

Developmental pharmacology of benzodiazepines under normal and pathological conditions

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ABSTRACT – Benzodiazepines are allosteric agonists of GABA_A receptors (GABA_AR), pentameric ligand-gated Cl⁻ channels, which serve both an important neurodevelopmental role but are also the principal inhibitory system in the brain. However, their subunit composition, channel properties, and function, as well as their region-specific expression patterns, change through development. These processes have been extensively studied in rodents and to some extent confirmed in higher species. Specifically, GABA_ARs acquire faster kinetics with age and their pharmacology changes rendering them more sensitive to drugs that have higher affinity for α 1 subunit-containing GABA_ARs, such as benzodiazepines, but also, their inhibitory function becomes more potent as they shift from having depolarising to hyperpolarising responses due to a shift in Cl⁻ gradient and cation chloride cotransporter expression. Concerns have been raised about possible pro-apoptotic and paradoxical effects of benzodiazepines in the neonatal normal rat brain, although it is unclear, as yet, whether this extends to brains exposed to seizures. Growing evidence indicates that the pharmacology and physiology of GABA_ARs may be altered in the brain of rats or humans with seizures or epilepsy, or different aetiologies that predispose to epilepsy. These changes follow different paths, depending on sex, age, region, cell type, aetiology, or time-point specific factors. Identification of dynamic biomarkers that could enable these changes *in vivo* to be monitored would greatly facilitate the selection of more effective agonists with fewer side effects.

Key words: GABA_AR subunit, epilepsy, chloride cotransporter, hyperthermia

Benzodiazepines are allosteric agonists of GABA_A receptors (GABA_ARs), pentameric ligand-activated Cl⁻ channels that typically mediate Cl⁻ inflow leading to neuronal hyperpolarisation and fast inhibitory postsynaptic currents. Benzodiazepines act in the pres-

ence of GABA, and their effects greatly depend upon the type of subunits present in the GABA_ARs. Their affinity is greatest for GABA_ARs containing α 1 and γ 2 subunits. The inhibitory effects of benzodiazepines in combination with their availability as formulations that

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permit rapid and flexible delivery (e.g. buccal, nasal, rectal), even in situations when intravenous access is not available, have established them as the first-line rescue drugs or treatment for rapid cessation of ongoing seizures throughout life.

However, the effects of GABA_AR agonists may change under certain normal or pathological situations, in which either the subunit composition is not optimal or the function of GABA_ARs is altered. Here, we will review the animal studies that have highlighted the developmental changes in GABA_AR physiology and pharmacology of their agonists, including benzodiazepines, and discuss these findings with regards to their potential relevance for the clinical use of benzodiazepines in the treatment of ongoing seizures, and especially in very young individuals.

GABA_AR structure and pharmacology

There are currently 16 known subunits in mammals (six α , three β , three γ , and one δ , ε , θ , and π). Each GABA_AR consists typically of five subunits, with the most commonly observed arrangement being a combination of two α , two β , and one γ subunit (*figure 1*). Subunit composition dictates a number of receptor characteristics, including localisation within each cell or in various brain regions, affinity for ligands and drugs, as well as regulation by specific signalling pathways. For example, typically (but not always), GABA_ARs that contain γ subunits are located post-synaptically, and their activation is recorded as phasic inhibitory postsynaptic currents (IPSCs) (Mody and Pearce, 2004; Farrant and Nusser, 2005; Mohler, 2006a). However, substitution of a γ for a δ subunit causes extrasynaptic localisation and results in tonic GABA_AR activation by ambient GABA molecules (tonic currents) (Nusser *et al.*, 1998; Farrant and Nusser, 2005). Specific subunit composition is not only responsible for subcellular localisation, but also impacts the kinetics of the receptors, as well as their affinity for certain ligands (Mohler, 2006a). As an example, $\alpha 3$ subunits located in extrasynaptic receptors have been shown to desensitize much more slowly than $\alpha 2$ subunits located in synaptic receptors (Devor *et al.*, 2001).

Whereas the GABA binding site lies between an α and a β subunit, the benzodiazepine binding pocket is between an α and a γ subunit, and their affinity is greatest for GABA_ARs which contain $\alpha 1$ and $\gamma 2$ subunits, intermediate for $\alpha 2$ or $\alpha 3$ -containing GABA_ARs, low for δ -containing GABA_ARs, and absent for those that contain $\alpha 4$ or $\alpha 6$ subunits. Consequently, the primary target of benzodiazepines is the potentiation of postsynaptic GABA_ARs, that are already activated by synaptically released GABA quanta, and which mediate phasic GABA inhibition.

Since specific subunit composition has such far-reaching effects on kinetics and pharmacology, the differential expression of GABA_AR subunits across brain regions, cell types, between genders, and as the brain develops, adds to the diversity of their final effects (Mohler, 2006a; Mohler, 2006b). The regional differences in subunit expression are also thought to contribute to the different functional effects of agonists that prefer certain subunit combinations (Mohler, 2006a). For example, $\alpha 1$ is found more in the thalamus, cortex, brainstem, hypothalamus, and hippocampus, and agonists may have antiseizure, sedative, and amnesic effects. In contrast, selective agonists for $\alpha 2$ and $\alpha 3$ -containing GABA_ARs exhibit anxiolytic or myorelaxant effects. However, most of these studies are largely on adult populations and as we will discuss later, the effects in the developing brain may be quite different.

GABA_AR structure and pharmacology change through development

During normal development, the affinity of GABA_ARs for agonists or antagonists changes due to age-, region- and sex-specific patterns of GABA_AR subunit expression, subcellular localisation, or mRNA editing. Most of these studies have been performed in rodents. The significantly shorter lifespan of rodents (approximately two years) warns that brain development in rodents occurs relatively faster than in humans. In general, a postnatal day (PN) 8-13 rat or mouse is considered to be equivalent to a human newborn full-term baby, based on gross measures of brain growth (weight, protein/DNA content). However, unlike human babies, eye opening only occurs between PN13-15 in rodents. Weaning from the dam usually takes place at around PN21 and puberty starts between PN32-35, whereas adulthood begins on PN60. In fact, when comparing rodent to human development, each developmental process may mature at its own tempo and cross-species extrapolation may not be very accurate.

GABA_AR subunit expression changes through development following cell-type and region-specific patterns. Although such developmental changes are widespread, there is considerable diversity across regions and cell types. However, a well-documented "subunit switch" between the $\alpha 2/\alpha 3$ and $\alpha 1$ subunits has attracted a lot of interest as it has been observed in multiple studies, regions, and species, and may potentially account for important age-related differences in GABA_AR inhibitory responses and their pharmacology. Laurie *et al.* described increasing $\alpha 1$ subunit expression with age in the telencephalic cortex, diencephalon, thalamus, hippocampus, and cerebellum (Laurie *et al.*, 1992). At the same time, a

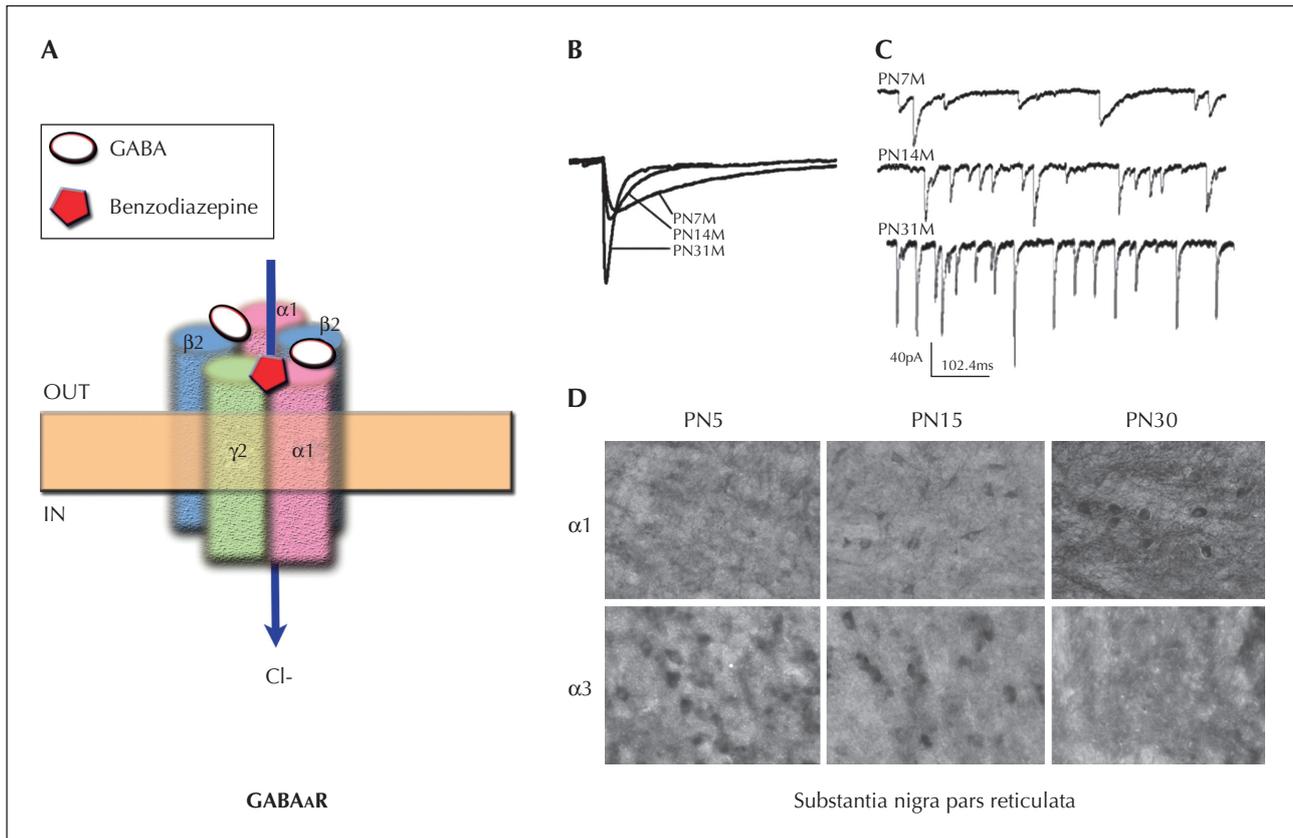


Figure 1. Developmental changes in GABA_AR composition and postsynaptic currents. (A) GABA_ARs are pentameric ligand-gated Cl⁻ channels that are typically comprised of 2 α and 2 β subunits, in addition to a fifth subunit which is usually a γ subunit, although other subunit combinations have been described. The GABA binding pocket lies between the α and β subunits, whereas the benzodiazepine binding site is between the α and γ subunits, with the $\alpha 1\gamma 2$ combination exhibiting greatest affinity. (B-D) During development, a gradual shift in the subunit composition of GABA_ARs has been described in several brain regions. Whole-cell patch clamp recordings from GABAergic SNR neurons are shown here to demonstrate that in older age groups, the kinetics of post-synaptic GABA_AR inhibitory currents acquire faster kinetics (faster rise and decay times) (B) and become more frequent (C). This can be explained by the gradual replacement of $\alpha 3$ subunits (highly present in immature neurons) and $\alpha 1$ subunits (highly expressed in mature neurons), as shown in the substantia nigra neurons (D), by performing immunofluorescence specific to either $\alpha 1$ or $\alpha 3$ subunits. Permission to reproduce panels B-D from Chudomel *et al.* (2009) was obtained by Elsevier.

simultaneous decline in $\alpha 3$ (telencephalon, cortex, diencephalon, thalamus, and cerebellum) and, in certain regions, $\alpha 2$ (diencephalon, thalamus) occurs. Similar observations have also been documented in the basolateral amygdala (Ehrlich *et al.*, 2013) and substantia nigra pars reticulata (SNR) (Chudomel *et al.*, 2009) (figure 1). The replacement of $\alpha 2/\alpha 3$ subunits by $\alpha 1$ alters not only the kinetics of phasic GABA_AR inhibitory postsynaptic currents (IPSCs), rendering them faster, but also decreases the affinity of these receptors to drugs such as zolpidem or benzodiazepines. For example, in the rat SNR, PN5-9 SNR GABAergic neurons had low frequency and slow GABA_AR-IPSCs, whereas in peripubertal PN28-32 rats, IPSCs were recorded with high frequencies and rapid kinetics and were more sensitive to zolpidem, a preferential agonist of $\alpha 1$ (Chudomel *et al.*, 2009). A similar switch has also been documented in the

monkey prefrontal cortex, with high levels of $\alpha 2$ mRNA in one-week-old monkeys and a gradual replacement by high $\alpha 1$ mRNA levels in adult monkeys (Hashimoto *et al.*, 2009). Studies in humans have been limited to post-mortem tissues, for obvious ethical reasons. Two independent studies in the human cerebral cortex reported increasing expression of $\alpha 1$ and $\gamma 2$ mRNA between the neonatal and adult ages, while the level of mRNA of other subunits, such as $\alpha 2$ (Fillman *et al.*, 2010) or $\alpha 4$ (Jansen *et al.*, 2010), declined. Another point of developmental regulation is at the mRNA editing level, which may change the function of the translated proteins. In the case of $\alpha 3$ subunit, the level of RNA editing leading to an isoleucine-to-methionine substitution in the region coding the third transmembrane domain was very low during mouse embryonic development, reached maximal levels at PN7, and was observed in 90% of adult mouse

brains (Rula *et al.*, 2008). The edited and non-edited forms of $\alpha 3$ subunit confer distinct channel properties. GABA_ARs containing non-edited $\alpha 3$ activate rapidly and deactivate more slowly than those that contain edited $\alpha 3$ subunits, but also exhibit stronger outward rectification (*i.e.* Cl⁻ influx). This particular feature renders the non-edited $\alpha 3$ subunit-containing GABA_ARs more capable of mediating shunt inhibition in the face of depolarising currents, and can therefore be protective early in development, when GABA_AR currents can be depolarising, as will be discussed in the following section.

Changes in depolarising to hyperpolarising GABA_AR signalling with age

The direction of GABA_AR responses depends upon the Cl⁻ gradient between the intra- and extracellular space and also, to a degree, on HCO₃⁻. The intracellular Cl⁻ concentration is controlled by cation-Cl⁻ cotransporters, of which the most widely studied is the Cl⁻ importer Na⁺/K⁺/Cl⁻ cotransporter 1 (NKCC1) and the Cl⁻ exporter K⁺/Cl⁻ cotransporter (KCC2) (*figure 2*). Early in life, in most studied neurons, there is a relatively high intracellular Cl⁻ concentration, due to a relative abundance of NKCC1 over KCC2. As a result, opening of the GABA_AR mediates Cl⁻ efflux, leading to depolarisation. The early immature depolarising effects of GABA_A receptors are important for development; nevertheless, they lead to calcium processes necessary for neuronal processes including cell migration and differentiation (Ben-Ari, 2002; Galanopoulou, 2008a). Precocious deprivation of depolarising GABA from developing neurons may lead to dysmature features, such as underdeveloped arborization of neuronal processes in cortical neurons (Cancedda *et al.*, 2007; Wang and Kriegstein, 2008; Wang and Kriegstein, 2011). It should be noted that these normal depolarising effects of GABA are not necessarily excitatory, since they may not always trigger action potentials. Furthermore, in the setting of excessive excitation, as during seizure activity, GABA_AR agonists may still exert inhibitory effects, through shunting inhibition, although this type of inhibition is less effective (Staley and Mody, 1992).

During development there is a gradual shift, from an NKCC1 dominant state, early on, to a KCC2 dominant state in more mature neurons. This eventually reduces the intracellular Cl⁻, resetting the Cl⁻ electrochemical reversal potential, and resulting in Cl⁻ influx and hyperpolarisation after GABA_AR activation (Plotkin *et al.*, 1997; Rivera *et al.*, 1999) (*figure 2*). The timing of shift from depolarising to hyperpolarising

GABA_AR signalling varies across brain regions, neuronal types, and between sexes, adding another level of complexity to the understanding of GABAergic signalling. Such examples are the substantia nigra pars reticulata (SNR) and CA1 pyramidal neurons of the hippocampus of Sprague-Dawley rats, where GABA_AR activation elicits sex-specific effects, with delayed disappearance of depolarising responses in males compared to females (Galanopoulou *et al.*, 2003; Kyrozis *et al.*, 2006; Galanopoulou, 2008b). Neurons in the SNR of male rats were shown to start manifesting hyperpolarising GABA_AR responses by postnatal day (PN) 17, while in females the switch occurred much earlier, at PN10 (Kyrozis *et al.*, 2006), consistent with increased KCC2 mRNA in the SNR of female rats at all ages (Galanopoulou *et al.*, 2003). This sexually dimorphic pattern continues across other brain regions, including CA1 pyramidal neurons. Again, female rats showed isoelectric or mildly hyperpolarised GABA_AR as early as PN4, while males started to have hyperpolarising responses around PN14 (Galanopoulou, 2008b). Systemic administration of GABA_AR agonists have also demonstrated sex-specific effects in intracellular processes, such as phosphorylation of the cAMP responsive element binding protein (pCREB), which are probably linked to the depolarisation-induced calcium rises (Auger *et al.*, 2001; Galanopoulou *et al.*, 2003; Perrot-Sinal *et al.*, 2003; Galanopoulou, 2006; Mantelas *et al.*, 2007; Galanopoulou, 2008c). Sex differences in KCC2 and/or NKCC1 have been proposed to underlie the earlier onset of hyperpolarising GABA_AR signalling in females (Galanopoulou *et al.*, 2003; Galanopoulou, 2008b; Damborsky and Winzer-Serhan, 2012; Murguía-Castillo *et al.*, 2013). KCC2 expression is also under the control of gonadal hormones in immature rat SNR neurons. According to a proposed hypothesis, the high endogenous levels of oestrogenic derivatives of testosterone aromatization in the newborn male rat brain may actually delay the developmental rise of KCC2 and the appearance of hyperpolarising GABA in certain brain regions, such as the SNR (Galanopoulou, 2008c). In humans, the only evidence corroborating the idea that there may be depolarising GABA in very immature neurons comes from post-mortem studies, where high NKCC1 and low KCC2 expression were shown in cortical extracts from neonates, followed by a rapid decline in NKCC1 and gradual increase in KCC2 during the first few months of life (Dzhala *et al.*, 2005; Jansen *et al.*, 2010).

GABA_AR agonists have age-specific effects

What is the impact of all these structural and functional changes of GABA_ARs upon the effects of drugs

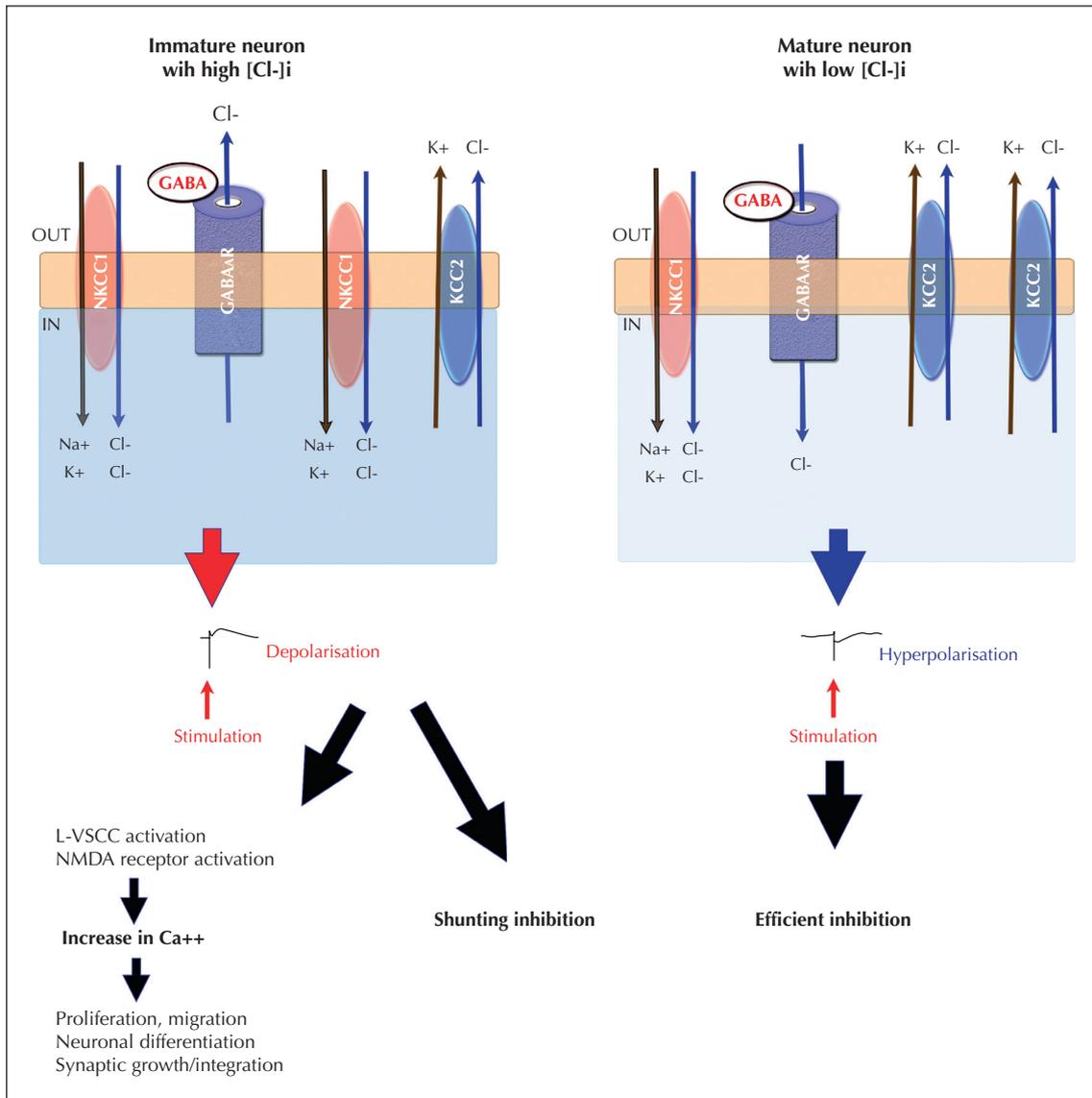


Figure 2. Developmental changes in the physiology of GABA_ARs. GABA_AR can normally elicit depolarising or hyperpolarising post-synaptic responses depending upon the electrochemical gradient of Cl⁻ (between the intra- and extra-cellular space). In immature neurons, there are abundant cation chloride cotransporters that import Cl⁻ (e.g. NKCC1) and scarce cation chloride cotransporters that export Cl⁻ (e.g. KCC2), creating relatively high intracellular Cl⁻ concentrations ([Cl⁻]_i). As a result, opening of GABA_ARs leads to efflux of Cl⁻, depolarising these neurons. GABA depolarisations are necessary early in life as they support calcium-sensitive processes that are important for neuronal proliferation, migration, differentiation, and synaptic growth and integration. GABA depolarisations are not necessarily excitatory, as they do not necessarily trigger action potentials. In the face of excessive neuronal excitation (e.g. during seizures), the open GABA_AR channels can still shunt excitatory currents, contributing a weak form of inhibition (shunting inhibition). In mature neurons, there is greater activity of KCC2 than NKCC1, which lowers [Cl⁻]_i and permits the hyperpolarising GABA_AR currents to emerge, once GABA_ARs open. This suggests that the efficacy of inhibitory effects of benzodiazepines may be enhanced in older age groups.

such as benzodiazepines or barbiturates through development? Animal studies have addressed this question in normal animals and have supported the idea of age-specific effects of GABAergic drugs. Increased apoptotic death and reduced neurogenesis is observed in one-week-old rats exposed to benzodiazepines or phenobarbital (Bittigau *et al.*, 2002;

Bittigau *et al.*, 2003; Stefovská *et al.*, 2008). Similar findings were observed with other antiseizure drugs, suggesting that the immature brain may be more dependent upon a higher level of neuronal activity than the adult for survival. Midazolam is reported to decrease the mechanical reflex threshold and increase the magnitude of mechanical and thermal reflexes in

neonatal rats, but had no effect in juvenile rats (Koch *et al.*, 2008). In the same study, midazolam had sedative effects in PN10 and PN21 rats but not in PN3 rats. Although it is tempting to associate these paradoxical effects of midazolam in newborn rodents with the early depolarising effects of GABA_AR signalling, the answer is not always as simple. The final output of the inhibition or activation of a neuronal cluster in the brain is largely dependent on the way a neuronal network is organised, interconnected, and functionally active. For example, infusions of the GABA_AR agonist muscimol in the SNR, which although ultimately silences local neuronal activity, may produce anticonvulsant or proconvulsant effects, depending on the age of the rat, whether it is male or female, and whether it is infused in the anterior or posterior SNR (Veliskova and Moshé, 2001; Veliskova and Moshé, 2006).

Even more importantly, drugs are typically given to target a specific disease process or symptom and their effects cannot be extrapolated to those observed in a diseased brain. For example, although benzodiazepines may potentially accentuate cell death in newborn rats, these studies were performed in normal rodents and not in animals that exhibit status epilepticus, which, on its own merit, may have adverse developmental outcomes. Therefore, careful evaluation, including evaluation of long-term outcomes, is warranted when assessing the efficacy and tolerability of these drugs in very young subjects.

Seizures and epilepsy can disrupt GABA_AR signalling

Multiple studies have investigated the association between either induced seizures, the epileptic state or different aetiology and the expression and function of GABA_AR subunits (reviewed in Galanopoulou and Moshé [2014]). Using the kainic acid or the pilocarpine (with or without lithium) models as methods to test the effects of status epilepticus on the expression of GABA_AR subunits, there is no singular pattern of changes that emerges. Rather, the effects of seizures depend upon the age at seizure induction, the time-point when subunit expression is studied, and subunit-specific, but also model and region-specific, factors (*figure 3*). For example, the expression of $\alpha 1$ subunit is increased in the dentate gyrus of adult rats exposed to early-life pilocarpine-induced status epilepticus (Zhang *et al.*, 2004; Raol *et al.*, 2006), regardless of whether these were epileptic or not, but reduced in those exposed to pilocarpine status epilepticus in adulthood (Brooks-Kayal *et al.*, 1998). Expression studies of GABA_AR subunits in post-surgical specimens from epilepsy patients with focal

cortical dysplasias also suggest a disrupted developmental expression profile of the GABA_AR subunits which is also dependent upon the type of dysplasia (Jansen *et al.*, 2010).

A study that has initiated a lot of interest into the potential pathogenic role of depolarising GABA_AR signalling in epilepsy reported an aberrant presence of depolarising GABA_AR signalling in human epileptic subiculum and paradoxical silencing of interictal epileptic discharges by GABA_AR inhibitors (Cohen *et al.*, 2002). This was attributed to reduction in KCC2 expression (Huberfeld *et al.*, 2007). Multiple reports confirmed similar observations in temporal lobe epilepsy (Palma *et al.*, 2006; Munoz *et al.*, 2007) or other pathological epileptic conditions, including hypothalamic hamartomas (Kim *et al.*, 2009), cortical dysplasias (Aronica *et al.*, 2007; Jansen *et al.*, 2010; Shimizu-Okabe *et al.*, 2011; Talos *et al.*, 2012), and tumour-associated epilepsy (Conti *et al.*, 2011). However, based on the human studies, it is not possible to differentiate between the contribution of epilepsy, aetiology, treatments, stressors or other factors on the observed changes in GABA_AR signalling. In animal studies, where some of these factors can be better controlled, pathological reversal to depolarising GABA_AR signalling with concomitant increase in NKCC1 or decrease in KCC2 have been documented in selected brain regions of adult rodents subjected to status epilepticus (Benini and Avoli, 2006; de Guzman *et al.*, 2006; Pathak *et al.*, 2007; Bragin *et al.*, 2009). On the other hand, the response of immature rats to seizures is again quite different and may be dependent upon sex-specific factors (Galanopoulou, 2008b) or the time after seizure occurrence (Galanopoulou, 2008b; Khirug *et al.*, 2010). For example, kainic acid status epilepticus in neonatal male rats with depolarising GABA_AR signalling in the hippocampus causes a premature appearance of hyperpolarising GABA_AR signalling, unlike that observed in adult rats (Galanopoulou, 2008b). It is currently, however, unclear whether this is a protective mechanism or if it contributes to the developmental deficits that ensue.

The biggest question therefore is how to use all this knowledge in a manner that will translate into more effective and safer use of GABAergic drugs in humans, particularly in very young individuals, with seizures or epilepsy. Even if we were to account for all the variables involved (age, sex, region, aetiology, cell type, etc.), the expression and function of GABA_ARs may change with time or in response to external stressors or internal comorbid or physiological factors that will require a live and dynamic predictive modelling method. The discovery and implementation of dynamic, safe biomarkers with sufficient temporospatial readout detail will be critical.

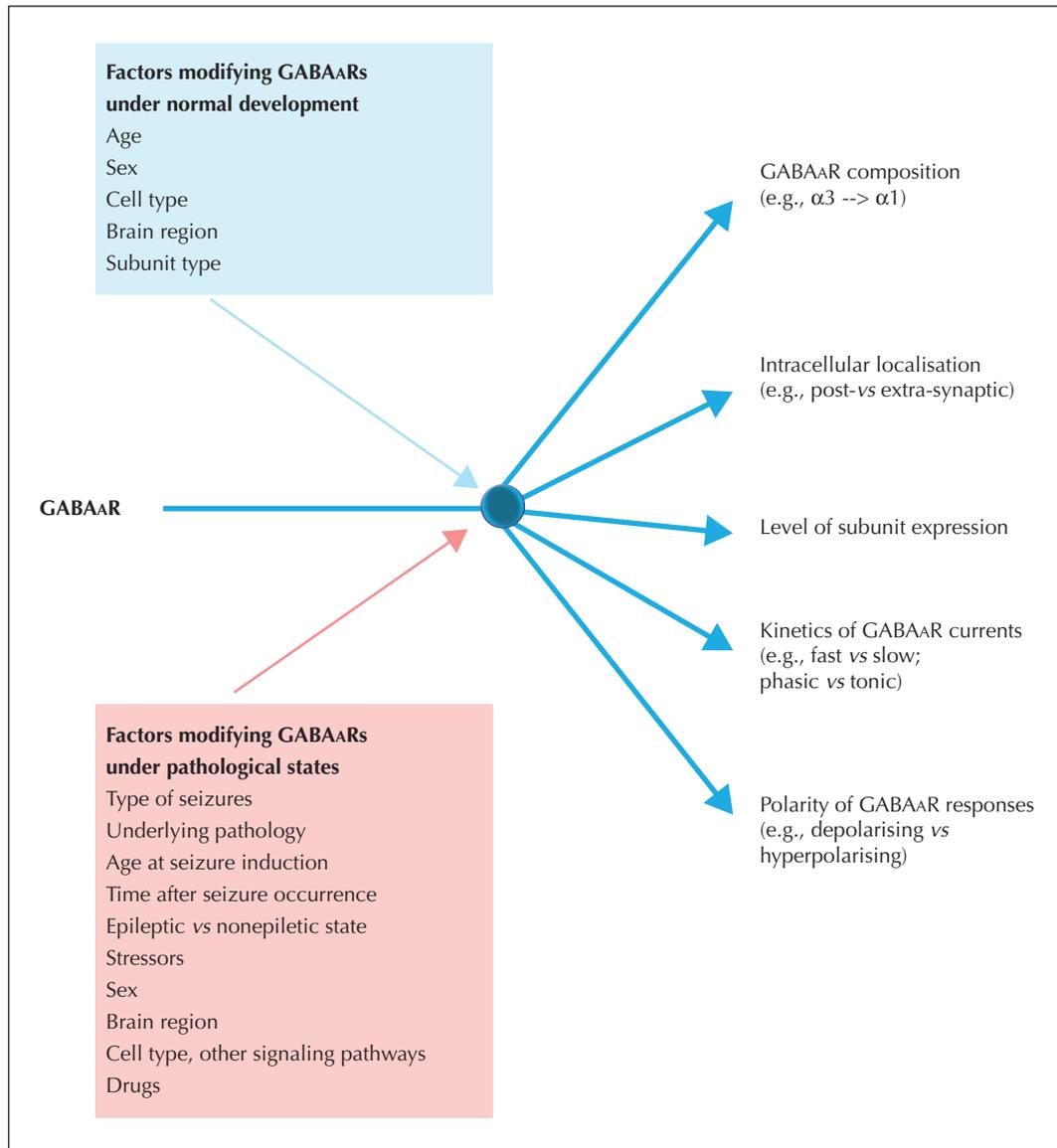


Figure 3. GABA_AR expression and functional changes during normal development and pathological states. Various factors contribute to the variable functions of GABA_ARs and strength of GABA_AR inhibition under normal conditions, including age, sex, cell type, brain region, and subunit types expressed. In addition, seizures, underlying pathological states (aetiology), age at seizure induction, time after seizures, the presence of epileptic state, and other stressors or drugs may further diversify the function of GABA_ARs. As a result, the end effect and efficacy of benzodiazepines may vary across brain regions, subjects, and under different normal or pathological states.

Hyperthermic seizures may alter GABA_AR signalling

Of particular interest is the role of fever on GABA_AR signalling, given the high prevalence of febrile seizures in the paediatric population. Several studies have looked specifically at the role of hyperthermia with variable observations. Hyperthermia can impair GABA_AR signalling by decreasing GABA_AR IPSCs, increasing GABA uptake, reducing selective α GABA_AR subunits, or cause retention of GABA_AR subunits in

the endoplasmic reticulum (Fujii *et al.*, 2001; Kang *et al.*, 2006; Sharma, 2006; Swijsen *et al.*, 2012). On the other hand, hyperthermia may enhance GABA_AR signalling in selective neuronal populations or increase benzodiazepine-sensitive GABA_ARs in certain brain regions (Gonzalez Ramirez *et al.*, 2007; Swijsen *et al.*, 2012). An interesting study has also proposed that reversal to depolarising GABA_AR signalling after hyperthermic seizures induces ectopic granule cells that may contribute to adult epilepsy (Koyama *et al.*, 2012). It should be noted, however, that the role of aberrant

seizure-induced neurogenesis in epileptogenesis has not been confirmed in other models (Sankar *et al.*, 2000).

Conclusions

In summary, the specific differences in expression and function of GABA_ARs related to age, anatomical region, sex, receptor subunit, and disease state make it clear that great care should be taken when it comes to using drugs, such as benzodiazepines, particularly early in development. Could more selective $\alpha 2/\alpha 3$ GABA_AR agonists offer a better efficacy profile for very young patients, given the lower levels of $\alpha 1$ subunit in many brain regions? Could selective enhancers of KCC2 enhance the therapeutic benefit of GABAergic agonists in very young patients? Would methods of tailoring such therapies to deliver them to the specific target areas, for the specific duration of the seizure, or other targeted symptoms prove to be safer? It should be noted, however, that most of the cited studies raising the concern that GABAergic drugs may have adverse effects in the naïve developing brain have been performed in naïve animals. There is no sufficient evidence to indicate that this may also be true for brains that have been exposed to GABAergic drugs with the intent to stop seizures, and it could prove to be challenging to design such a controlled study, given that benzodiazepines have been established as a first-line rescue treatment at all age groups. Yet, as we go forward and more clinical studies are being developed to test the efficacy and tolerability of benzodiazepines as rescue treatments for seizures, it would be worth evaluating not only the immediate efficacy and tolerability but also the longer-lasting consequences of exposure to GABAergic drugs, in both developmental and behavioural outcomes (Galanopoulou, 2008c). The experience in the NEMO (NEonatal seizures using Medication Off-patent) study with the observed ototoxicity from the use of bumetanide, an NKCC1 inhibitor, as adjunctive medication to barbiturates to stop neonatal seizures highlights the concern of safety (Pressler *et al.*, 2013). This is particularly true for regions that are not necessarily within the epileptogenic zone and may become exposed to the drug. □

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