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Sexually dimorphic expression of KCC2 and GABA function

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Abstract

GABA_A receptors have an age-adapted function in the brain. During early development, they mediate depolarizing effects, which result in activation of calcium-sensitive signaling processes that are important for the differentiation of the brain. In more mature stages of development and in adults, GABA_A receptors acquire their classical hyperpolarizing signaling. The switch from depolarizing to hyperpolarizing GABA_A-ergic signaling is triggered through the developmental shift in the balance of chloride cotransporters that either increase (ie NKCC1) or decrease (ie KCC2) intracellular chloride. The maturation of GABA_A signaling follows sex-specific patterns, which correlate with the developmental expression profiles of chloride cotransporters. This has first been demonstrated in the substantia nigra, where the switch occurs earlier in females than in males. As a result, there are sensitive periods during development when drugs or conditions that activate GABA_A receptors mediate different transcriptional effects in males and females. Furthermore, neurons with depolarizing or hyperpolarizing GABA_A-ergic signaling respond differently to neurotrophic factors like estrogens. Consequently, during sensitive developmental periods, GABA_A receptors may act as broadcasters of sexually differentiating signals, promoting gender-appropriate brain development. This has particular implications in epilepsy, where both the pathophysiology and treatment of epileptic seizures involve GABA_A receptor activation. It is important therefore to study separately the effects of these factors not only on the course of epilepsy but also design new treatments that may not necessarily disturb the gender-appropriate brain development.

Keywords

GABA; substantia nigra; hippocampus; sex; autism; epilepsy

1.1 INTRODUCTION

Over the last few decades, many studies have highlighted the differences between the male and female brain and showed that they are not just limited to structures involved primarily in sexual behavior. These include morphological, functional and ontogenetic differences in a variety of neurotransmitter signaling systems and cell types that influence the way the male and female brains operate under normal and pathological conditions. Such differences are also applicable during the very early stages of life, even before gonadal function fully matures. In epilepsy, epidemiological and clinical studies have suggested that males and females may have

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different susceptibility to specific seizure types and epileptic syndromes, especially those first manifesting during the early postnatal life. In this review, I will focus on the GABA_A receptor signaling system, known both as an important neurotrophic factor during development but also as the major inhibitory neurotransmitter signaling system in the mature brain. I will introduce why sex differences in the physiology of GABA_Aergic system are important for normal, sex-appropriate brain development and how they influence the consequences of seizures and antiepileptic drugs.

1.2 GABA_A receptors and chloride cotransporters in normal brain development

1.2.1 Molecular biology of developmental changes in GABA_A receptor physiology

GABA_A receptors are pentameric ligand-gated ion channels that are permeable to chloride ions and to a lesser extent bicarbonate [reviewed in (Farrant and Kaila 2007; Galanopoulou 2008b)]. The subunit composition of each receptor complex determines its subcellular localization, pharmacological, and kinetic properties. Albeit GABA_A receptors are largely known as the principal inhibitory receptors in the mature brain, early in life they depolarize immature neurons (Ben-Ari et al. 1989; Cherubini et al. 1990). These paradoxical depolarizing effects of GABA are promoted by the relative accumulation of chloride inside the immature neurons, leading to chloride efflux once GABA_A receptor channels open.

Chloride regulation and the control of GABA_A receptor signaling are effected through chloride cotransporters and channels (Table I) (Payne et al. 1996; Delpire 2000; Farrant and Kaila 2007; Galanopoulou 2008b). The main proteins that increase intracellular chloride are the sodium-potassium-chloride cotransporters (NKCCs) and sodium-chloride cotransporters (NCCs), which import chloride along with the respective cations (sodium with or without potassium) in an electroneutral manner. To counteract their effects, the potassium chloride cotransporters (KCCs) export these ions decreasing intracellular chloride (Figure I). The best-studied representatives of these proteins are the neuronal-specific KCC2 isoform (KCC class) and the almost ubiquitous NKCC1 (NKCC class). During development, there is a gradual shift in the balance of these cotransporters from a NKCC1-dominant to a KCC2-dominant state, that ultimately favors the maintenance of reduced chloride concentration in more mature neurons and appearance of hyperpolarizing GABA_A responses (Plotkin et al. 1997; Rivera et al. 1999; Mikawa et al. 2002; Wang et al. 2002; Galanopoulou et al. 2003). In most studied rodent neuronal cells, this shift occurs during the first four weeks of life, a time that coincides with the switch of GABA_A receptor signaling from depolarizing to hyperpolarizing (herein referred as *GABA_A switch*) (Rivera et al. 1999; Galanopoulou et al. 2003; Khazipov et al. 2004; Banke and McBain 2006; Kyzozis et al. 2006; Galanopoulou 2008a). The GABA_A switch follows region and cell type specific tempos that can be gender-specific. It may also differ across the somatodendritic space (Romo-Parra et al. 2008).

1.2.2 Implications for normal brain development

GABA_A-mediated depolarizations are essential for normal brain development. The physiological processes affected by them have been detailed previously [reviewed in (Galanopoulou 2005; Akerman and Cline 2007; Ben-Ari et al. 2007; Farrant and Kaila 2007)]. In brief, GABA_A receptor mediated depolarizations activate voltage-sensitive calcium and sodium channels and relieve the blockade from N-methyl-D-aspartate (NMDA) receptors (Ben-Ari et al. 1997). Such effects influence the neuronal activity but also trigger cascades of calcium-sensitive signaling processes that ultimately control DNA synthesis, proliferation and migration, neuronal differentiation and communication (Reichling et al. 1994; Owens et al. 1996; Ben-Ari et al. 1997; Ganguly et al. 2001; Ben-Ari 2002; Kandler et al. 2002; Galanopoulou et al. 2003; Cancedda et al. 2007).

It is becoming increasingly more evident that disruption of the normal developmental patterns of depolarizing or hyperpolarizing GABA_A signaling can result in developmental abnormalities. Precocious termination of depolarizing GABA_A signaling in maturing neurons may have severe consequences for brain development. This is supported by a number of *in vitro* and *in vivo* studies that emphasize the importance of correct timing of the GABA_A switch. Precocious termination of depolarizing GABA_A receptor signaling via selective overexpression of KCC2 *in utero* in rat embryonic (E17–18) ventricular progenitors impairs their morphological differentiation and dendritic arborization, although it does not affect cortical migration (Cancedda et al. 2007). Similarly, global overexpression of KCC2 in zebrafish embryos decreased the number of spinal motoneurons and interneurons, decreased the axonal tracts and the size of the brain and spinal cord (Reynolds et al. 2008). Premature switch of GABA_A responses to hyperpolarizing by overexpression of KCC2 may shift the balance of glutamatergic versus GABA_Aergic postsynaptic responses in favor of the GABA_A-mediated inhibitory input (Chudotvorova et al. 2005; Akerman and Cline 2006). NKCC1 knockout mice exhibit a variety of deficits including impaired locomotion and pain perception, deafness, problems with fertility, salivation, and intestinal function (Delpire et al. 1999; Sung et al. 2000; Delpire and Mount 2002).

In contrast, prolongation of the developmental periods with depolarizing GABA_A receptor signaling, in KCC2 knockout mice, is lethal soon after birth due to motor and respiratory impairments (Hubner et al. 2001). Partial deficit in KCC2 increases the susceptibility to seizures and seizure-related hippocampal injury (Delpire and Mount 2002). To further corroborate the experimental data, mutations in chloride cotransporters and channels can be pathogenic in humans. Mutations in another KCC isoform, KCC3, have been linked with *Agenesis of Corpus Callosum and Peripheral Neuropathy* (ACCPN), a syndrome also associated with bipolar disease, and at least in transgenic mice lowers the threshold for seizures (Howard et al. 2002; Meyer et al. 2005) (Table I).

The importance of the age-related changes in the direction of GABA_A receptor signaling in normal development has long been known. In the current review, I will rather present more recent data implicating chloride cotransporters and GABA_A receptor signaling in the sexual differentiation of the brain and will discuss how these sex-specific developmental processes modify the effects of seizures and other early life stressors.

1.3 GABA_A receptors as mediators of sex-specific differentiating signals

The GABAergic system has been a favorite research target in studies of sexual dimorphism in the brain. Several sex differences in GABAergic indices have been documented in both developing animals (Table II) and adults (Table III). These refer to the numbers of GABAergic neurons, expression of proteins involved in GABA synthesis or metabolism or GABA_A receptors, as well as the responsiveness to GABA-acting drugs. The sex-specific phenotypic and functional differences in the GABAergic system may play key roles in the way the male and female mature brains operate. In adulthood, however, many of these differences are under the control of sex hormones and can often be reversed by gonadectomy or hormonal manipulations (Segovia and Guillamon 1993; Becu-Villalobos et al. 1997; Cooke et al. 1998; Knickmeyer and Baron-Cohen 2006a). To better understand how the sexual differentiation of the GABAergic system evolves, it is important to trace it back to the early developmental stages. Sexual differentiation of the brain starts very early, driven by the genomic differences, such as those dictated by the sex-determining region of the Y chromosome (*sry*) (Sinclair et al. 1990). The perinatal and early postnatal periods are viewed as critical or sensitive periods for defining the sexual differentiation of several brain regions. Such effects are usually linked to the perinatal testosterone surge and the influence of its estrogenic or androgenic metabolites. Among the signaling pathways involved in the sexual

differentiation of the brain is the GABA_Aergic. In the following paragraphs, I will present recent findings from the substantia nigra (SN) and hippocampus detailing how differences in the timing of GABA_A switch contribute to this process.

1.3.1 Sex-specific patterns of GABA_Aergic signaling in infantile rat SN

Although the SN is not among the classical nuclei involved in reproduction, it has a central role in networks involved in movement and information processing (Deniau et al. 2007; Field and Pellis 2007), functions known to be distinctly different in males and females. The SN is also involved in the pathophysiology of diseases that exhibit age and sex-specific patterns of vulnerability, like Parkinson's, Tourette syndrome, and epilepsy (Peterson et al. 1992; Moshé 1997; Kotsopoulos et al. 2005; Shulman 2007; Baym et al. 2008). It consists of two main compartments. The SN pars compacta (SNC) contains almost exclusively dopaminergic neurons, which are affected in Parkinson's disease and also participate in the networks implicated in Tourette's syndrome. The SN pars reticulata (SNR) consists of predominantly GABAergic neurons and a minority of dopaminergic neurons located in its posterior region (Gonzalez-Hernandez and Rodriguez 2000). It is now widely accepted that the GABAergic SN neurons form an important subcortical center controlling seizure propagation, as demonstrated in a variety of animal models of seizures (Iadarola and Gale 1982; Gonzalez and Hettinger 1984; McNamara et al. 1984; Moshé and Albala 1984) (De Sarro et al. 1984; Turski et al. 1990). Experiments in which intra-SNR infusions of GABA_Aergic drugs were done, revealed that the function of the SNR in seizure control changes with age (Moshé and Albala 1984; Sperber et al. 1987; Sperber et al. 1989) but is also different between sexes (Veliskova and Moshé 2001). A number of studies have outlined the age- and sex-specific features of SNR neurons, which might relate to its changing role in seizure control. These differences start very early during the embryonic stage, when neurogenesis appears to occur later in male than in female SNR (Galanopoulou et al. 2001) and continue postnatally. The developmental patterns in the expression of gonadal hormone receptors, at least as early as the day of birth in the rat, are sex-specific, suggesting different levels of responsiveness to sex hormones (Ravizza et al. 2002). During early postnatal development, the responsiveness of the SN neurons to gonadal hormones and GABA appears to be different in male and female pups, based on the documented differences in the expression of their receptors (Ravizza et al. 2002; Ravizza et al. 2003).

Given the abundant GABA_Aergic afferent input on the SN and the important role of depolarizing actions of GABA in neuronal differentiation, our first experiments explored whether the phenotypic differences between the male and female SNR might relate to differences in GABA_A receptor signaling. In situ hybridization experiments confirmed that KCC2 mRNA expression increased through development in both sexes, between PN15 (rat infantile stage) and PN30 (rat prepubertal stage) (Galanopoulou et al. 2003), similar to what had previously been reported for other structures (Rivera et al. 1999). However, at any given age, it was always higher in the female than in the male SNR (Galanopoulou et al. 2003). The earlier developmental rise in KCC2 expression in females correlated indeed with important physiological differences. In male PN15 rat SNR neurons, the GABA_A receptor agonist muscimol triggered depolarizing currents, increased calcium intracellularly, as well as the expression of the phosphorylated and active form of CREB (pCREB-ir; *cAMP responsive element binding protein-immunoreactivity*), and the expression of calcium-regulated mRNAs, like KCC2 (Galanopoulou et al. 2003; Galanopoulou 2006) (Figure I). In contrast, muscimol caused hyperpolarizing currents in female PN15 SNR neurons and failed to activate calcium signaling, as assessed by the lack in activation of pCREB or KCC2 transcription (Galanopoulou et al. 2003; Galanopoulou 2006). Muscimol, however, decreased KCC2 mRNA in female PN15 SNR neurons, probably through inhibition of a yet unknown regulatory pathway (Galanopoulou et al. 2003). These sex differences are not just pharmacological effects but can also be triggered by physiologic stimuli, such as synaptic stimulation of GABA_Aergic

synapses. Using synaptic stimulation of GABAergic SNR neurons in the presence of glutamatergic inhibitors, which leave only the GABA_Aergic synapses operative, the timing of GABA_A receptor switch from depolarizing to hyperpolarizing was found to occur earlier in female (PN10) than in male (PN17) SNR (Kyrozis et al. 2006). In terms of ontogenesis, and considering the faster development and shorter life span of rats, this 7-day difference may have enormous repercussions for brain development. Definitely, this is not the beginning of sexual differentiation. However, physiological activities, drugs or experiences that lead to activation of GABA_Aergic synapses in the infantile SNR may have different translational effects in males and females, influencing calcium-sensitive differentiation.

1.3.2 GABA_A receptor signaling as sex-specific modifier of estradiol effects

To further understand the mechanisms underlying the higher expression of KCC2 in the female SNR, we examined the *in vivo* regulation of KCC2 mRNA by gonadal hormones. As previously stated, the perinatal surge of testosterone in male rats is required for the masculinization of most studied sexually brain structures. Unlike humans, in rats, this is usually through the estrogenic derivatives of testosterone, produced through aromatization, and less often through the androgenic metabolites, like dihydrotestosterone (DHT) (Cooke et al. 1998). To determine whether KCC2 is regulated by gonadal hormones, the effects of systemic administration of testosterone, 17 β -estradiol or DHT on KCC2 mRNA expression in PN15 SNR were studied (Galanopoulou and Moshé 2003). Testosterone and DHT increased KCC2 mRNA expression in both male and female PN15 SNR neurons. In contrast, 17 β -estradiol decreased KCC2 mRNA in males but not in females. These effects were seen both after short (4 hours) or long periods (52 hours) of exposure to the hormones. However, they occurred only in neurons in which active GABA_A-mediated depolarizations were operative (naïve male PN15 SNR neurons). Estradiol failed to downregulate KCC2 in neurons in which GABA_A receptors or L-type voltage sensitive calcium channels (L-VSCCs) were blocked (bicuculline or nifedipine pretreated PN15 male rat SNR), and in those that had already hyperpolarizing GABA_A signaling (female PN15 SNR neurons). This indicated that 17 β -estradiol-mediated downregulation of certain calcium-regulated genes, like KCC2, shows a requirement for active GABA_A-mediated activation of L-VSCCs (Galanopoulou and Moshé 2003). In agreement with this model, *in vivo* administration of 17 β -estradiol decreased pCREB-ir in male but not in female PN15 SNR neurons (Galanopoulou 2006). The idea that the effects of estradiol on chloride cotransporters or GABA_A signaling may depend upon the direction of GABA_A responses is also reverberated in other publications. In hippocampal pyramidal neurons of adult ovariectomized female rats, where GABA_A signaling is thought to be hyperpolarizing, 17 β -estradiol had no effect on KCC2 expression (Nakamura et al. 2004). In contrast, in cultured neonatal hypothalamic neurons that still respond with muscimol-triggered calcium rises, thought to be due to the depolarizing effects of GABA_A receptors, 17 β -estradiol delays the period with excitatory GABA_A signaling (Perrot-Sinal et al. 2001). However, a direct involvement of KCC2 in this process has not been demonstrated yet. Such findings indicate that GABA_A signaling can not only augment the existing sex differences through pathways directly regulated by its own receptors, but can also interact indirectly and modify the effects of important neurotrophic and morphogenetic factors, like estradiol, at least in some neuronal types (Galanopoulou 2005; Galanopoulou 2006). It is possible that perinatal exposure to higher levels of the estrogenic metabolites produced by the testosterone surge in male pups could be one factor that maintains KCC2 expression lower in males. In agreement, daily administration of 17 β -estradiol in neonatal female rat pups, during the first 5 days of life, reduces KCC2 mRNA at postnatal day 15. This does not occur if 17 β -estradiol is given only during the first 3 days of postnatal life (personal unpublished data).

1.3.3 Applicability to other sexually dimorphic regions

Such signaling differences are not unique to the rat substantia nigra. In the CA1 pyramidal region of the male hippocampus, most prior studies done on male rats or rats of unspecified sex had placed the switch of GABA_A signaling at PN14 (Khazipov et al. 2004; Banke and McBain 2006; Tyzio et al. 2007). In our recent work on CA1 pyramidal neurons of the hippocampus of Sprague-Dawley rats, we found that GABA_A signaling acquired its hyperpolarizing mode of signaling at PN14 in males only. Female CA1 pyramidal neurons exhibit, however, earlier onset of hyperpolarizing GABA_A responses to physiological synaptic stimulation, which are already present by PN4 (Galanopoulou 2008a). This correlates with the higher expression of KCC2 and decreased activity of NKCC1-like, bumetanide-sensitive chloride cotransporters in females (Galanopoulou 2008a). The finding that GABA_A inhibition matures earlier in the female than in the male CA1 pyramidal region is also in agreement with earlier reports that *in vivo* muscimol administration increases pCREB-ir in male but decreases it in female rat hippocampus on the day of their birth (Auger 2003). On the other hand, Tyzio et al reported that at the time of birth, oxytocin triggers the transient appearance of hyperpolarizing GABA_A signaling in CA3 pyramidal neurons from rats of unspecified sex, by decreasing the activity of NKCC1 (Tyzio et al. 2006). Direct electrophysiological experiments are therefore warranted for that time period to determine whether sex or regional differences may explain these observations.

In the bed nucleus stria terminalis of PN5 Wistar rats, *in vivo* diazepam administration increased pCREB-ir and neuronal nitric oxide synthase (nNOS) only in male but not in female pups (Mantelas et al. 2007). This was blocked by L-VSCC inhibitors, implicating depolarizing GABA_A signaling in males (Mantelas et al. 2007). No such sex differences in response to diazepam were observed in the anterior or posterior rat PN5 hypothalamus (Mantelas et al. 2007). In a different study, high doses of muscimol, injected in Sprague-Dawley rats on the day of their birth, increased pCREB-ir in the median preoptic and mediobasal hypothalamic nuclei in males but decreased it in females (Auger et al. 2001). The same group of investigators identified differences in KCC2 mRNA (higher in females) and NKCC1 mRNA and protein (higher in males) that could potentially be linked with these pharmacological effects (Perrot-Sinal et al. 2007). However, similar levels of activated NKCC1 (phosphorylated form) was found (Perrot-Sinal et al. 2007).

In support of the sexually differentiating effects of GABA_A signaling in the developing brain are a number of studies that confirm that prenatal or early postnatal exposure of the developing brain to GABA_Aergic drugs may alter the behavior of the offspring in adulthood (Table IV). Further studies are however needed to directly test whether such effects are mediated by sex-specific patterns of GABA_A responses in the pertinent brain regions.

1.4 GABA_A receptors as sex-specific modifiers of the consequences of seizures

Depolarizing GABA_A signaling is thought to contribute to the enhanced excitability of the immature brain. In certain *in vitro* models of neonatal seizures, inhibitors of GABA_A receptors or of NKCC1 also inhibit or decrease the ictal epileptiform discharges (Khalilov et al. 2003; Khazipov et al. 2004; Dzhalal et al. 2005), although model-specific differences have been suggested (Kilb et al. 2007). These raise the possibility that the longer maintenance of depolarizing GABA_A signaling in certain brain regions of the developing male brain that participate in seizure networks may be one of the biological factors that render males more susceptible to seizures (Hauser et al. 1993; Kotsopoulos et al. 2005). However, direct proof of this hypothesis has not yet been offered by the available *in vivo* models of seizure induction, which test the behaviors of seizure networks rather than of individual neuronal structures. This

can partly be attributed to the fact that, until recently, males and females were used indifferently in studies involving developing rats. When focusing on individual brain regions, like the SNR, sex differences in their function in seizure control start to become evident. Pharmacological activation of GABA_Aergic SNR receptors has proconvulsant effects in male but not female PN15 rats (Moshé and Albala 1984; Sperber et al. 1987; Veliskova and Moshé 2001). Multiple factors appear to contribute to these sex specific effects, including the type and level of GABA_A receptor expression (Ravizza et al. 2003), hormonal influences (Veliskova and Moshé 2001), and possibly GABA_A-driven sexual differentiation (Galanopoulou et al. 2003; Galanopoulou and Moshé 2003; Galanopoulou 2005).

Of special importance is the need to clarify the mechanisms underlying the adverse outcomes of neonatal seizures that include cognitive deficits and epilepsy. In agreement with the human studies, late cognitive and developmental deficits have been demonstrated in a number of animal models of early life seizures (Holmes 1991; de Rogalski Landrot et al. 2001; Lee et al. 2001; Hoffmann et al. 2004; Sayin et al. 2004) (Wasterlain 1977; Hesdorffer et al. 1998; Pisani et al. 2007). Could such adverse effects be at least partially mediated by the disruption of GABA_A-sensitive brain development from the neonatal SE? The existence of sex-specific patterns of GABA_A signaling in the neonatal hippocampus (Galanopoulou 2008a) presented the opportunity to determine, in neurons with the same chronological age, whether the direction of GABA_A signaling influences the effects of neonatal SE. Three episodes of KA-SE, each induced at PN4–6 were utilized to simulate severe seizures. In the absence of neuronal injury in the hippocampus, 3KA-SE reversed the direction of GABA_A signaling in both sexes (Galanopoulou 2007; Galanopoulou 2008a). In male pups, 3KA-SE at PN4–6 led to a precocious switch of E_{GABA} to hyperpolarizing values. This was attributed to increased KCC2 expression and decrease in bumetanide-sensitive NKCC1-like activity. On the other hand, in female pups, 3KA-SE led to a transient re-appearance of depolarizing GABA_A signaling between PN8–14, associated with an increase in NKCC1-like activity (Galanopoulou 2008a). Considering the rapid rate of brain development, the disruption of GABA_A-driven neuronal differentiation in 3KA-SE pups during the second postnatal week may have potentially severe neurodevelopmental consequences. However, there is no evidence yet that 3KA-SE pups develop epilepsy, as no spontaneous seizures were seen in young adulthood (Galanopoulou 2008a). Unlike the findings with neonatal 3KA-SE, repetitive but brief flurothyl-induced seizures (5 per day, PN1–PN5) did not alter the timing of GABA_A switch in the CA3 region of the hippocampus of rats of unspecified sex (Isaeva et al. 2006). Although model or sex-specific differences may be involved, these findings corroborate the epidemiological data that indicate that prolonged seizures may have graver outcomes than recurrent brief seizures do (Hesdorffer et al. 1998; Pisani et al. 2007).

Interestingly, in our study, prolonged maternal separation also influenced the timing of GABA_A receptor switch. Both male and female pups subjected to prolonged sessions of maternal separation (6 hours daily, between PN4–6) manifested earlier switch of GABA_A signaling to hyperpolarizing, in the CA1 pyramidal region (Galanopoulou 2008a). Decreased bumetanide-sensitive NKCC1-like activity was noted in stressed pups, of both sexes, whereas stressed males also showed an increase in KCC2-ir (Galanopoulou 2008a). Duration of maternal separation appears to be important, as short daily periods of separation did not influence GABA_A switch (Isaeva et al. 2006). Although the effects of prolonged maternal separation were different than the effects of 3KA-SE, both were mediated by GABA_A receptors, as they were not observed in pups pretreated with the GABA_A receptor antagonist bicuculline.

1.5 Sex-specific GABA_A signaling: clinical implications

How relevant are these findings in the clinical field? Are there certain situations where sex-specific patterns in GABA_A signaling may influence the function of the human brain under normal or pathological conditions? The limitations of experimentation in humans have been an obstacle in obtaining direct evidence for or against any such associations. I will discuss therefore various situations where possible links may be worth exploring, as technology advances, as well as the challenges in doing so.

First, which is the developmental stage in humans that may be considered as equivalent to the time when GABA_A signaling may still exhibit its immature patterns? The only indirect evidence so far, suggesting that the switch probably occurs around the perinatal period, has been derived from post-mortem analysis of chloride cotransporter expression in the parietal cortices of patients dying from non-neurological causes (Dzhala et al. 2005). This study demonstrated a shift from a NKCC1-dominant state to a KCC2-dominant state around the time of birth (Dzhala et al. 2005).

It is tempting to associate such findings with occasional reports of ictogenic effects of GABA_A-acting drugs, like midazolam in low-birth-weight premature neonates (Montenegro et al. 2001; Ng et al. 2002). These are only sporadic reports and, at present, GABA_A-acting drugs, including benzodiazepines and barbiturates still remain the first line treatment for neonatal seizures. Even in cases where the reversal potential of GABA_A responses is depolarizing, it can still shunt excess excitation, as it occurs during seizures (Staley and Mody 1992). However, GABA_A-mediated shunting inhibition is less efficient than GABA_A inhibition occurring in more mature neurons with hyperpolarizing GABA_A signaling. If indeed depolarizing GABA_A signaling also occurs for longer developmental periods in the brain of male neonates, it could contribute to their higher susceptibility to early life seizures (Hauser et al. 1993). Specialized *in vivo* functional tests of GABA_A signaling would be however needed to test these associations.

Are disease syndromes with sex-specific predominance attributable to the reported differences in GABA_A signaling? For certain diseases, like Tourette's syndrome or autism, where clear distinction exists in their prevalence in males versus females, such associations appear meaningful. Autism, for instance, has been proposed as a paradigm of "extreme male brain", based on psychometric assessments that suggest that the male-type of thinking and functioning, i.e. "systemizing", are overrepresented over the female-type attributes, i.e. "empathizing" (Baron-Cohen 2002). Perinatal exposure to high levels of testosterone has been proposed to contribute to the exaggeration of the male-type features in autism (Knickmeyer and Baron-Cohen 2006b). Polymorphisms in GABA_A receptor subunits have been proposed as susceptibility traits for autism, suggesting that factors that control GABA_A signaling may be important in the pathogenesis of the disease (Maddox et al. 1999; Maestrini et al. 1999; Bass et al. 2000; Martin et al. 2000; Menold et al. 2001; Nurmi et al. 2001; Buxbaum et al. 2002; Hellings et al. 2002; McCauley et al. 2004; Muhle et al. 2004; Ma et al. 2005; Collins et al. 2006; Vincent et al. 2006).

Among the epileptic or seizure syndromes linked with mutations of GABA_A receptor subunits or chloride cotransporters, we can identify a few with mild male predominance. These include the autosomal dominant epilepsy with febrile seizure plus, febrile seizures, rare cases with severe myoclonic epilepsy of infancy, and epilepsy with grand mal upon awakening (Cossette et al. 2002; Harkin et al. 2002; Kananura et al. 2002; Wallace 2002; Haug et al. 2003; Marini et al. 2003; Dibbens et al. 2004; Macdonald et al. 2004; Audenaert et al. 2006). However, the same mutations have also been linked with syndromes without clear preference towards males or females. The contrast between the easiness in identifying biological sex differences in the

experimental literature and the often underwhelming findings of the clinical studies may be because the observational studies are strongly influenced by a combination of factors, including genetic, biological, epigenetic that cannot be as easily isolated as in experimental animals. Awareness though about the diversity of these processes between sexes will allow us to design studies and functional tests sensitive in identifying different sex-specific patterns in the expression, natural course, semiology, and response to treatments of these syndromes.

A direct implication of the finding that GABA_Aergic signaling is involved in the sexual differentiation of the brain is that GABA_A-acting drugs may have sex-specific effects in brain development. In the experimental literature, this has been demonstrated with multiple studies (Table II, Table IV). In humans, this is relatively unexplored and it is therefore important to further evaluate not only the acute but also the late and sex-specific effects of exposure to GABA_A-acting drugs that are commonly used for episodic or chronic disorders of early life or pregnancy, to better evaluate their consequences. If the primary goal in our practice and research is to cure epilepsy without side effects, as has been emphasized in the Epilepsy Research Benchmarks set during the “*Curing Epilepsy 2007: Translating Discoveries into Therapies*” NINDS-funded conference (Bethesda, MD), it will be important to design and implement therapies that take into account not only the developmental stage of the patient but also respect the specific needs of the male and female brain.

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Neurons with high $[Cl^-]_i$ (i.e. PN15 male SNR)

Neurons with low $[Cl^-]_i$ (i.e. PN15 female SNR)

Figure I. Sex-specific patterns in GABA_A signaling and ensuing control of KCC2 expression in PN15 rat GABAergic SNR neurons

In PN15 male SNR (left panel), there is relatively low expression of KCC2 compared to NKCC1, leading to high intracellular Cl^- ($[Cl^-]_i$). As a result, activation of GABA_A receptors depolarizes them and activates L-VSCCs, increasing intracellular calcium. This leads to increased expression of pCREB-ir and KCC2 mRNA expression. In these neurons, estradiol (E) downregulates KCC2 mRNA, through pathways interacting with GABA_A-mediated activation of L-VSCCs.

In PN15 female SNR (right panel), there is relative abundance of KCC2 expression, decreasing $[Cl^-]_i$. GABA_A receptor activation results in hyperpolarization, without altering intracellular

calcium or calcium-sensitive processes. Estradiol also cannot influence KCC2 expression in these neurons.

Table I
Selected proteins involved in Cl⁻ homeostasis with known effects on GABA_A signaling.

Protein	Function	Disease linkage	References
Favoring hyperpolarizing GABA_A responses			
Potassium Chloride co-transporters (KCCs)	Efflux of K ⁺ and Cl ⁻	<i>Humans</i> : Agenesis of corpus callosum with peripheral neuropathy and variants with bipolar disease <i>Knockout mice</i> epilepsy	(Payne et al. 1996; Delpire and Mount 2002; Howard et al. 2002; Meyer et al. 2005; Salin-Cantegrel et al. 2007)
Chloride channel 2	Efflux of Cl ⁻	<i>Humans</i> : Idiopathic generalized epilepsy; Lesion-related epilepsy. <i>Knockout mice</i> : Vacuolar leukoencephalopathy, blindness, decreased conduction velocity in central auditory paths, normal neuronal morphology.	(Haug et al. 2003; D'Agostino et al. 2004; Niemeyer et al. 2004; Bertelli et al. 2007; Blanz et al. 2007; Everett et al. 2007)
Na ⁺ dependent anion exchanger	Influx of Na ⁺ , HCO ₃ ⁻ ; Efflux of H ⁺ , Cl ⁻ .	N/A	(Kintner et al. 2007)
Favoring depolarizing GABA_A responses			
Sodium potassium chloride cotransporters (NKCCs)	Influx of Na ⁺ , K ⁺ and 2Cl ⁻	<i>Humans</i> : Bartter's syndrome type 1	(Delpire and Mount 2002)
Sodium chloride cotransporters (NCCs)	Influx of Na ⁺ , and Cl ⁻	<i>Humans</i> : Gitelman's syndrome	(Nicolet-Barousse et al. 2005)
Na ⁺ independent anion exchanger	Influx of Cl ⁻ ; Efflux of HCO ₃ ⁻ .	<i>Humans</i> : small susceptibility to Idiopathic generalized epilepsy (AE3). <i>Knockout mice</i> : Reduced seizure threshold	(Sander et al. 2002; Hentschke et al. 2006)

Table II

Sex differences in the developing brain, as they relate to the GABAergic system.

Parameter	Species / Age / brain region	Male vs female	Reference
GAD mRNA	Rat Sprague Dawley, dorsomedial nucleus, arcuate nucleus, CA1.	PN1 : M>F; PN15: M=F	(Davis et al. 1996)
GABA _A receptor $\alpha 1$ subunit mRNA	Rat Sprague-Dawley SNR (PN15 and PN30)	M<F	(Ravizza et al. 2003)
Number of GABA-ir neurons	Rat Sprague-Dawley striatum (E16 – E21); SNR (PN15, PN30);	M<F	(Ovtscharoff et al. 1992; Galanopoulou et al. 2001; Ravizza et al. 2003)
GABA _A switch to hyperpolarizing	Sprague-Dawley rats: SNR, SNC, CA1 pyramidal neurons of the hippocampus	Earlier in females	(Galanopoulou et al. 2003; Galanopoulou 2006; Kyrozis et al. 2006; Galanopoulou 2008a)
KCC2 expression	Sprague-Dawley rats: PN15–30 SNR, PN10 CA1 pyramidal neurons of the hippocampus	M<F	(Galanopoulou 2008a)
	Sprague-Dawley rats: Medialbasal hypothalamic nucleus (neonatal)	M<F	(Perrot-Sinal et al. 2007).
NKCC1 expression or activity	Sprague-Dawley rats: SNR, CA1 pyramidal neurons of the hippocampus	bumetanide-sensitive NKCC1-like activity: M>F	(Galanopoulou 2008a)
	Sprague-Dawley rats: Medialbasal hypothalamic nucleus (neonatal)	mRNA: M>F active protein: M=F	(Perrot-Sinal et al. 2007)
Muscimol effects	Neonatal Rat hypothalamus, CA1 pyramidal neurons	M: increase in pCREB F: decrease in pCREB	(Auger et al. 2001)
	Sprague-Dawley rat SN, (PN15)	M: increase in pCREB-ir, KCC2 mRNA. F: no effect	(Galanopoulou et al. 2003; Galanopoulou 2006)
Effects of infusions of GABA _A -acting drugs in the SNR	Sprague-Dawley rat SN, (PN15–30)	Sex specific changes in the function of SNR-mediated seizure control	(Veliskova and Moshé 2001)
Effects of pre- or perinatal exposure to GABA _A ergic drugs	Rats, mice	Sex and region specific effects on neurosteroid system, behavior, BDNF levels, accessory olfactory bulb, locus coeruleus, susceptibility to PTZ seizures	(Kellogg et al. 2006) (Sobrian and Nandedkar 1986; Pankaj and Brain 1991; Rodriguez-Zafra et al. 1993; Perez-Laso et al. 1994; Kellogg et al. 2000; Cannizzaro et al. 2001; Christensen et al. 2003; Nicosia et al. 2003)
Bicuculline effects on somatostatin.	Rats (periventricular median eminence)	M: stimulates somatostatin release (PN5-PN17, PN75) except for transient inhibitory effects at PN40). F: decreases somatostatin, starting at PN25.	(Murray et al. 1999)

M: males; F: females. PTZ: pentylentetrazole. BDNF: brain derived neurotrophic factor.

Table III

Sex differences in the adult brain, as they relate to the GABAergic system.

Parameter	Species / Age / brain region	Male vs female	Reference
Rodents			
GAD activity	Rat hypothalamus, substantia nigra	M=F	(Pericic et al. 1986; Manev and Pericic 1987)
GAD67 mRNA	Adult Sprague-Dawley rats: Medial amygdaloid nucleus	M>F.	(Searles et al. 2000)
GAD65 mRNA	Diagonal Band of Broca: Dorsomedial nucleus:	M>F; M<F.	
GABA content	Adult Sprague-Dawley rats: Nucleus accumbens, Medial preoptic area, Ventromedial hypothalamus.	M>F (diestrous)	(Frankfurt et al. 1984)
	Diagonal Band of Broca,	M>F (diestrous)	
GABA turnover	Adult Sprague Dawley rat hypothalamus	M>F	(Searles et al. 2000)
Number of GABA-ir neurons	Adult rat Sprague-Dawley: Bed Nucleus Stria Terminalis, amygdala.	M>F	(Stefanova 1998)
GABA transaminase	Rat brain	M<F	(Sherif et al. 1991)
Muscimol binding	Adult wood mice: Caudate-putamen, Anterior Hypothalamic Nucleus.	M>F	(Canonaco et al. 1996)
	Bed Nucleus of the Stria Terminalis, Ventromedial hypothalamic nucleus, Ventral lateral Thalamic nucleus, Pontine Central Gray, SNR.	M<F	
	Adult Sprague Dawley rats: Cortex	M>F	(Kokka et al. 1992)
Benzodiazepine (BZ) binding (flunitrazepam)	Adult Wistar rats: Frontal Cortex	Affinity: M>F BZ receptors: M<F	(Farabollini et al. 1996)
Picrotoxin or bicuculline-induced seizures or EEG changes	Adult Sprague-Dawley and Wistar rats, cats, CBA/H and swiss mice	Sex and species-specific effects on latencies to seizures, ED50, mortality, EEG findings	(Pericic et al. 1986) (Bujas et al. 1997; Pericic and Bujas 1997; Matejovska et al. 1998; Tan and Tan 2001)
Bicuculline induced inhibition of post-gonadectomy rise in LH	Adult rats	M: no effect F: present	(Hood and Schwartz 2000)
Bicuculline induced inhibition of post-gonadectomy rise in LH	Adult rats	M: no effect F: present	(Hood and Schwartz 2000)
Pentylenetetrazole-induced seizures	Adult Sprague Dawley rats, Adult Swiss mice	Threshold to seizures: M≤F	(Kokka et al. 1992; Medina et al. 2001)
Humans			
GABA levels (Magnetic resonance spectroscopy)	Humans, occipital cortex	M<F	(Sanacora et al. 1999)
sDiazepam	Humans	Sex differences in EEG power and coherent activity	(Romano-Torres et al. 2002)
Time to recovery from anesthesia	Humans	M>F	(Bajaj et al. 2007)
Zolpidem, triazolam effects	Humans	No significant sex difference on sedation, poor recall, impaired	(Greenblatt et al. 2000)

Parameter	Species / Age / brain region	Male vs female	Reference
Rodents			
		psychomotor performance, beta EEG	

M: males; F: females. GAD: glutamic acid decarboxylase.

Table IV

Sex-specific effects of perinatal exposure to GABAergic drugs on brain development and behavior.

Treatment	Endpoint	Male vs female	Reference
Diazepam (DZ), 1.5mg/kg/day SC, E14–20, Wistar rats	Open field activity (OFA) and acoustic startle reflex in adulthood.	<u>M-DZ</u> : reduced total distance traveled, higher rearing frequency. <u>F-DZ</u> : less time spent in central squares. <u>M-DZ and F-DZ</u> : higher acoustic startle amplitude	(Cannizzaro et al. 2001)
DZ 2.5 – 10 mg/kg/day, E14–20, rats.	Dopamine- β -hydroxylase (DBH)-ir and corticotropin releasing factor (CRF) neurons in adult males.	<u>M-DZ</u> : Decreased DBH-ir and CRF neurons in paraventricular nucleus of the hypothalamus.	(Inglefield et al. 1993)
1–2.5 mg/kg/day, E14–20, rats	Elevated plus maze DZ	<u>M-DZ</u> : more time spent in open arms; <u>F-DZ</u> : no difference.	(Kellogg et al. 1991)
	Social interaction in adult rats.	<u>M-DZ</u> : increased in unfamiliar but decreased in familiar environment. <u>F-DZ</u> : decreased in unfamiliar environment.	
DZ 10 mg/kg/day prenatally, rats	Acquisition or retention of a simultaneous choice discrimination task in adults.	<u>DZ in 2nd gestational week</u> : No effect. <u>DZ in 3rd gestational week</u> : impaired in males. <u>DZ given E3–18</u> : impaired in both sexes, worse in F.	(Frieder et al. 1984)
DZ 2.5 mg/kg/day prenatally or postnatally.	OFA, continuously reinforced lever-pressing responses in adults.	<u>DZ in 3rd gestational week</u> : no sex differences in adulthood. <u>DZ at PN0–16</u> : no sex differences in OFA; impaired level-pressing behavior in M only.	(Guillamon et al. 1990)
DZ 1–2.5 mg/kg/day, postnatally, Wistar rats	Maternal behavior.	<u>DZ at PN0–16</u> : Positive effects in both F and M.	(Del Cerro et al. 1995; Segovia et al. 1996)
DZ 2.5 mg/kg/day E14–21, Wistar rats	Neonatal reflexes (cliff aversion, forelimb pacing and grasping, bar holding).	<u>Offspring-DZ</u> : delayed appearance of neonatal reflexes.	(Nicosia et al. 2003)
DZ prenatal (E14–21) or postnatal (PN0–21) Wistar rats	PTZ seizures (PN50).	<u>Offspring-DZ</u> : M>F	(Nicosia et al. 2003)
Prenatal DZ.	Sensitivity of GABA _A receptors to Cl ⁻ , sensitivity to bicuculline.	<u>Offspring-DZ</u> : Altered sensitivity in adults; in M environmental stressors further alter these effects.	Reviewed in (Kellogg 1999)
Prenatal (E15–20) exposure to antiepileptics (AEDs: Phenobarbital 2mg/kg/day, clonazepam 0.15mg/kg/day, valproic acid 20 mg/kg/day), Sprague-Dawley rats.	Spontaneous alternation (PN43–45).	<u>Controls</u> : M: persevered, F: random alternations. <u>AED in utero</u> : M: random alternations, F: persevered.	(Sobrian and Nandedkar 1986)
	Spontaneous activity (PN43–45)	<u>M-clonazepam</u> : hypoactive. <u>F-phenobarbital/valproic acid</u> : hyperactive.	(Sobrian and Nandedkar 1986)
	Righting reflex (PN1–14); Resident-intruder test (PN21).	<u>Righting reflex</u> : prenatal treatments resulted in sporadic days with significant changes. <u>M-Ro</u> : immobility, more attacks. <u>F-Ro</u> : more digging, immobility. <u>M-CGSP</u> : less grooming. <u>F-CGS</u> : less defensive.	(Pankaj and Brain 1991)

Treatment	Endpoint	Male vs female	Reference
Alprazolam (AL) 0.32 mg/kg/day E18, C57BL/6 mice.	Social play, sleep/wake patterns, aggression at prejuvenile (PN17), juvenile (PN25), adults.	<u>Offspring-AL (PN17)</u> : Less desire to escape, isolated behavior, shorter awake periods. <u>M-AL (juvenile, adult)</u> : more aggression on food restriction and cage changing.	(Christensen et al. 2003)

M: male; F: female; AED: antiepileptic; AL: alprazolam; BZ: benzodiazepine; CRF: corticotropin releasing factor; DBH: dopamine- β -hydroxylase; DZ: diazepam; E: embryonic day; OFA: open field activity; PN: postnatal day; PTZ: pentylentetrazole; M-DZ or F-DZ: male or female offspring exposed to DZ; M-AL or F-AL: male or female offspring exposed to AL; M-Ro or F-Ro: male or female offspring exposed to Ro15-1788; M-CGS or F-CGS: male or female offspring exposed to CGS8216.