **(c)** Formation of GABAergic synapses: early effects of activity deprivation. During early maturation (P0–P2), the synaptogenesis rate was impaired by treatment with the Na^+ channel blocker tetrodotoxin (TTX), with glutamate receptor antagonists [2-amino-5-phosphonovaleric acid (APV) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)] or with a $GABA_A$ receptor antagonist [bicuculline (Bicu)], as compared with application of normal artificial cerebrospinal fluid (ACSF) [32]. In this histogram, the frequencies of miniature $GABA_A$-receptor-mediated postsynaptic currents ($mGABA_A$-PSCs) – recorded in CA3 pyramidal cells (*in toto* preparation from P0 hippocampus) after 24 h *in vitro* in the different conditions – are reported relative to the frequencies at P0. **(d)** Formation of GABAergic synapses: later effects of bicuculline. Chronic blockade of $GABA_A$ receptors in hippocampal organotypic explants from P7 pups resulted in increased numbers of GABAergic synapses. Numbers were measured using immunoreactivity (IR) for GABA, glutamic acid decarboxylase (GAD)65, $GABA_A$ receptor α1 subunits ($GABA_A$ α1) and $GABA_A$ receptor α2 subunits ($GABA_A$ α2), and were expressed as percentages of control values, according to data from Refs [33,69].

inhibition. Thus, GABA excites immature neurons and acts during development in synergy with – not in opposition to – NMDA receptors [30]. GABAergic synapses are also formed before glutamatergic synapses, suggesting that dynamic control of the formation of GABA mechanisms must be independent of glutamate synapses, but that formation of glutamate mechanisms need not be controlled independently of GABA synapses. In addition, GABAergic interneurons become postmitotic and form synapses before the principal glutamatergic neurons [26,31], and they generate network-driven activities at a stage when most pyramidal neurons have no functional synapses [26]. Because GABA receptors are also operative before synapse formation (see preceding discussion), the excitatory actions of GABA have a programmed developmental role that does not simply inhibit glutamate synapses; this raises the possibility that there are other mechanisms of regulating GABA – and/or glutamate – that are not interdependent. In keeping with this, applications of tetrodotoxin (TTX) or antagonists of GABA or glutamate receptors for 24 h in intact postnatal day (P)0 rat hippocampus *in vitro* have a similar effect on the formation of GABAergic synapses: blocking the frequency increase of miniature GABA-receptor-mediated EPSCs that occurs spontaneously *in vivo* and *in vitro* [32]. This suggests that the formation of GABAergic synapses requires action potentials and ongoing activity generated by GABA or glutamate, but not both. By contrast, in organotypic hippocampal explants from P7 rat pups, at a stage when GABA switches from being excitatory to inhibitory, chronic treatment with antagonists of GABA and glutamate receptors respectively increase and decrease the number of GABA-immunoreactive synapses on hippocampal pyramidal cells [33] (Figure 1). These observations suggest that during the earlier phases of maturation the activity-dependent homeostatic device is not aimed at equilibrating excitation and inhibition, but that this is the case during the later phases of maturation.

When the GABA shift is activity dependent

In maturing brain, GABA exerts a depolarizing action related namely to a reverse gradient of Cl^-. This transient effect is essentially due to a low expression of the neuronal Cl^--extruding K^+/Cl^- co-transporter KCC2 [34]. There is general agreement that the GABA switch from excitatory to inhibitory action is mediated by upregulation of the co-transporter KCC2, which extrudes Cl^- and has delayed expression [35]. Whether this shift is activity dependent is at present controversial. Ganguly *et al.* [36] reported that the GABA switch is delayed by chronic blockade of $GABA_A$ receptors, and accelerated by increased $GABA_A$ receptor activation. These authors suggested that GABA activity modulated the levels of KCC2 mRNA, acting as a self-limiting trophic factor during neural development.

However, this notion was challenged by Titz *et al.* [37], who showed that this switch occurred in cultured midbrain neurons treated with $GABA_A$ receptor antagonists, and by Ludwig *et al.* [35], who showed that chronic treatment of cultured hippocampal explants with the $GABA_A$ antagonist picrotoxin did not affect the developmental upregulation of KCC2 protein expression. Interestingly, these authors also demonstrated that TTX and glutamate receptor antagonists affected neither the developmental pattern of KCC2 expression (suggesting that the maturation of GABA system itself does not require neuronal spiking) nor ionotropic transmission *in vitro*.

When knocking-out GADs does not affect brain development

This plethora of actions of GABA stands in contrast to the lack of effects of genetically deleting the enzymes that synthesize GABA. Thus, double knockdown of the GABA-synthesizing enzymes glutamic acid decarboxylase (GAD)65 and GAD67 did not produce discernible disorders of brain histogenesis, including cortical layering [38]. Although this observation might suggest that neurogenesis and cell migration do not require GABAergic systems, it bears stressing that the redundancy in knockout animals is well documented and that other transmitter systems could compensate for GABA loss, including modulation of cortical cell proliferation [2,3] and migration [12] by glutamate. Clearly, other studies measuring the effects on activity of knocking out these GADs, and more detailed determination of the morphological properties of these networks, are required.

Analysis of mice with knockout mutations affecting GABA receptors, namely the $GABA_A$ receptor $\alpha 1$ subunit, did not provide significant evidence for the trophic effects of GABA because changes to synaptic GABA transmission were only minor. However, reduced outgrowth of dendritic spines in the juvenile visual cortex of these mice has been reported [39].

Concluding remarks

Recent observations suggest that GABA has a variety of important functions during maturation. This role is not restricted to GABA because several other transmitters can modulate essential functions in developing brain [40]. The uniqueness of GABA is epitomized by its early operation – before glutamate synapses are functional – indicating that, at least during a restricted period, GABA provides all the excitatory drive. In addition, the possibly activity-dependent shift of GABA actions following upregulation of KCC2 provides a remarkable modulation of the set-point at which GABA will resume its classical inhibitory effects. Interestingly, GABA becomes excitatory in adult patients with epilepsy [41], owing to a shift in the Cl^- reversal potential. Similar changes have been observed in acute preparations *in vitro*, implicating them in the genesis of epileptic foci [42] and suggesting that they are related to seizure-induced downregulation of KCC2 expression [43]. Therefore, recapitulation of GABA developmental mechanisms might have physiological and/or pathological consequences on adult brain. The pivotal role of GABA and its strong relationship with other transmitters and trophic factors (Box 2) provide a feedback mechanism to reinforce the role of GABA (e.g. by synergistic excitatory actions of $GABA_A$ and NMDA receptors [30]). In this respect, GABA would have a pivotal role in coordinating convergent factors and actions aimed at sculpting neurons and networks.

Study of the developmental actions of GABA has far improved our understanding of how activity can modulate brain formation and also exert pathogenic effects. However, little attention has been paid to the repercussions of neurological drug therapy on brain maturation. Clearly, many antiepileptic, psychotropic and sedative drugs could perturb brain maturation by their actions on GABA function. In the developmental saga of GABA actions, there is still much to be discovered and many surprises to be found.

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