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Sex dimorphism in seizure-controlling networks

Fillippo Sean Giorgi^{a,*}, Aristea S. Galanopoulou^b, and Solomon L. Moshé^{b,c}

^a Department of Clinical and Experimental Medicine, Section of Neurology, University of Pisa–Pisa University Hospital, I56126 Pisa, Italy

^b Saul R. Korey Department of Neurology, Laboratory of Developmental Epilepsy, Montefiore Epilepsy Management Center, and Dominick P. Purpura Department of Neuroscience, Bronx, NY, 10461, USA

^c Department of Pediatrics, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY, 10461, USA

Abstract

Males and females show a different predisposition to certain types of seizures in clinical studies. Animal studies have provided growing evidence for sexual dimorphism of certain brain regions, including those that control seizures. Seizures are modulated by networks involving subcortical structures, including thalamus, reticular formation nuclei, and structures belonging to the basal ganglia. In animal models, the substantia nigra pars reticulata (SNR) is the best studied of these areas, given its relevant role in the expression and control of seizures throughout development in the rat. Studies with bilateral infusions of the GABA_A receptor agonist muscimol have identified distinct roles of the anterior or posterior rat SNR in flurothyl seizure control, that follow sex-specific maturational patterns during development. These studies indicate that (a) the regional functional compartmentalization of the SNR appears only after the third week of life, (b) only the male SNR exhibits muscimol-sensitive proconvulsant effects which, in older animals, is confined to the posterior SNR, and (c) the expression of the muscimol-sensitive anticonvulsant effects become apparent earlier in females than in males. The first three postnatal days are crucial in determining the expression of the muscimol-sensitive proconvulsant effects of the immature male SNR, depending on the gonadal hormone setting. Activation of the androgen receptors during this early period seems to be important for the formation of this proconvulsant SNR region. We describe molecular/anatomical candidates underlying these age- and sex-related differences, as derived from *in vitro* and *in vivo* experiments, as well as by [¹⁴C]2-deoxyglucose autoradiography. These involve sex-specific patterns in the developmental changes in the structure or physiology or GABA_A receptors or of other subcortical structures (e.g., locus coeruleus, hippocampus) that may affect the function of seizure-controlling networks.

Keywords

Substantia nigra pars reticulata; GABA receptor; Dimorphism; Androgen receptor; Estrogen receptor; Critical period; KCC2; Rat; Immature; Locus Coeruleus; Hippocampus; Seizures; Epilepsy

Introduction

Various subcortical structures play a critical role in modulating seizures. A great deal of data has been provided concerning a role for thalamus, especially in models of primary generalized epilepsy (see the review by Avanzini et al., 2000), and several structures belonging to the basal ganglia–cortical circuits have been extensively studied. Among them a pivotal role in seizure modulation seems to be played by the substantia nigra pars reticulata (SNR) as well as the superior colliculus (SC) and subthalamic nucleus (STN) through their connections with the SNR (Garant and Gale, 1987; Lado et al., 2003; Velísková et al., 1996a). There is also evidence concerning the role of monoaminergic subcortical nuclei, and in particular for the noradrenergic (NE) locus coeruleus (LC), and the serotonergic (5HT) raphe nuclei. These structures are part of the brainstem reticular formation, are formed by neurons whose axons widely innervate the cortical mantle, such as the noradrenergic axons, and exert a modulatory role on their targets.

In the last two decades, it has been clearly shown that the SNR has significant sex-related effects on different types of seizures, and that some of these vary significantly during development. Most of the present review will primarily address the sex-specific features of the SNR, including its differentiation, morphology and function in seizure control, and will discuss potential mechanisms and implications of such a dimorphism. Lastly, we will also address the existing evidence for a potential dimorphic role of other seizure-controlling structures.

The SNR: a predominantly GABAergic basal ganglia nucleus

The SNR constitutes the main part of substantia nigra (SN). The SNR is formed almost exclusively of fast-spiking GABAergic cells (Gerfen, 2004; Schultz, 1986), which are much less densely packed than their dopaminergic counterpart of the pars compacta of the SN (SNpc). A few dopaminergic cells can also be found in the posterior SNR (SNRp) (González-Hernández and Rodríguez, 2000). The SNR together with the globus pallidus internal (GPi), which in rodents is represented by the entopeduncular nucleus, is the main output structure of the basal ganglia.

SNR neurons receive afferents from the STN, lateral globus pallidus (GP) and from the striatum. They send axon terminals to structures outside of the basal ganglia, namely the thalamus, the pedunculopontine tegmental nucleus (PPT) and the SC (Gerfen, 2004); in addition, each GABAergic SNR neuron sends axon collaterals to neighboring SNR neurons (Deniau et al., 1982; Grofova et al., 1982). Specific subregions of the SNR receive projections from specific subregions of the afferent nuclei, and such a topographical segregation is maintained also in the targets of SNR efferents (reviewed in Gerfen, 2004),

The thalamic targets of SNR include bilateral projections to the ventromedial (VM), parafascicular, centromedian and paracentral nuclei and unilateral projections to the centrolateral, mediodorsal and thalamic reticular nucleus (Gulcebi et al., 2012).

Communication via GABA_A receptors (GABA_AR) helps orchestrate the net activity of SNR neurons, although other neurotransmitter systems such as glutamatergic receptors play a role too (Zhou and Lee, 2011). GABA_ARs are pentameric ligand-activated ionic channels permeable to chloride ions and, to a lesser extent, to bicarbonate (Farrant and Kaila, 2007; Galanopoulou, 2008b). The precise composition of GABA_ARs in terms of subunits types determines their kinetics, affinity to drugs, sub-cellular localization (extra- or post-synaptic), or region-specific expression in a complex manner (Galanopoulou, 2008b; Mohler, 2006). Usually, functional channels comprise two α and two β subunits. When the fifth subunit is a δ subunit, GABA_AR are extrasynaptic and responsible for tonic current generation. In contrast, GABA_AR with a γ subunit are most commonly post-synaptic, generating phasic inhibitory post-synaptic currents (IPSCs), but can also be found at extrasynaptic sites (reviewed in Galanopoulou, 2008b).

GABA_AR signaling classically induces neuronal hyperpolarization, due to an influx of Cl⁻, which follows the electrochemical Cl⁻ gradient between the extracellular and the intracellular compartments. The intracellular Cl⁻ concentration ([Cl⁻]_i) is regulated by cation Cl⁻ cotransporters. These include Cl⁻ exporters, like K⁺/Cl⁻ cotransporters (KCCs), and Cl⁻ importers like the Na⁺/K⁺/Cl⁻ cotransporters (NKCCs). During development, there is a gradual shift in the expression and activity of two main representatives, mainly NKCC1 which declines (Plotkin et al., 1997) and KCC2 that increases with age (Rivera et al., 1999). As a result, the [Cl⁻]_i is considerably higher in most studied immature neurons compared to the mature ones. Thus, activation of GABA_ARs in immature neurons with high [Cl⁻]_i triggers depolarizing potentials due to the efflux of Cl⁻ and hyperpolarizing potentials in mature neurons with low [Cl⁻]_i, due to Cl⁻ influx. The early depolarizing GABA_AR signaling is essential for normal brain development, as it results in depolarization-induced activation of L-type voltage-sensitive calcium channels (L-VSCCs) and NMDA receptors (reviewed in (Ben-Ari, 2002; Farrant and Kaila, 2007; Galanopoulou, 2008b). In fact, precocious termination of the depolarizing GABA effects may have serious adverse effects in the way normal neurons develop and arborize to form synaptic connections (Cancedda et al., 2007; Wang and Kriegstein, 2008, 2011). It is worth noting that in the normal brain the GABA_AR-mediated depolarizations are not necessarily excitatory, as they can still induce a weaker form of inhibition, shunting inhibition, when neuronal depolarization exceeds the reversal potential of GABA_ARs (Staley and Mody, 1992).

The role of SNR in seizure control in males

In the early 1980s, it was shown that in male adult rats, microinfusions of muscimol (a GABA_AR agonist) in the SNR exert anticonvulsant effects toward different types of experimental seizures such as tonic hindlimb extension in the maximal electroshock test and tonic and clonic seizures produced by pentylenetetrazole and bicuculline (Iadarola and Gale, 1982), as well as toward flurothyl-induced clonic seizures (Okada et al., 1986), a seizure model which allows a precise quantification of the seizure threshold. [¹⁴C]2-deoxyglucose

(2-DG) auto-radiographic studies showed that the SNR was differently involved in kainic acid-induced seizures in adult male rats versus male rat pups (Albala et al., 1984) and, surprisingly, that bilateral muscimol microinfusions into the SNR of 15-17 postnatal days (PN) old male rat pups were proconvulsant in flurothyl-induced clonic seizures (Garant et al., 1995; Moshé and Albala, 1984; Okada et al., 1986; Sperber et al., 1987). The age-related differences in the modulation of the nigral output systems following various pharmacological manipulations were documented in several studies summarized in Table 1 and point out that there is an age-related role of the SNR in the control of seizures but also in motor control. Indeed, given the known higher propensity of the immature CNS to seizures and status epilepticus (Moshé and Albala, 1983; Moshé et al., 1983), the SNR has been considered as an important candidate to justify such an age-related shift in seizure susceptibility and expression that may be involved in human seizures too.

In parallel with the growing amount of data on the role of SNR on seizures, another important observation was made in the mid-90s. Detailed analysis on the effects of the spatial features relating the microinfusions of pharmacologic agents into specific regions of SNR along its anterior–posterior axis led to the discovery that actually the SNR of adult male rats can be divided into two functionally separate regions, an anterior (SNRa) and a posterior one (SNRp), bearing different effects in the flurothyl-induced clonic seizures (Moshé et al., 1994; Sperber et al., 1999; Velísková et al., 1996b; Velísková and Moshé, 2001). In particular, in adult male rats, bilateral muscimol infusions are anticonvulsant if infused in the SNRa and proconvulsant if infused in the SNRp (Sperber et al., 1999). Region-specific effects were also noted for the GABA_AR agonist (Z)-3-[(aminoiminomethyl)thio] 2-propenoic acid (ZAPA) and γ -vinyl-GABA which increases local GABA levels (Velísková et al., 1996b). In contrast, bicuculline (a GABA_AR antagonist) infusions were proconvulsant into the SNRa and without any effect in the SNRp (Velísková et al., 1996b) (see also Table 1).

A remarkable observation was that in male rat pups, up to PN21 (see also below), such a regional differentiation was not present, and muscimol microinfusions were always proconvulsant, irrespective of the site of SNR infusion (Moshé et al., 1994; Sperber et al., 1999; Velísková and Moshé, 2001).

***In vivo* evidence for a gender-related effect of SNR toward seizures**

For almost two decades since the discovery of a prominent role of SNR in seizure susceptibility, all of the above-mentioned studies, either in adult or immature rats, were performed in males, as it is usually done in rodent research. The biasing role of such aspect became clear only many years later, when the role of sex was analyzed for the first time. Such studies, together with an in-depth analysis of the interaction of sex with brain development, provided unexpected results.

The first studies in which the role of sex was analyzed were performed by Moshé's group using bilateral muscimol infusions either in the SNRa or SNRp. It was shown that although muscimol infusions in the SNRa had anticonvulsant effects in both male and female adult rats, there was a clear sex difference when muscimol was infused in the SNRp, eliciting

proconvulsant effects in males and having no effect in females (Moshé, 1997; Velísková and Moshé, 2001). In fact, a detailed developmental study on the effects of bilateral muscimol infusions in flurothyl seizure control revealed the following.

- (a) The functional differentiation between the SNRa and SNRp in seizure control starts to appear after PN21, in both sexes, but the effects of muscimol are sex-specific in PN15–21: proconvulsant in PN15–21 males, without effect in PN15–21 females.
- (b) The muscimol-sensitive proconvulsant SNR region is present in males only. It is present irrespective of SNR region between PN15–21 and only by the SNRp infusions in older male rats.
- (c) The muscimol-sensitive anticonvulsant SNR region is localized to the SNRa, in both sexes, but emerges earlier in females (PN25) than in males (PN30).

The role of sex hormones in the development of SNR sex dimorphic effects on seizures

Sex hormones have a key role in orchestrating sexual dimorphic features (for review, see Shah et al., 2012). During brain development, sex hormones have organizational effects which induce permanent anatomical differences between males and females in specific brain structures (McEwen, 1999). In early development, the levels of testosterone (T) are critical for the formation of male or female phenotypic features of the brain that eventually will be expressed in adulthood (McEwen, 1999). T can affect brain development, either a) through the action of T or its metabolite dihydrotestosterone (DHT) on androgen receptors (AR) or, b) through the action of the aromatase-mediated metabolite 17 β -estradiol (E) on estrogen receptors (ER) (McEwen et al., 1982; McEwen and Parsons, 1982).

Most of these dimorphic effects are linked to the perinatal surge in T levels in males (Arnold and Gorski, 1984), which occurs around gestational days 18–19 (Ward and Weisz, 1984; Weisz and Ward, 1980) and during the first few hours following birth (Baum et al., 1988; Corbier et al., 1978; Slob et al., 1980). Regional differences in hormonal levels have been found across brain regions and these may be even higher in sexually dimorphic structures (Konkle and McCarthy, 2011). In fact, there is a so-called “critical period” during which sex hormones exert their organizational effects in many brain structures and in rats the first 6 days of life are considered particularly critical for the effects of T and its metabolites (Goy and McEwen, 1980). The levels of gonadal hormones in the brain are not however only defined by the peripherally circulating hormones but also by the local steroidogenesis in certain brain regions and the metabolism of existing steroids. For example, the levels of E at birth vary significantly across brain regions, suggesting region-specific levels of local estradiol synthesis in the brain (Konkle and McCarthy, 2011). Furthermore, in the hippocampus or hypothalamus, the local steroid levels (T, DHT, E) are not affected by neonatal (PN0) adrenal-ectomy or gonadectomy, suggesting that local steroidogenesis in these regions utilizes substrates different than the peripherally circulating T (Konkle and McCarthy, 2011). In fact, the idea that only T can serve as substrate for E production has

been contested, and the possibility that other C19 steroids can also be converted to E has been proposed (Lieberman, 2008).

Concerning the role of SNR on seizures, Velísková and Moshé (2001) showed that postnatal T is responsible for the male proconvulsant phenotype of SNR muscimol effects on flurothyl seizures. They showed that: a) castration of males at PN0 produces the female-like responses of SNR muscimol on clonic flurothyl-induced seizures at PN15 (i.e., lack of any effect on seizures); since this change did not occur in sham operated males a causative role for perinatal stress could be ruled out (Velísková and Moshé, 2001); b) daily administration of T from PN0 to PN14 to either females or to PN0-castrated males induces the appearance of the SNR proconvulsant phenotype, a characteristic of intact males (Velísková and Moshé, 2001).

Subsequent studies investigated which T metabolite (i.e., DHT or E) was responsible for this maturational effect, and which was the precise duration of this critical period (Giorgi et al., 2007). The data showed that: (a) PN0-2 seems to be the “gonadal hormone sensitive period” for the formation of the muscimol-sensitive proconvulsant phenotype of the male SNR and (b) both estrogenic [diethylstilbestrol (DES)] and androgenic [T or DHT] compounds given between PN0 and PN2 either in females or in PN0-castrated males, can induce the appearance of the muscimol-sensitive proconvulsant SNR function. A further series of experiments, by Heida et al. (2008) strengthened this *scenario*, by showing that, in males, the early postnatal (PN0–2) systemic administration of flutamide (an AR antagonist) is sufficient to lead to the disappearance of the proconvulsant muscimol effects at P15. The latter observation was somehow unexpected, since it had been previously observed that the masculinization of the brain is mainly mediated by the action of estrogen (reviewed in Shah et al., 2012), while an exclusive role of androgens has been considered for a long time to be implicated in few brain structures, and especially in the spinal cord (i.e. nucleus bulbocavernosus (Breedlove et al., 1982)). However, there are complex interactions between the AR and ER responsive pathways that could further complicate these interpretations. For instance, perinatal flutamide administration has been shown to increase the ovarian production of E in pigs, by enhancing aromatization (Grzesiak et al, 2012). Indeed, a shared effect of androgens and estrogens seems to be relevant for some masculinizing effects (Breedlove, 1997; Han and De Vries, 2003; Shughrue and Dorsa, 1994).

Thus, activation of both AR and ER appears to be important for the presence of proconvulsant SNR muscimol responses. Interestingly, a relevant role of androgens for the sexual differentiation in the brain, has been observed in other brain regions such as amygdala, hippocampus, hypothalamus, and LC (Garcia-Falgueras et al., 2005; Jones and Watson, 2005; Morris et al., 2005), as witnessed by studies using male rats with testicular feminization mutation. The effects of early sex hormone manipulations on the SNR have been tested also in terms of the gonadal hormone receptor expression. In particular, Ravizza et al. (2003a) analyzed the gonadal receptor expression in the rat SNR at PN1, and found that, while SNR expression of ER α is similar in the two sexes, female rats showed a higher AR and ER β immunoreactivity (-ir) compared with males. Females treated with T at PN1 had lower levels of both AR and ER β than controls. Interestingly, the number of AR- and ER-expressing cells was not affected by this treatment. Even though these data do not allow

to extrapolate precise functional effects, they suggest that the hormonal receptor expression itself in the SNR differs in males and females, is dependent on the early postnatal hormonal setting, and might self-perpetuate a structural/functional maturation of SNR in a sex-specific manner.

Evidence for sexual dimorphism of the GABA_AR expression in the rat SNR

The molecular basis for the differences of SNR response *in vivo* and *in vitro* (see below) to GABAergic agents is still under investigation. The GABA_AR subunit composition is a potential candidate, given its role in the functional effects of GABA (reviewed in Galanopoulou, 2008b; Olsen and Sieghart, 2009) (Table 2). Thus, it has been shown by *in-situ* hybridization, that the distribution of $\alpha 1$ and $\gamma 2$ GABA_AR subunit positive cells was homogeneous across the SNRa and SNRp in PN15 male rats. However, in PN21 and older animals, the SNRa appeared to be less densely populated by $\alpha 1$ - or $\gamma 2$ -positive cells, but individually stained SNRa cells contained more silver grains than those in the SNRp (Velísková et al., 1998a). Similar regional differences were reported for the $\alpha 1$ mRNA expression in the female SNR (Ravizza et al., 2003b). Furthermore, when comparing males and females, within each SNR region, expression of $\alpha 1$ subunit mRNA was always higher in females compared to age-matched males (Ravizza et al., 2003b).

In 2009, Chudomel et al. reported the protein expression of $\alpha 1$ and $\alpha 3$ subunits in the SNRa of PN5-30 male and female rats, using immunohistochemistry and compared it with the developmental changes in the kinetics of GABA_AR-IPSCs. A developmental increase in perisomatic $\alpha 1$ -ir and decrease in $\alpha 3$ -ir was observed in both males and females, between PN5 and PN30 (Table 2). This developmental swap of $\alpha 3$ by $\alpha 1$ subunits is well known to occur in several brain regions, affecting the kinetics and drug sensitivity of GABA_AR-IPSCs (Chudomel et al., 2009; Gingrich et al., 1995; Lavoie et al., 1997; Verdoorn, 1994). Indeed, in whole cell patch clamp experiments, the kinetics of IPSCs became faster with age and more responsive to the $\alpha 1$ -preferring agonist zolpidem, consistent with the increase in $\alpha 1$ -ir (Chudomel et al., 2009). The only sex differences observed were a higher expression of $\alpha 1$ -ir and $\alpha 3$ -ir in female PN5 SNRa paralleled by a greater sensitivity of female PN5-9 SNRa neurons to zolpidem, compared with same age males.

A pattern similar to the one above described for the $\alpha 1$ subunit applies for the content of GABA within PN15 and PN30 SNR neurons (Ravizza et al., 2003b): this is lower, in general, in males than in same age females, and is also higher in SNRa than in SNRp neurons of PN30 rats (Ravizza et al., 2003b) (see Table 2).

Earlier appearance of hyperpolarizing GABA_AR signaling in female SNR: is it relevant to its muscimol-sensitive anticonvulsant function?

To investigate whether the sexually dimorphic function of the SNR involved sex differences in the physiology of local SNR neurons, Galanopoulou et al. (2003) studied the electrophysiological properties of GABA_AR of PN14-17 SNR GABAergic neurons using gramicidin perforated patch clamp recordings, which preserve the $[Cl^-]_i$ (Kyrodis and Reichling, 1995). Bath application of muscimol depolarized GABAergic SNR neurons from

male pups, while it hyperpolarized female SNR neurons (Galanopoulou et al., 2003). In parallel, fura2AM imaging of SNR GABAergic neurons demonstrated that muscimol bath application induced an increase in $[Ca^{++}]_i$ in male SNR neurons, but not in female SNR neurons. These observations altogether were exciting since they seemed to show that single cell SNR phenomena could underlie at least in part a complex sex-related difference occurring *in vivo* (i.e. the modulatory role of GABA SNR manipulation toward seizures), which is likely to involve several structures apart from SNR itself. By using the same experimental protocol, it was also demonstrated that in the SNRa the switch of GABA_AR-mediated postsynaptic currents occurs around PN17 in males and around PN10 in females (Kyrozis et al., 2006).

Additional studies demonstrated that GABA_AR agonists such as muscimol can only activate the transcription of calcium-sensitive genes, like KCC2, or increase the phosphorylation of the transcription factor *cAMP responsive element binding protein* at Ser133 (pCREB) in PN15 male but not in PN15 female SNR neurons (Galanopoulou et al., 2003; Galanopoulou, 2006), confirming that this sexual dimorphism in GABA_AR signaling has broader repercussions on gene transcription and potentially in neuronal differentiation and sexual differentiation of the SNR.

Sex hormones may influence the mRNA expression of KCC2 in PN15 male and female SNR (Galanopoulou and Moshé, 2003). Administration of 17 β -E reduced KCC2 mRNA expression in only PN15 male SNR neurons and this effect occurred in the presence of GABA_AR depolarization-induced L-VSCC activation. In contrast, T or DHT increased KCC2 mRNA expression in PN15 rat SNR, regardless of the direction of GABA_AR signaling. These observations suggest that the higher perinatal E levels in males could suppress KCC2 gene expression, delaying the developmental increase of KCC2 and appearance of hyperpolarizing GABA_AR signaling. Although the local levels of E in the neonatal male SNR have not been measured, it is possible that the local aromatization of the elevated circulated T in males could result into more estrogenic derivatives.

Neonatal hormonal manipulations that feminized the function of the PN15 SNR in seizure control were also capable of switching the responses of GABA_ARs in PN13-16 SNR neurons (Table 3) (Giorgi et al., 2007). Castration of neonatal male rats rendered GABA_ARs in PN13-16 SNR neurons hyperpolarizing, as in females, regardless of the age the castration was performed (PN0 or PN3). Furthermore, early hormonal administration (DES or DHT) between PN0-PN2, in PN0-castrated males or intact females, was not sufficient for the depolarizing GABA_AR responses characteristic of PN13-16 SNR neurons to emerge. Parallel studies showed that more prolonged daily exposure to E (PN0-4 but not PN0-2) was needed to reduce KCC2 mRNA expression and permit the depolarizing GABA_A receptor responses to appear in the PN15 SNR (Galanopoulou, unpublished data) (Galanopoulou, 2008a).

The above findings suggest that, in PN15 male SNR, the muscimol-sensitive proconvulsant function is not directly mediated by the depolarizing GABA_AR signaling of SNR neurons. In further support is the observation that the disappearance of the muscimol-sensitive proconvulsant SNRa role occurs after PN21 (Velísková and Moshé, 2001), while the

depolarizing GABA_AR responses of SNRa neurons disappear by PN17 (Kyrozis et al., 2006). However, these observations cannot exclude that the presence of depolarizing GABA during the neonatal period, could promote the differentiation of the muscimol-sensitive proconvulsant SNR region, through activation of downstream cascades of calcium-sensitive signaling processes. Indeed, as will be described in the subsequent review (Akman et al., in this issue), insults that cause precocious termination of depolarizing GABA_ARs in the male SNR can abolish the proconvulsant function of the PN15 male SNR.

Sex differences in SNR-related output connections as a function of age

The SNR is part of a larger network that includes the primary seizure focus – when such a focus can be identified – regions controlling seizure onset or propagation (“gateways”), as well as seizure termination (Avanzini et al., 2012; Gale, 1992; Lado and Moshé, 2008; Velísková and Moshé, 2006). Several lines of evidence have demonstrated that components of the basal ganglia, including the SNR, as well as other interacting brain regions (e.g., thalamus, cortical regions or brainstem nuclei) can modulate the susceptibility to and propagation of seizures. The SNR is connected with the remaining basal ganglia structures, receiving input mainly from the striatum and STN, and projecting towards the SC and VM thalamus, the parafascicular, centromedian and paracentral nuclei, but also to the centrolateral, mediodorsal and thalamic reticular nucleus (Gulcebi et al., 2012) thus affecting, indirectly, many telencephalic structures (Fig. 1).

It is likely that the age- and sex- specific differences in the way individual network components mature may impact the way and timing when different regions become connected or respond to each other. As a result, the functional output of a network may follow age- and sex-specific maturation patterns that are distinct from those of its individual components.

To delineate the structures that are affected by muscimol intranigral infusions, 2-DG autoradiography studies were performed. Moshé et al. (1994) injected 2-DG 30 min after unilateral muscimol infusions, a timepoint that corresponds to the time when muscimol-infused rats are exposed to flurothyl. It was demonstrated that in adult male rats, muscimol infusions into the SNRa induced specific regional metabolic changes depending on the infusion site. While infusions into the SNRa are associated with decreased glucose utilization into the ipsilateral striatum, the sensorimotor cortex and VM, infusions into the SNRp do not affect the sensorimotor cortex and increase glucose uptake at the level in the ipsilateral GP, and in the dorsal striatum (Moshé et al., 1994). These data suggest that this network could be important in mediating the proconvulsant effect of muscimol infusions into the SNRp. The timing between administration of muscimol infusions and 2-DG is crucial in demonstrating these networks (Velíšek et al., 2005).

The components of a network are not exclusively utilized for a single function and within each region there may be region-specific functional specifications, which may follow different age and sex specific patterns of maturation. Furthermore, the context within which a network operates (e.g., under normal conditions or in the presence of a chemoconvulsant) may change its final output. For example, unilateral muscimol infusions in the SNR of

normal rats can elicit circling behavior towards the contralateral side (Moshé et al., 1994; Velíšek et al., 2005). However, in this experimental paradigm, muscimol had sex-specific effects on circling frequency when infused in the SNRa of adult male and female rats, but not when infused in the SNRp. This is quite different from the effects of intranigral muscimol on flurothyl seizure control, where sex differences are observed with the infusions in the SNRp but not in the SNRa.

Are there other subcortical seizure controlling structures which are sexually dimorphic?

Other sites involved in seizure phenomenology and onset have sex-related features, and among them one cannot forget the amygdala (see, for instance, Cooke and Woolley, 2005; Stefanova and Ovtcharoff, 2000). The list of potential candidates apart from SNR involves several areas (for reviews, see for instance Galanopoulou, 2008a; Gale, 1992; Lado and Moshé, 2008), including the hippocampus (Galanopoulou, 2008b), thalamic structures (which can be especially relevant in generalized and in limbic epilepsies), as well as serotonergic and noradrenergic nuclei giving rise to a more diffuse effect through a widespread innervations of the brain (Jacobs and Azmitia, 1992; Szabadi, 2013). In the hippocampus, female CA1 pyramidal neurons begin manifesting hyperpolarizing GABA_AR responses to physiological synaptic stimulation much earlier than males (i.e., already at PN4 versus PN14 in males) (Akman et al., in this issue; Galanopoulou, 2008a,c), and this correlates with the higher expression of KCC2 and decreased activity of NKCC1-like, bumetanide-sensitive, cation chloride cotransporters in females (Galanopoulou, 2008a,c).

5HT may have a relevant role in models of epilepsy and, likely, in human epilepsy (Dailey et al., 1992; Jobe, 2003). There is a report showing a higher content for 5HT and its metabolites in the hypothalamus of female rats compared to male rats; however, this may be transient and not sufficient to produce changes in postsynaptic 5-HT receptors (Ferrari et al., 1999). To date, no data exist on sex differences in 5HT innervations of cortical regions, which are likely to be an important site for the effects of 5HT on seizures (Jobe, 2003).

The hypothalamus seems to be involved in subcortical spreading of different types of seizures (Mirski and Fisher, 1994; Shehab et al., 1992; Silveira et al., 2000), as well as to play a role as a seizure-triggering area in some patients and typically those affected by hypothalamic hamartomas (Pati et al., 2013). Thus, sex-related differences in this area might be relevant for epilepsy sex-related differences as well. In line with this, it is worth noting that many studies have shown that there are clear sex-related differences in GABAergic parameters in rats. For instance, hypothalamic GABA content and turnover are higher in males than females (Frankfurt et al., 1984; Searles et al., 2000), the expression of KCC2 is lower in neonatal males vs females, while the opposite occurs for NKCC1 (Perrot-Sinal et al., 2007), and neonatal hypothalamic muscimol infusions increase pCREB in males while decreasing it in females (Auger et al., 2001).

There are interesting hints for potential dimorphic features of the LC in seizure modulation. LC sends direct projections to the whole cortex (either archi-, paleo- or neocortex), as well as to several sub-cortical structures and it is the main source for norepinephrine (NE) in the

brain, and especially for limbic structures (Fallon et al., 1978; Giorgi et al., 2003), where it is considered to exert modulatory effects which might be relevant for mood, emotions and stress responses (Aston-Jones et al., 1996; Itoi and Sugimoto, 2010). Concerning seizures, a pivotal role for LC has been confirmed for all of the models of limbic seizures tested so far, including amygdala kindling (McIntyre, 1980; McIntyre and Edson, 1981), seizures evoked focally from the piriform cortex (Giorgi et al., 2003, 2006, 2008), or by systemic chemoconvulsants (Szot et al., 1999). Furthermore, LC plays also a crucial role in extralimbic seizure models, including pentylenetetrazol and maximal electroshock-induced seizures (Mason and Corcoran, 1978, 1979; Mishra et al., 1994), as well as spontaneous seizures occurring in GEPR-3 and 9 rats (Jobe et al., 1994).

LC exhibits sex-related features. In females, LC neurons possess a dendritic tree with longer and more branched processes, giving rise also to a denser and wider innervation (extending also to peri-LC region) (Bangasser et al., 2011), and receive a denser and probably more “efficient” input from different afferent structures, especially limbic ones (Bangasser et al., 2011). Electrophysiological studies have shown that corticotropin-releasing factor, a neuropeptide cotransmitter of LC afferents from limbic structures, exerts a higher activating effect on female than in male LC neurons (Curtis et al., 2006). The above quoted dimorphic features are permanent and due to early dimorphic differentiation, since sex-hormonal manipulation in adult rats does not affect them (Bangasser et al., 2011; Curtis et al., 2006), even though there are no specific data on the extent of the critical period responsible for this sexual dimorphism. Even though these aspects have been interpreted as crucial in the proposed different role of emotional stimuli in affecting arousal in women vs. men, with an emphasis on gender-related differences of stress-related psychiatric disorders (Bangasser and Valentino, 2012), they might have implications for epileptogenesis too. To delineate whether there are dimorphic differences in the role of LC in seizures, it should be assessed whether there is a difference in NE release in crucial sites during seizures, in males versus females, since an increased release of NE in limbic structures has been observed during seizures, and might have self-inhibitory effects (Giorgi et al., 2003). Furthermore, it should also be assessed whether the LC produces different effects in different seizures models in males and females.

Concluding remarks

There is emerging evidence that gender differences may be important in the etiology and expression of epilepsy in humans (Perucca et al, this issue; van Luijckelaar et al, this issue). The pathophysiological basis for the sex-related differences is still far from being understood, but the dimorphism of seizure-controlling structures might have a role. Thus far, the SNR is the only seizure-modulating structure for which the sexually dimorphic functional role on seizures (i.e. the anti- or proconvulsant effects of GABAergic SNR infusions) have been clearly established and confirmed. Most of the available data on the role of sex in SNR effects have been obtained in rats, in the flurothyl clonic-tonic model, since it allows a precise quantification of the seizure threshold. It would be interesting to assess its sex and age-dependent role in other animal models of seizures too, in which SNR has been shown to play an important role in male adult rats [e.g., amygdala kindling (Löscher et al., 1987), maximal electroshock model (Iadarola and Gale, 1982)]. In rats, it is

now known that there is a critical postnatal period during which the gonadal hormones and their various metabolites determine the sex- and age-related maturation of the GABAergic system and seizure-controlling function of the SNR. These findings also suggest the importance of testing for possible age- and sex-related differences in the effects of systemically administered GABAergic drugs in seizures (see Akman et al, in this issue). However, the net result of systemic GABAergic drug administration cannot be predicted, because the same drug may affect various brain regions with different – and even opposite – effects toward seizures. This has been, for example, shown with the muscimol infusions in the SC versus SNR (Gale, 1992). The potential occurrence of age- and/or sex-related differences in the effects of GABAergic drugs should be investigated in detail in other subcortical structures as well. In the clinical setting, rigorous comparison of the effect of GABAergic drug in the two sexes, at different ages, and in specific epilepsy syndromes (e.g. focal symptomatic, generalized symptomatic, or genetic epilepsies) may disclose potentially relevant differences which, at least in part, could be related to the degree of age- and sex-related involvement of SNR in these syndromes.

Recently, the loop diuretic drug bumetanide (a blocker of NKCC1) has been evaluated for its potential anticonvulsant effect, in models of neonatal seizures (e.g. Dzhalal et al., 2008; Cleary et al., 2013; Mazarati et al., 2009) and in one patient (Kahle et al., 2009). As mentioned above, NKCC1 has an age-dependent expression in SNR and hippocampus. While the effects of bumetanide on seizures need to be interpreted cautiously (Vanhatalo et al., 2009; Chabwine and Vanden Eijnden, 2011), it would be worth testing also the effects of loop diuretic drugs into the SNR to assess their potential age- and sex-related role in SNR-mediated epilepsy modulation.

The potential dimorphic effects of other powerful seizure-modulating systems/nuclei, and involving neurotransmitters other than GABA, deserve further investigations, too. The noradrenergic nucleus LC is a strong potential candidate, and even in this case disclosing such differences might help understanding the male/female and age-dependent differences in the susceptibility to seizures, as well as to design specific treatments tailored according to personalized features of the patients.

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Abbreviations

AR	androgen receptors
DES	diethylstilbestrol
2-DG	[¹⁴ C]2-deoxyglucose

DHT	dihydrotestosterone
E	17 β -estradiol
ER	estrogen receptors
GABA_AR	GABA _A receptors
GP	lateral globus pallidus
KCC	K ⁺ /Cl ⁻ cotransporter
IPSC	inhibitory post-synaptic current
-ir	immunoreactivity
LC	locus coeruleus
L-VSCC	L-type voltage sensitive calcium channel
NE	norepinephrine
NKCC	Na ⁺ /K ⁺ /Cl ⁻ cotransporter
PN	postnatal day
PPT	pedunculopontine tegmental nucleus
SC	superior colliculus
SNpc	pars compacta of the substantia nigra
SNR	pars reticulata of the substantia nigra
SNRa	anterior part of SNR
SNRp	posterior part of SNR
STN	subthalamic nucleus
T	testosterone
VM	ventromedial thalamic nucleus

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Fig. 1.

Schematic representation of connections of male SNR in relation with seizure modulation. A: The SNR receives GABAergic afferents from the GP, as well as from the striatum; it also receives direct dopaminergic afferents from the SNpc, and glutamatergic projections from the STN. SNR, together with the globus pallidus internal (not shown in the figure) is the only output structure from the basal ganglia. It sends GABAergic efferents to its target structures: the thalamus, the SC and PPT. All of these three structures play a crucial role in seizure modulation. The cortex, thalamus and STN mainly send glutamatergic projections to their targets (dark red solid arrows); SNR, striatum and GP send GABAergic inhibitory efferents to their targets (orange dashed arrow); dotted gray arrows represent SNpc dopaminergic efferents. The PPT is formed by cholinergic neurons. Panels B and C show the effects of unilateral infusions of muscimol in the male SNR in terms of increased (red arrows) or decreased (blue arrows) [^{14}C]2-deoxyglucose (2-DG) uptake in target regions (from Moshé et al., 1994, and unpublished observations from Moshé's Lab). The SNR sites are schematized as circles: the SNRa with an anticonvulsant effect of muscimol is depicted in white, while the proconvulsant site is in black. B: In adult male rats, unilateral muscimol infusion in the SNRp (where bilateral muscimol infusions are proconvulsant) increases 2-DG uptake in the ipsilateral GP and striatum; unilateral muscimol infusion into the SNRa

(where bilateral muscimol infusions are anticonvulsant) decreases 2-DG uptake in the ipsilateral VM and GP, while it increases 2-DG uptake in the ipsilateral SC. C: in PN15 male rat SNR (where bilateral intranigral muscimol infusions are proconvulsant), unilateral muscimol infusions increase 2-DG uptake in the ipsilateral GP and striatum. Abbreviations: GP: lateral globus pallidus; PPT: pedunculopontine tegmental nucleus; SC: superior colliculus; SNpc: pars compacta of the substantia nigra; SNR: pars reticulata of the substantia nigra; SNRa: anterior part of SNR; SNRp: posterior part of SNR; STN: subthalamic nucleus; VM: ventro-medial thalamic nucleus.

Table 1

Early data on the developmental effects of bilateral SNR infusions of GABAergic or glutamatergic agents toward flurothyl-induced seizures in male rats.

Chemical	Mechanism	PN15 male SNR	Adult male SNRa	Adult male SNRp	Reference
Muscimol	GABA _A R agonist (low-/high-affinity site)	Proconvulsant	Anticonvulsant	Proconvulsant	Moshé et al., 1983,1994; Velísková and Moshé, 2001
THIP	GABA _A R agonist	Biphasic effects	Anticonvulsant	Not tested	Garant et al., 1995; Xu et al., 1992
GVG	Inhibitor of GABA aminotransferase	Anticonvulsant	Anticonvulsant	Proconvulsant	Xu et al., 1991a,b Velísková et al., 1996b
Bicuculline	GABA _A R antagonist (low-affinity site)	Proconvulsant	Proconvulsant	No effect	Sperber et al., 1987 Velísková et al., 1996b
ZAPA	GABA _A R agonist (low-/high-affinity site)	Biphasic effects	Anticonvulsant	Proconvulsant	Sperber et al., 1999 Velísková et al., 1996b
Zolpidem	GABA _A R agonist (α selective agonist)	Anticonvulsant	Anticonvulsant	No effect	Velísková et al., 1998b
Baclofen	GABA _B R agonist	Anticonvulsant	No effect	Not tested	Sperber et al., 1989a,b
CGP 35,348	GABA _B R antagonist	Proconvulsant	No effect	Not tested	Velísková et al., 1994
AP7	NMDA receptor antagonist	No effect	Anticonvulsant	Proconvulsant	Wurpel et al., 1992

Modified from Scantlebury et al., 2009. Biphasic effects indicate anticonvulsant effect with low doses and proconvulsant with high doses
Abbreviations: GVG: γ-vinyl-GABA; SNR: substantia nigra pars reticulata; SNRa: anterior part of substantia nigra pars reticulata; SNRp: posterior part of substantia nigra pars reticulata; PN15: postnatal day15; THIP: 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol; ZAPA: (Z)-3-[(aminoiminomethyl)thio]prop-2-enoic acid; AP7: DL-2-amino-7-phosphonoheptanoic acid.

Table 2GABAergic in vivo and in *vitro* parameters in SNR cells in relation to sex and developmental stage.

SNR parameter	Age sex	Findings	Reference
GABA-ir cells/section	PN15, PN30	Males < females	Ravizza et al., 2003 a
	Males-females	SNRa < SNRp (all groups except PN30 males)	
Density of GABA-ir/cell	PN15, PN30	Males < females	Ravizza et al., 2003 a
	Males-females	PN15: SNRa = SNRp; PN30: SNRa > SNRp	
GABA _A α 1 mRNA/cell	PN15, PN30	males < females	Ravizza et al., 2003a
	Males-females	PN15: SNRa = SNRp; PN30: SNRa > SNRp	
	PN15-adults	SNRa < SNRp in adults, SNRa = SNRp at PN15	Moshé et al., 1994
	Males		
	PN15-PN21-PN60	PN15: SNRa = SNRp; PN21: SNRa > SNRp	Velísková et al., 1998a
	Males		
GABA _A α 1 mRNA ex pressing cells	PN15, PN21, PN60	PN15: SNRa = SNRp; PN21 and PN60: SNRa < SNRp	Velísková et al., 1998a
	Males		Moshé et al., 1994
Density of GABA _A α 1-ir/SNRa cell	PN5, PN15, PN30	PN5: males < females;	Chudomel et al., 2009
	Males-females	PN15 < PN30 (both sexes)	
Density of GABA _A α 3-ir/SNRa cell	PN5, PN15, PN30	PN5: males < females, PN5 > PN15 > PN30;	Chudomel et al., 2009
	Males-females; SNRa	males: PN5, PN15 > PN30; females: PN5 > PN15 > PN30	
	only		
GABA _A γ 2L mRNA/cell	PN15, PN21, PN60	PN15: SNRa = SNRp;	Velísková et al., 1998a
	Males	PN21, PN60: SNRa > SNRp	
GABA _A γ 2 L subunit expressing cells	PN15, PN21, PN60	PN15:SNRa = SNRp;	Velísková et al., 1998a
	Males	PN21, PN60: SNRa < SNRp	
KCC2 mRNA	PN15, PN30, adults	PN15, PN30: males < females	Galanopoulou et al.
	Males-females	Males: PN15 < PN30 < adult; females: PN15 < PN30	2003
		PN15: SNRa = SNRp at PN15; PN30, adult: SNRa > SNRp	
GABAergic cells neurogenesis	BrdU: E13-14-15;	GABAergic cell density: males < females	Galanopoulou et al.
	Tested at PN30;	Earlier peak of neurogenesis of SNRa (E13) than SNRp (E13-14)	2001
	Males-females	Earlier peak ofneurogenesis offemales	
Spontaneous IPSCs	PN5-9; PN12-15;	IPSCs frequency, amplitude, charge transfer: increase with age;	Chudomel et al., 2009
	PN28-32		
	Males-females	IPSC rise and decay times: decrease with age;	
		PN5-9: males have higher IPSC frequencies, amplitudes, and charge transfers than PN5-9 females;	
		PN28-32 males have	
		Higher IPSC amplitudes, and shorter rise/decay times than PN28-32 females;	

SNR parameter	Age sex	Findings	Reference
α 1 GABAAR-mediated IPSCs	PN5–9; PN12–15;	Zolpidem-induced increase in decay time increases with age.	Chudomel et al., 2009
	PN28–32		
	Males–females	Zolpidem-induced increase in charge/IPSCs is lower in PN5–9 males than the rest of groups.	

Abbreviations: BrdU: bromo-deoxy-uridine; DES: diethylstilbestrol; DHT: 5 α -dihydrotestosterone; GABAA R: GABAAR; ir: immunoreactive; PN: postnatal day; SNRa: anterior part of substantia nigra pars reticulata; IPSCs: inhibitory post-synaptic currents.

Table 3 GABA_AR *in vivo* and *in vitro* responses of SNRa neurons in relation to sex, developmental stage and early gonadal hormone manipulation.

Group	Effects of muscimol infusion in SNRa on flurothyl-induced seizures <i>in vivo</i>	GABA _A R responses of SNRa neurons <i>in vitro</i>
PN15 males		
Intact	Proconvulsant	Depolarization
PN0-castrated	No effect	Hyperpolarization
PN0-castrated + TP (PN0-2)	Proconvulsant	Hyperpolarization
PN0-castrated + DES (PN0-2)	Proconvulsant	
PN0-castrated + DHT (PN0-2)	Proconvulsant	
PN15 females		
Intact	No effect	Hyperpolarization
TP (PN0-15)	Proconvulsant	
TP (PN0-2)	Proconvulsant	Hyperpolarization
DES(PN0-2)	Proconvulsant	Hyperpolarization
DHT (PN0-2)	Proconvulsant	Hyperpolarization
PN21 males	Proconvulsant	Hyperpolarization
PN21 females	No effect	Hyperpolarization
PN30 males	Anticonvulsant	
PN30 females	Anticonvulsant	

Abbreviations: DES: diethylstilbestrol; DHT: 5 α -dihydrotestosterone; GABA_AR: GABA_A receptor; PN: postnatal day; SNRa: anterior part of substantia nigra pars reticulata; TP: testosterone propionate.