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Age and sex differences in GABAergic transmission in the substantia nigra pars reticulata in the rat

Dizertační práce

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List of abbreviations:

aCSF – artificial cerebrospinal fluid

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA - analysis of variance

BIM - bicuculline methobromide

BGT-1 - betaine-GABA transporter 1

CNQX - 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline

D-AP5 - D-(-)-2-Amino-5-phosphonopentanoic acid

DMSO - dimethyl sulfoxide

GABA - γ-aminobutyric acid

$GABA_ARs$ – $GABA_A$ receptors

$GABA_CRs$ – $GABA_C$ receptors

gabazine - SR 95531 hydrobromide

GABRD - gene encoding the δ subunit

GAT 1-3 - GABA transporters 1-3

GEFS+ - generalized epilepsy with febrile seizures plus

IGE - idiopathic generalized epilepsy

Ihold - holding current

-ir – immunoreactivity

JME - juvenile myoclonic epilepsy

K-S test – Kolmogorov-Smirnov test

NGS – normal goat serum

NMDA - N-methyl D-aspartate

PN – postnatal days

Rs – series resistance

RT – room temperature

SE – standard error

sIPSCs – spontaneous inhibitory postsynaptic currents

SNR_A - anterior part of the substantia nigra pars reticulata

SNR – substantia nigra pars reticulata

TBS – tris based saline

THIP – 4,5,6,7-tetrahydroisoxazolo (5,4-c)pyridin-3-ol

TTX - tetrodotoxin

1. Introduction

Epilepsy is a serious chronic brain disorder characterized by recurrent unprovoked epileptic seizures. The prevalence of active epilepsy in population is about 0.5-1%. It is a disabling disease which can severely decrease the quality of life and can result in serious health impairment or death. Therefore, it is of highest importance to find an adequate treatment of epileptic seizures. Epilepsy and epilepsy syndromes are markedly heterogenous entities with variable responses to treatment. About 2/3 of seizures sufficiently respond to a first antiepileptic drug, while the remaining 30 percent of patients continue to have seizures even with multiple medications and despite maximal effort of healthcare professionals. Thus, elucidating pathophysiological mechanisms underlying seizures is necessary to find new avenues to tackle this serious illness.

1.1. Brief pathophysiology of epileptic seizures and epilepsy

Pathophysiology of epileptic seizures and epilepsy is quite a complex issue. An epileptic seizure is by the ILAE (International League Against Epilepsy) and IBE (International Bureau for Epilepsy) definition „a transient occurence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain“ whereas epilepsy is „a disorder of the brain characterized by an enduring predispozition to generate epileptic seizures“.

For the purpose of this thesis are discussed only pathophysiological mechanisms of epileptic seizures resulting from an acute focal brain injury bearing in mind, however, that the issue of genetically caused epilepsies/epileptic syndromes and epilepsies due to developmental disorders are equally important.

There is a vast array of acute insults that can cause epileptic seizures including ischemic stroke (Bladin et al., 2000), intracranial hemorrhage (Weisberg et al., 1991), vascular malformations (Kraemer and Awad, 1994), tumors (Oberndorfer et al., 2002, Rosati et al., 2009), trauma (Singh and Pathak, 1990, Annegers et al., 1998), central nervous system infection (Solbrig et al., 2006, Hjalmarsson et al., 2007), status epilepticus (Scantlebury et al., 2010, Reddy and Kuruba, 2013) or prolonged febrile seizures (Dube et al., 2006). After the injury follows a latent period without seizures (also called a seizure-free period) during which evolve alterations on the subcellular, cellular and neuronal network levels. Histological and immunohistochemical findings from animal models of temporal lobe epilepsy and patients after brain surgery for temporal lobe epilepsy show significant neuronal cell loss in the hippocampal formation, gliosis, sprouting of mossy fibers and forming reccurent curcuits, synaptic reorganization, altered receptor subunit expression and activation of cascades of changes in gene expression (Buckmaster and Dudek, 1997, Loup et al., 2000, Proper et al., 2000, Peng et al., 2004, Pitkanen et al., 2007, Zhang et al., 2007). These complex processes that transform a healthy non-epileptic brain into an epileptic one are called epileptogenesis. Despite multiple and often different factors leading to creating of a hyperexcitable enviroment in an injured brain tissue, the final common pathway is a pathological synchronous electrical activity stemming from the disbalance between inhibitory and excitatory mechanisms leading to an epileptic seizure.

It is now clear that an epileptogenic focus consists of multiple small hyperexcitable microdomains where an asymptomatic electrical activity or „microseizures“ may occur. The microseizures are usually self-limited but once the pathological electrical activity

spreads to other parts of the brain, a clinical epileptic seizure becomes apparent (Bragin et al., 2000).

Vast majority of epileptic seizures are self-limited and stop spontaneously within a few minutes (Lado and Moshe, 2008). Thus, intrinsic mechanisms leading to cessation of seizures are also widely studied apart from those pathological processes involved in seizure generation. Mechanisms that lead to termination of seizures can be divided into those acting at the subcelular level (membrane, synapses), local networks of neurons and complex subcortical circuits (Lado and Moshe, 2008). One of the integral parts of subcortical circuits is the substantia nigra pars reticulata and its involvement in seizure control will be discussed further in the text.

1.2. The substantia nigra pars reticulata

1.2.1. Concise anatomy of the basal ganglia complex

The substantia nigra pars reticulata (SNR) is a subcortical deep brain structure anatomically belonging to the basal ganglia system (Fig. 1).

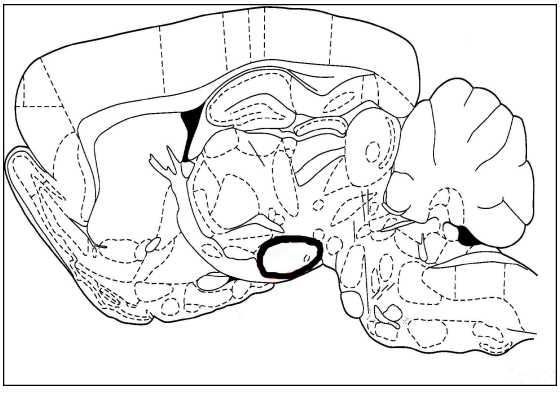

Figure 1. Substantia nigra on sagittal section in the rat (black elipse)

The basal ganglia are composed of several interconected nuclei and are functionally

involved, among other functions, in the control of movement through its ample somatotopically organized connections with the primary motor cortex, premotor and supplemental motor area. A signal to a voluntary movement enters the basal ganglia through the input nuclei composed by the caudate nucleus and putamen (together forming the striatum). The information is processed in the basal ganglia circuitries and exits through the output nuclei formed by the internal globus pallidum and substantia nigra pars reticulata to the motor nuclei of the thalamus and subsequently to the cerebral cortex (Afifi, 2003, McHaffie et al., 2005).

1.2.2. Role of the SNR in seizure control

In addition to its well defined role in the motor control, the SNR was also identified as a structure involved in the control of experimentally induced generalized seizures (Iadarola and Gale, 1982, Moshe and Albala, 1984, Gale, 1985, Deransart et al., 1998, Veliskova and Moshe, 2006). The SNR receives electrical signals via a direct and indirect pathways, which are anatomically and functionally distinct. Through the direct pathway, it receives a monosynaptic inhibitory input from the GABAergic neurons located in the striatum and, via the indirect pathway, arrives a polysynaptic excitatory input from glutamatergic neurons of the subthalamic nucleus (Smith et al., 1998). The axons of SNR neurons project into several brain structures including the superior colliculus, pedunculopontine nucleus and ventromedial thalamus (Chevalier et al., 1985, Deniau and Chevalier, 1985). Since the SNR is populated with predominantly GABAergic cells, its major electrical output is inhibitory.

The SNR is not a functionally homogenous nucleus regarding seizure control and

modulation but its electrophysiological properties largely depend on the sex and maturational stage. Several chemical manipulations revealed two topographically distinct regions within the SNR, an anterior and a posterior part (Veliskova et al., 2005). Bilateral infusions of muscimol, a $GABA_A$ receptor agonist, into the anterior part of adult rat SNR (SNR_A), increase the seizure threshold (i.e., has an anticonvulsant effect) in both sexes in the flurothyl model of generalized clonic seizures (Garant et al., 1995, Moshe and Garant, 1996, Veliskova and Moshe, 2001). The anticonvulsant effect is thus caused by attenuation of the firing activity of nigral neurons, reduced nigral output and subsequent disinhibition of target structures including the superior colliculus, pedunculopontine tegmental nucleus and ventromedial thalamus (Chevalier et al., 1985, Deniau and Chevalier, 1985, Dean and Gale, 1989). Similarly, decrease of the firing rate of SNR neurons can be achieved by blocking the excitatory glutamatergic input from the subthalamic nucleus using NMDA (N-methyl D-aspartate) receptor antagonists (e.g., 2-amino-7-phosphonoheptanoic acid, AP-7) resulting in seizure inhibition (Turski et al., 1986, Deransart et al., 1996). Identical anticonvulsant effect was obtained when neurons in the superior colliculus were disinhibited with local injections of bicuculline, a $GABA_A$ receptor antagonist (Dean and Gale, 1989). Conversely, enhancement of neuronal firing activity in the anterior SNR by intranigral microinfusions of bicuculline (Sperber et al., 1989) or glutamatergic receptors agonist NMDA (Turski et al., 1987), produced a stronger electrical output resulting in proconvulsant effects. Bilateral muscimol application into the pedunculopontine nucleus led to significant increase of the proconvulsant activity (Okada et al., 1989).

In the posterior part of the SNR (SNR_P), the muscimol effect is sex-dependent as it

decreases the seizure threshold (a proconvulsant effect) in adult male rats whereas it has no effect in females (Veliskova and Moshe, 2001).

Several *in vivo* studies also imply that the SNR is an important part of the subcortical system suppressing generalized absence seizures (Deransart et al., 1996, Deransart et al., 1998, Paz et al., 2007) thus making the basal ganglia complex and other subcortical structures potential targets for new therapeutic approaches. Indeed, high-frequency electrical stimulation of the SNR had an anticonvulsant effect in flurothyl model of generalized seizures (Velisek et al., 2002) and amygdala-kindled seizures in adult male rats (Shi et al., 2006).

Some promising therapeutic implications in reducing seizure frequency by affecting subcortical circuits were demonstrated in a few observational studies in patients with intractable generalized and partial epilepsies who benefited from the deep brain stimulation (DBS) of the SNR (Wille et al., 2011), subthalamic nucleus (Loddenkemper et al., 2001), (Handforth et al., 2006, Lee et al., 2006) or thalamic nuclei (Lim et al., 2007).

1.2.3. SNR properties change with maturation and sex

The propensity of the SNR to set a seizure threshold changes with maturational stages and sex (Moshe et al., 1994, Veliskova and Moshe, 2001). Localized bilateral intranigral muscimol microinfusions in the 30-day-old male rats produces two sets of results. Muscimol application in the anterior part of the SNR has anticonvulsant effects on flurothyl induced seizures, whereas injection in the posterior part results in proconvulsant effects. However, muscimol infusions in 15-day-old male rats lead to only

proconvulsant actions irrespective of the site of application (Moshe et al., 1994). In 30-day-old female rats, muscimol increases the seizure threshold, i.e. has anticonvulsant effects, in the SNR anterior, whereas has no impact on seizures when injected into the posterior part of the substantia nigra pars reticulata. Surprisingly no effects on seizures compared to controls were observed following muscimol application in either region in 15-day-old female rats (Veliskova and Moshe, 2001). The key factor in determining the SNR responsiveness to muscimol is its exposure to testosterone in early development (Giorgi et al., 2007) and switch to the adult phenotype occurs around the third postnatal week (Veliskova and Moshe, 2001, Veliskova et al., 2004).

1.3. Subunit composition of $GABA_A$Rs in the SNR_A

The majority of cells in the SNR_A are fast spiking GABAergic neurons (Richards et al., 1997), which receive the inhibitory input via postsynaptic $GABA_A$Rs from the striatum and globus pallidus (Smith and Bolam, 1989, Smith and Bolam, 1991, Bolam et al., 2000, Misgeld, 2004). GABA acts on two different types of receptors, ligand-gated $GABA_A$ and $GABA_C$ receptors and metabotropic $GABA_B$ receptors coupled with G-proteins. Activation of $GABA_A$ and $GABA_C$ receptors, which function as chloride channels, results in the influx of negatively charged chloride ions into neurons and subsequent hyperpolarization of the cell membrane. GABA (γ-aminobutiric acid) binding on $GABA_B$ receptors leads to opening of G-protein controlled potassium channels. Membrane hyperpolarization is achieved via efflux of positively charged potassium ions from inside the cell.

$GABA_A$ receptors are ligand-gated channels composed of five different

subunits (α1-6, β1-3, γ1-3, δ, ε, π, θ) permeable for chloride and HCO_3^- ions (Levitan et al., 1988, Olsen et al., 1991, Barnard et al., 1998, Benarroch, 2007). Although the subunits could theoretically form innumerable combinations of receptors, only a limited number of receptor subtypes are found in living organisms, the most frequent one in the brain being α1β2γ2 (Sieghart and Sperk, 2002, Farrant and Kaila, 2007).

Distinct subunit composition of nigral $GABA_A$Rs appears to underlie observed age- and sex-related differences in the control of seizures and sensitivity to drugs modulating GABAergic system such as muscimol. Many studies have shown that in particular the α subunit subtypes undergo significant changes in expression during brain development. In most studied brain regions, including the SNR, the α2 and α3 subunits are highly expressed in the early stages of postnatal maturation and their levels gradually decrease with age while the α1 subunit mRNA and protein levels rise (Laurie et al., 1992, Fritschy et al., 1994, Veliskova et al., 1998a). α1 mRNA expression in the SNR showed higher levels in postnatal day (PN) 30 than in PN15 rats (Moshe et al., 1994) and in female rats than age-matched male rats (Veliskova et al., 1998a, Ravizza et al., 2003).

Regional differences in α1 subunit mRNA distribution were also found between anterior and posterior part of the SNR depending on maturational stage. *In situ hybridization* technique revealed homogeneous expression of α1 subunit mRNA throughout the SNR at the age of PN15 in both sexes while at PN30, α1 mRNA levels were significantly higher in the SNR_A than SNR_P (Ravizza et al., 2003).

1.3.1. Synaptic $GABA_A$ receptors and postsynaptic currents

The subunit composition of $GABA_A$Rs determines not only the kinetic and pharmacological properties of receptors but also their localization on the cell membrane. For example, presence of the γ2 subunit targets newly formed $GABA_A$ receptors to the postsynaptic compartment of the synaptic cleft (Essrich et al., 1998) where they mediate a "phasic" GABA inhibition. Synaptic receptors are briefly exposed (<1ms) to relatively high concentrations (milimolar range) of GABA (Clements, 1996, Mody and Pearce, 2004). After being activated by GABA, $GABA_A$Rs open for a very short period of time and chloride ions are allowed to flow down their electrochemical gradient across the membrane into the cell. Accumulation of negatively charged ions in the intracellular compartment results in hyperpolarization of the cell membrane and increases the threshold for action potentials generation. The flow of chloride anions through the channel can be detected on the membrane of the postsynaptic neuron as inhibitory postsynaptic currents (IPSCs). Biophysical properties of IPSCs (such as the rise and decay times) are largely determined by the $GABA_A$Rs subunit composition. The decay time of IPSCs is faster when the α1 subunit is present, whereas it slows down if $GABA_A$Rs contain α2 or α3 subunits instead (Verdoorn, 1994, Gingrich et al., 1995, Lavoie et al., 1997). Different subunit assemblies also underlie distinct pharmacological properties of GABAergic receptors. For instance, γ subunit-containing receptors are much less sensitive to GABA and desensitize at a markedly higher rate compared to δ subunit-containing receptors (Saxena and Macdonald, 1994). The high deactivation rate is necessary for receptors to rapidly regain the ready-to-open state after GABA is cleared away by diffusion out of the synaptic cleft and subsequently taken up

by the selective active transport. γ subunits along with α subunits are also essential for benzodiazepine sensitivity (Pritchett et al., 1989), whereas δ subunit-containing receptors are insensintive to benzodiazepines (Sieghart, 1995).

1.3.2. Extrasynaptic $GABA_A$ receptors

1.3.2.1. Definition

Extrasynaptic $GABA_A$ receptors are essential for mediating a "tonic" GABA inhibition (Bai et al., 2001, Nusser and Mody, 2002, Stell and Mody, 2002, Semyanov et al., 2004). One of the key features of extrasynaptic receptors is the presence of a δ subunit (Nusser et al., 1998, Wei et al., 2003), which gives them characteristic kinetic and pharmacological properties in comparison with synaptic, γ subunit-containing, $GABA_A$Rs (Saxena and Macdonald, 1994). The δ subunit markedly increases affinity for extracellular GABA (Barnard et al., 1998, Sur et al., 1999), which is present, unlike the synapse, in very low concentrations (Lerma et al., 1986). Furthermore, δ-containing $GABA_A$Rs desensitize at much slower rate than synaptic receptors containing γ subunit (Saxena and Macdonald, 1994) and remain open despite continuing exposure to ambient GABA (Nusser et al., 1998).

Delta subunit containing $GABA_A$ receptors have been described in many animal brain structures including cerebellar granule cells (Brickley et al., 1996, Nusser et al., 1998), dentate gyrus granule cells (Wei et al., 2003), hippocampal interneurons (Semyanov et al., 2003), thalamus (Jia et al., 2005), cortex (Drasbek et al., 2007), etc. but also in human brain (Scimemi et al., 2006).

Immunohistochemical colocalization studies proved that in cerebellar granule cells the δ subunit preferentially co-assembles with α6 and β2/3 subunits (Nusser et al., 1998). In other brain structures such as the dentate gyrus or thalamus, the δ subunit forms receptors with α4 and β subunits (Wei et al., 2003, Jia et al., 2005). α6β2/3δ receptors were found exclusively extrasynaptically (Nusser et al., 1998) while α4βδ receptors in the dentate gyrus were present also perisynaptically (Wei et al., 2003). The reason why the δ-containing $GABA_A$Rs are not in synapses is due to the lack of a γ subunit, which is mandatory for anchoring the receptors in synapses (Essrich et al., 1998).

1.3.2.2. Sources of ambient GABA

Several mechanisms were proposed to explain the presence of extrasynaptic GABA. The possible sources were described in great detail in cerebellar granule cells (Rossi et al., 2003). Firstly, GABA released from presynaptic vesicles by incoming action potential is promptly taken up from the synaptic cleft by efficient transporter mechanisms in order to make the postsynaptic $GABA_A$ receptors responsive to another binding of a neurotransmitter. Certain amount of GABA, however, spills over and diffuses from the synaptic cleft and is capable of activating perisynaptic and extrasynaptic $GABA_A$Rs (Nusser et al., 1998, Wei et al., 2003). This action-potential (AP) dependent source of GABA can be thus blocked by TTX (tetrodotoxin), a potent inhibitor of voltage-dependent sodium channels necessary for generation of action potentials (Petrini et al., 2004). This source of GABA is most important in young animals (Rossi et al., 2003). Secondly, GABA can be released from Golgi cells terminals or astrocytes by a non-vesicular Ca^{2+}- and AP- independent mechanism (Liu et al., 2000). The AP-

independent GABA source becomes dominant in adult animals over AP-evoked vesicular release (Rossi et al., 2003). Lastly, the same group suggests that levels of extracellular GABA can also rise after stimulation of presynaptic nicotinic receptors (Rossi et al., 2003).

GABA uptake transporters strictly regulate GABA presence in the synaptic cleft (Borden, 1996, Overstreet and Westbrook, 2003, Keros and Hablitz, 2005). Four transporters have been described so far including GAT-1, GAT-2, GAT-3 (GABA transporters 1-3) and BGT-1 (betaine-GABA transporter 1) (Borden, 1996, Dalby, 2003, Conti et al., 2004). *In situ* hybridization studies showed that the GAT-1 mRNA is the most abundant transporter in the brain and moderate labeling was also found both in nigral neurons (Swan et al., 1994) (Durkin et al., 1995, Bahena-Trujillo and Arias-Montano, 1999) and glial cells (Radian et al., 1990). Under certain circumstances, such as depolarization of the neuronal membrane with extracellular potassium, the GABA transporters may, however, reverse their action and expel GABA into the extracellular space and become yet another source of ambient GABA (Gaspary et al., 1998).

1.3.2.3. Functional role of the tonic GABA inhibiton

Permanent activation of extrasynaptic $GABA_A$Rs result in prolonged opening of chloride channels. The increased permeability of the cell membrane for chloride ions gives rise to a „shunting“ effect which counteracts excitatory inputs and thus increases the threshold for iniciating of action potentials (Mitchell and Silver, 2003, Farrant and Nusser, 2005). On the contrary, blocking the tonic inhibition with a $GABA_A$ antagonist bicuculline increases excitability of the neuron and decreases amplitude of current injection

necessary to evoke action potentials (Brickley et al., 1996).

Multiple studies have brought numerous pieces of evidence that alterations in the δ subunit expression and changes in the magnitude of tonic GABA currents are closely involved in several experimental models of epilepsy. Mice with a targeted mutation of the gene encoding the δ subunit develop spontaneous seizures and are more susceptible to pentylenetetrazol-induced seizures (Spigelman et al., 2002). The δ subunit levels were altered following pilocarpine treatment in a mouse model of temporal lobe epilepsy (Peng et al., 2004). Dentate gyrus granule cells of the mice with recurrent spontaneous seizures exhibited weaker staining of the δ subunit and, in keeping with the immunohistochemical findings, *in vitro* extracelullar recording demonstrated increased excitability of the dentate gyrus (Peng et al., 2004).

A link between a defective δ subunit and higher propensity for developing spontaneous seizures was also observed in humans. Mutations in the gene encoding the δ subunit (GABRD) were found in patients with generalized epilepsy with febrile seizures plus (GEFS+) and idiopathic generalized epilepsy (IGE) (Dibbens et al., 2004). Human recombinant α4β2δ $GABA_A$Rs containing either of the two types of mutated δ subunit variants associated with GEFS+ and juvenile myoclonic epilepsy (JME) generated tonic currents with significantly lower amplitudes than wild type receptors (Dibbens et al., 2004, Feng et al., 2006).

Extrasynaptic $GABA_A$ receptors can be modulated by several compounds including alcohol (Liang et al., 2004), anaesthetic and sedative drugs (Bai et al., 2001, Belelli et al., 2005) or metabolites of steroid hormones (neurosteroids) (Brown et al., 2002, Stell et al., 2003). Neurosteroids are metabolites of sex hormones (such as progesterone or

testosterone) and corticosteroids (deoxycorticosterone) (Reddy, 2010). They are produced localy in the brain and as positive allosteric modulators increase efficacy of GABA on extrasynaptic $GABA_ARs$ and thus neurosteroids have potent anticonvulsant effects. One experimental study found a relationship between stages of ovarian cycle, the δ subunit expression and seizure frequency in the mouse. During late diestrus when concentrations of progesterone and neuroactive steroids reached their peaks, the δ subunit expression and tonic inhibition in the dentate gyrus granule cells increased and mice were less prone to develop seizures (Maguire et al., 2005). On the contrary, when progesterone levels decreased in early stages of the ovarian cycle, the δ subunit expression was downregulated resulting in the reduction of the tonic inhibition and the mice developed high frequency of seizures (Maguire et al., 2005). Similar observations were made in patients with catamenial epilepsy as the occurence of seizures is linked to certain stages of the menstrual cycle. When progesterone and neurosteroid levels are elevated in the luteal phase, the seizure threshold is high. Once progesterone levels drop, the seizure occurence increases (Reddy, 2009, Verrotti et al., 2010).

1.3.2.4. Developmental aspects of the tonic inhibition

The magnitude of $GABA_ARs$ mediated tonic current is not constant but changes during development in some brain structures. For instance, the tonic inhibition increase with age in cerebellar granule cells (Brickley et al., 1996, Tia et al., 1996) while declines in CA1 pyramidal cell in the hippocampus (Demarque et al., 2002, Semyanov et al., 2003). The underlying causes may be related to age-specific $GABA_ARs$ subunit composition, different anatomical arrangements of synapses (Brickley et al., 1996, Tia et al.,

1996) or more efficient GABA uptake from the synaptic cleft (Jensen et al., 2003, Semyanov et al., 2003).

2. Aims and hypothesis

2.1.

Aim #1: To study whether there are any differences in the α1 and α3 subunit expression in the SNR_A as a function of age and sex.

To study whether these subunit changes affect synaptic GABAergic transmission.

Hypothesis #1: The expression of the α subunit subtypes in the synaptic $GABA_A$Rs in SNR_A neurons is age- and sex-related and underlies changes in the properties of the spontaneous inhibitory postsynaptic currents (sIPSCs).

2.2.

Aim #2: To investigate whether there is any expression of the δ subunit in the SNR_A, whether there are present any age- and sex-related differences and whether there is an electrophysiological correlate in a GABA tonic current.

Hypothesis #2: The magnitude of the tonic GABA current in SNR_A neurons is closely linked to the presence of the δ subunit.

2.3.

Aim #3: To study whether the tonic GABA inhibition can be influenced by specific $GABA_A$ receptor agonists and modulators and whether there are any age- and sex-related patterns.

Hypothesis #3: Different responsiveness of male and female SNR_A neurons to modulatory effects of $GABA_A$ mimetic drugs during development depends on the $GABA_A$Rs subunit composition.

3. Experimental procedures

3.1. Animals

For IPSCs analysis, we used Sprague-Dawley rats of both sexes divided into 3 different age groups PN5-9, PN12-15 and PN28-32, with the date of birth taken as PN0 (Taconic Farms, New York, USA). For the tonic current study, the age ranges were distended to PN5-9, PN11-16 and PN25-32. Rats were kept at constant temperature (21 - 23°C), relative humidity (40 - 60%) and a 12 h dark/12 h light cycle (lights on at 7:00am) in an animal facility accredited by the American Association for the Accreditation of Laboratory Animal Care. Rats younger than 21 days were kept with a dam. After weaning, rats were kept in cages of 3-4 same sex rats with water and food *ad libitum*. All procedures and experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

3.2. Immunohistochemistry

Sprague –Dawley male and female rats were transcardially perfused with saline and then formalin at PN5, PN15 and PN30. Their brains were collected, fixed overnight in formalin, immersed in 30% sucrose and when they sank they were frozen and kept at -80°C till use. Sagittal 40μm sections were cut in a MICROM cryostat (MICROM International, Walldorf, Germany) and were stained with rabbit antibodies specific for the α1 and α3 (Millipore, Billerica MA). The anti-α1 antibody recognizes the aminoacid sequence 1-16 of the rat α1 subunit protein, whereas the anti-α3 antibody recognizes the aminoacid sequence 1-15 of the rat α3 subunit protein. Both were used at a dilution of 1:800. and δ $GABA_A$Rs subunits. As for the δ subunit, we used 2 sources of rabbit

anti-δ antibodies to confirm that the observed expression changes are independent of the δ subunit specific antibody used. Because the results regarding the developmental changes in δ subunit expression were comparable, the data were combined. The first antibody was developed by Dr. Gunther Sperk (Innsbruck Medical University, Austria) and recognized an epitope consisting of the 44 amino-terminal amino acids of the rat $GABA_AR$ δ subunit (Pirker et al., 2000). The second was a commercial polyclonal rabbit anti-δ antibody, also recognizing the amino-terminus of the rat $GABA_AR$ δ subunit (Millipore, Billerica Massachusetts).

Immunochemistries were done in free-floating sections. The steps included incubation with 1% H_2O_2 in tris based saline (TBS) for 30 minutes at room temperature (RT); blocking in TBS with 10% normal goat serum (NGS) and 0.4% Triton-X-100 (90 minutes, RT); incubation with the primary antibody in TBS with Triton X100 0.4% and 3% NGS (2-3 days, 4°C with shaking); incubation with secondary biotinylated anti-rabbit antibody (Vector Labs, Burlingame, CA) (1:200 dilution) in TBS with 0.4% Triton-X-100 and 3% NGS, RT; and further peroxidase based staining as per manufacturer's protocols (Vector Labs; ABC Elite kit and 3,3'-diaminobenzidine/nickel substrate kit). In every assay a representative brain from all groups was included to minimize inter-assay variability. Brains were coded to permit blinded assessment of the values.

Because the SNR is sparsely populated, densitometric analysis of cellular perisomatic immunostaining was done by sampling representative stained cells from 4-5 anterior SNR sections per rat, using the Image J software (Wayne Rasband, Research Services Branch, NIMH, Bethesda Maryland, USA). The densitometry values of these cells were averaged for each brain and results were used in the statistical analysis. Similar

densitometric analysis of immunochemically stained sections has been used extensively to provide semi-quantitative assessment of the level of protein expression in the stained cells (Rieux et al., 2002, Galanopoulou, 2006, Galanopoulou, 2008).

A mean value for the cellular δ-ir signal density was obtained per rat and was used in the statistics. In the cell count experiments, the number of total δ-ir SNR_A neurons was counted from 1 section per brain in sagittal SNR sections at the level of the subthalamic nucleus. Similar cell counts were done on adjacent Nissl-stained SNR_A sections. Statistics were performed on the "total numbers of δ-ir SNR_A neurons per section" as well as the percent of δ-ir neurons among the total Nissl-stained SNR_A neurons expressed as "[(total numbers of δ-ir SNR_A neurons) / (total numbers of Nissl-stained SNR_A neurons) * 100]".

3.3. Drugs

BIM (bicuculline methobromide), SR 95531 hydrobromide (gabazine), tetrodotoxine (TTX), 4,5,6,7-tetrahydroisoxazolo (5,4-c)pyridin-3-ol (THIP), muscimol, and D-AP5 were dissolved in distilled water whereas CNQX and zolpidem were dissolved in DMSO (final dilution 1:1000). Bicuculline (and its water soluble preparations such as BIM) is a competitive $GABA_A$Rs antagonist. Gabazine is a selective high-affinity antagonist binding at low-affinity $GABA_A$Rs (Heaulme et al., 1987, McCabe et al., 1988). TTX is a highly selective neuronal Na^+ channel blocker (Catterall et al., 2005), which completely inhibits firing action potentials (Atherton and Bevan, 2005). D-AP5 blocks glutamatergic NMDA receptors-mediated currents whereas CNQX inhibits AMPA receptors (King and Lopez-Garcia, 1993). Muscimol is a $GABA_A$R agonist and a partial $GABA_C$R

agonist. THIP is an agonist for α4δ-containing $GABA_A$Rs and $GABA_C$R antagonist. All drugs were diluted to the desired concentration after bath applied in aCSF and washed in the recording chamber at a flow rate of 4 ml/min. BIM, and zolpidem were purchased from Sigma-Aldrich, St. Louis, MO; gabazine, TTX, THIP, muscimol, D-AP5 and CNQX from Tocris Bioscience, Ellisville, MO.

3.4. Slice preparation

Slices containing SNR for IPSCs study were prepared from rats of either sex at PN5-9, 12-15 and 28-32, slices for tonic current recordings from age groups PN5-9, PN11-16 and PN25-32. Rats were deeply anesthetized with isoflurane and decapitated. The brain was quickly removed and placed in oxygenated (95% O_2/5% CO_2) ice-cold sucrose slicing solution containing (in mM): 187 sucrose, 3 KCl, 2 $CaCl_2$, 1.9 $MgCl_2$, 1.2 NaH_2PO_4, 26 $NaHCO_3$ and 20 D-glucose, pH 7.4, 300-310 mOsm. 300 μM thick sagittal slices were cut using a vibratome (Leica, VT1000S). Slices were transferred into oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl; 2.5 KCl; 1 NaH_2PO_4; 26 $NaHCO_3$; 2 $CaCl_2$; 1.3 $MgSO_4$ and 20 glucose, pH 7.3-7.4, 290-300 mOsm, and allowed to recover at room temperature for at least 1 hour before recording.

3.5. Electrophysiology

Cells were visualized with an upright Eclipse E600-FN microscope (Nikon) in the SNR_A (Veliskova and Moshe, 2001). Whole-cell patch clamp recordings were made from electrophysiologically identified GABAergic neurons using an Axopatch 200B amplifier (Molecular Devices, Union City, CA). Patch pipettes were pulled using

Flaming/Brown micropipette puller (Sutter Instruments Co, Novato, CA) from thin-wall borosilicate glass tubing (1.5 mm OD; World Precision Instruments, Sarasota, FL) and had open tip resistance 2-3 MΩ when filled with an intracellular solution containing (in mM): 140 CsCl, 4 NaCl, 1 $MgCl_2$, 10 HEPES, 10 EGTA, 2 Mg-ATP, 290 mOsm, pH 7.3 adjusted with CsOH. No correction was made for the liquid junction potential of +4.3 mV. Slices were continuously perfused at a rate of 4 ml/min with oxygenated aCSF solution. All recordings were performed at room temperature.

Neurons were voltage-clamped at a holding potential of -70 mV and broken in to establish whole cell configuration recordings. We waited 3-5 minutes after breaking-in until the holding current stabilized.

The SNR consists predominantly of GABAergic neurons but it also contains a small portion of dopaminergic cells. The two populations can be distinguished by their electrophysiological responses to hyperpolarizing current. To determine whether neurons were GABAergic, they were stepwise hyperpolarized in current clamp configuration by injection of negative current (from -70 mV to -130 mV) and the decisive parameter for accepting a cell as GABAergic was lack of hyperpolarization-induced inward rectification or sag (Richards et al., 1997, Radnikow and Misgeld, 1998) (Fig. 2 A, B).

A) B)

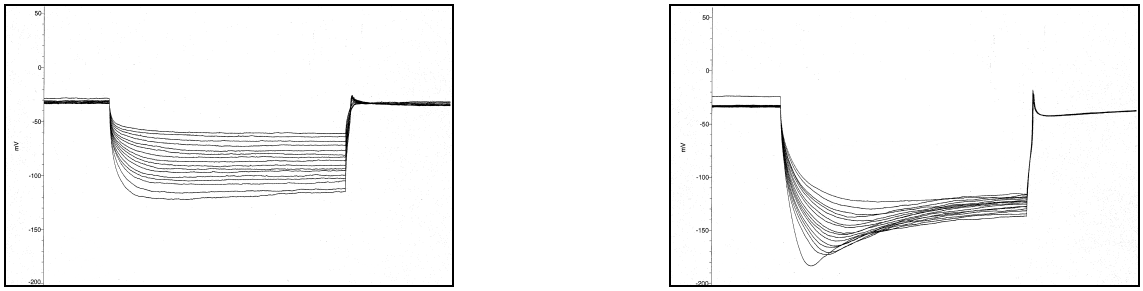

Figure 2. Electrophysiological signature of GABAergic (A) and dopaminergic cell (B).

After determining the cell phenotype, we switched back to the voltage clamp mode and held the cell at -70 mV. All GABAergic events were thus observed as inward currents. Series resistance was estimated by measuring the transient current in response to 1- to 5-mV 200 ms-long hyperpolarizing voltage steps. Cells were accepted for further analysis provided that the series resistance after 40-60% compensation did not exceed 15 MΩ and/or did not change by more than 15% during data acquisition. The input resistance could not be exactly measured due to the high intracellular Cs^+ concentration, which blocks K^+ channels (Shao and Dudek, 2005). All recordings were performed in the presence of glutamate antagonists D-AP5 (50 μM) and CNQX (10 μM) to block excitatory amino acid-mediated transmission. Recorded data were filtered at 2 kHz (low-pass Bessel filter) and sampled at 10 kHz. The bandwidth was sufficient enough to include all fast frequencies of interest. All data were recorded with pClamp 8 analysis software (Molecular Devices Co, Sunnyvale, CA) through a Digidata 1322A digitizer (Molecular Devices Co, Sunnyvale, CA).

3.5.1. Spontaneous inhibitory postsynaptic currents recording

Spontaneous inhibitory postsynaptic currents (sIPSCs) were analyzed offline using Mini Analysis Program (Synaptosoft, Decatur, GA). Individual events were automatically selected if their amplitude and area under curve were 5-fold higher than the set threshold detection parameters. All recordings were subsequently visually checked to remove artifacts. Both single and multiple peaked events were included into the analysis. A minimum of 50 accepted events per cell was analyzed (on average 450 events) and

averaged to obtain mean values. The amplitude was measured from the baseline to the peak of the synaptic current. We further analyzed the 10-90% rise time, the 37 % decay time (measured as a time required for the current to decay to 37% of its peak amplitude) and the charge transferred by a single sIPSC (calculated by the software as an integrated area under curve). The charge transfer was calculated as a product of the mean sIPSC frequency and charge transferred by averaged sIPSC ($q = f_{mean} \times q_{averaged\ sIPSC}$). Baseline sIPSCs were analyzed before zolpidem 0.5 μM was applied and compared with events when the drug was present in the slice for at least 5 minutes.

3.5.2. Tonic current recording

Baseline and post-drug holding currents (Ihold) were measured by averaging the Ihold from 20 epochs (50-100 ms each), 1 epoch per second, over a 20 second period. For baseline Ihold, the 20 second period immediately prior to the time of drug application was used. For post-drug Iholds, 20 second periods during the time of peak or trough drug responses were used, which was usually 80-100 second from the time of the drug administration. Gaussian all-point histograms were constructed from these epochs using 0.5 pA bins. The datapoints not contaminated by IPSCs were fitted according to the Levenberg-Marquardt method to obtain the mean Ihold amplitudes. The difference between baseline and post-drug Iholds expressed magnitude of the tonic current. In order to eliminate the cell size as a confounding factor in measurements, all drug-induced changes in Ihold were related to the cell capacitance and expressed as a tonic current density (pA/pF) and this value was eventually used for definite comparison of age- and sex-related differences. The cell capacitance was calculated from current

transients recorded in response to 5 mV hyperpolarizing voltage steps. In the first set of experiments, BIM (100 μM) was used to reveal the tonic current measured as the change in the Ihold. TTX (1 μM) and gabazine (500 nM) were used to eliminate IPSCs prior to BIM application to determine baseline Ihold as used in other papers to separate synaptic and extrasynaptic responses (Bai et al., 2001, Liang et al., 2004).

In the remaining pharmacological studies (muscimol, THIP, zolpidem), TTX and gabazine were not used to simulate our previous *in vivo* studies (Moshe and Albala, 1984, Garant et al., 1995, Veliskova and Moshe, 2001). In order to obtain the mean Ihold and tonic current density changes, only all point histograms of episodes uncontaminated by IPSCs were used.

3.6. Statistics

Statistics on the densitometry measurements were carried on with repeated measures multiple factor ANOVA (analysis of variance) and Tukey HSD post hoc comparisons, using Statview and JMP softwares (SAS Institute, Cary, NC, USA).

Two-way ANOVA followed by post hoc Tukey's HSD and Fisher's LSD *t*-tests were used to compare age and sex differences in sIPSCs properties and in tonic current changes, respectively. Because the sensitivity of the two-way ANOVA and post hoc tests decrease as the number of inter-group comparisons increases, we utilized unpaired *t*-test to explore whether significant differences in the studied variables existed in specific same age groups that demonstrated visible sex-related differences. The paired *t*-test was used to assess zolpidem effects. The Kolmogorov-Smirnov (K-S) two-sample, two-tailed test was used to compare cumulative amplitude, 10- 90% rise time and decay time distributions.

All values are expressed as least square mean value±standard error (SE). F values for each variable are given as $F_{variable}$ (degrees of freedom, residuals).

4. Results

4.1. α1 and α3 subunits expression in the SNR_A

In the first set of experiments, we compared the expression of the α1 and α3 $GABA_A$Rs subunits in the anterior SNR of PN5, PN15, and PN30 male and female rats, using specific immunochemistries. Representative photos and results of the statistical comparisons of the densitometric comparison of perisomatic α1-immunoreactivity (-ir) and α3-ir are presented in Figures 3 and 4 and Table 1. The $GABA_A$Rs α1-ir increased between PN15 and PN30 in both sexes (Table 1, Fig. 3). Sex had a significant overall effect, with females expressing more $GABA_A$Rs α1-ir than males (Table 1, Fig. 3). However, in inter-group comparisons of the same age groups, only the PN5 females had statistically higher expression of $GABA_A$Rs α1-ir than males (Table 1, Fig. 3). In contrast, the $GABA_A$Rs α3-ir decreased between PN5 and PN30 in both sexes, without any significant sex differences (Table 1, Fig. 4). The decrease was steeper in females, with significant statistical differences among all 3 age groups. In males, significant differences were observed only between PN5 and PN30 rats (Table 1, Fig. 4). Once again, sex differences were only observed at PN5, when females expressed more $GABA_A$Rs α3-ir than males (Table 1, Fig. 4).

Table 1. Age and sex specific effects on the expression of α1 and α3 $GABA_A$R-ir in rat anterior SNR.

A. Main effects, two-way ANOVA, repeated measures.

Groups	n (rats/group)	Age (F-value)	Sex (F-value)	Age*Sex (F-value)
α1 $GABA_A$R-ir	5	8.4*	6.5*	0.6
α3 $GABA_A$R-ir	4	12.2*	0.2	1.8

*P<0.05

B. Least squares means tables.

Groups	α1 $GABA_A$R-ir somatic expression		α3 $GABA_A$R-ir somatic expression	
	n (rats)	Least Squares Means (% of PN15 male group)	n (rats)	Least Squares Means (% of PN15 male group)
PN5 M	5	93.1±4.7#	4	115.4±6.1#
PN15 M	5	100±4.3#	4	100±5.5
PN30 M	5	124.8±4.5	4	86±5.5
PN5 F	5	116.7±4.8§	4	131.9±6#§
PN15 F	5	109.4±4.3#	4	100.9±5.6 #¶
PN30 F	5	133.2±4.6	4	75.56±5.5

#P<0.05 vs PN30 same sex group; §P<0.05 vs PN5 males; ¶P<0.05 vs PN5 females; Tukey post hoc comparisons.

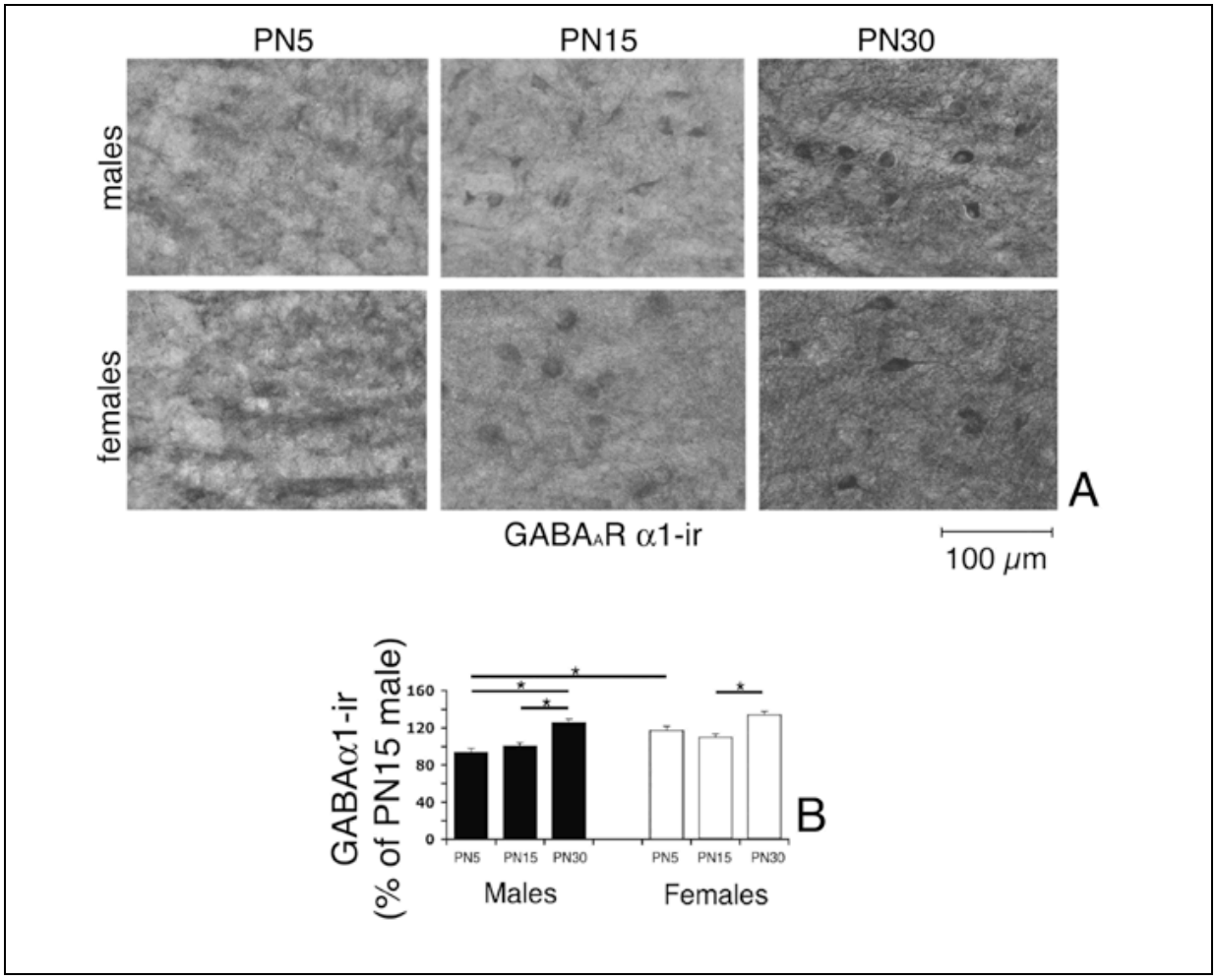

Figure 3. Expression of $GABA_A$Rs α1-ir in the anterior SNR of PN5, PN15, and PN30 male and female rats. **Panel A:** Representative photographs of anterior SNR neurons stained with anti- $GABA_A$Rs α1 specific antibody. The α1-ir increases with age in both genders, in both the somata and the dendritic processes. The scale bar indicates 100μm distance. **Panels B:** Densitometric comparisons of perisomatic α1-ir confirmed the developmental increase in $GABA_A$Rs α1-ir in both male and female rats between PN5 and PN30. As the SNR is sparsely populated, densitometry was done on individual α1-ir anterior SNR cells and was averaged for each brain. At PN5, females expressed more $GABA_A$Rs α1-ir than PN5 males. The asterisks indicate statistically significant differences ($P<0.05$) between the groups linked with bars.

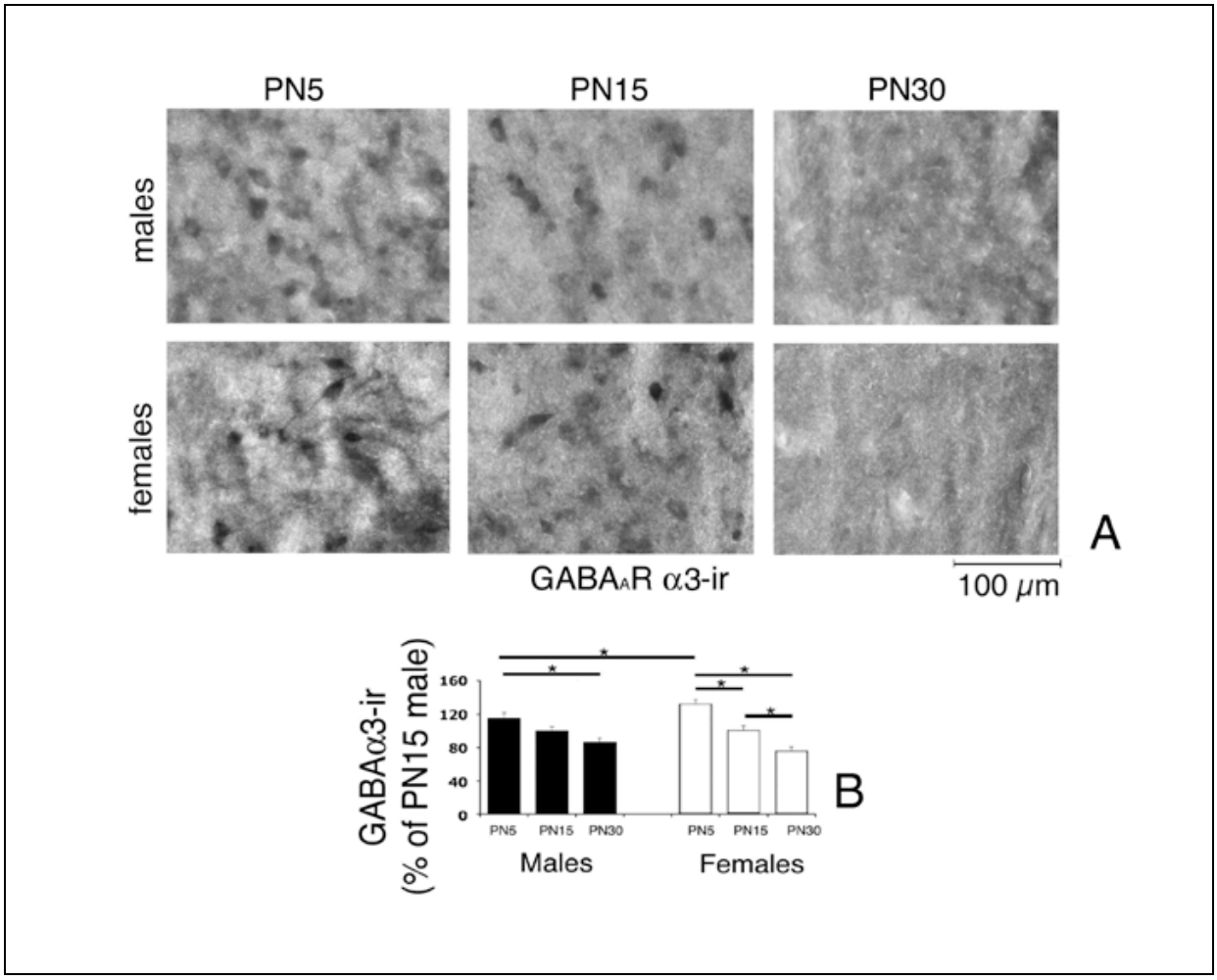

Figure 4. Expression of $GABA_AR$ α3-ir in the anterior SNR of PN5, PN15, and PN30 male and female rats. **Panel A:** Representative photographs of anterior SNR neurons stained with anti-$GABA_ARs$ α3 specific antibody. The α3-ir decreases with age. The scale bar indicates 100μm distance. **Panels B:** Densitometric comparisons of perisomatic α3-ir confirmed the developmental decrease in $GABA_ARs$ α3-ir in both male and female rats between PN5 and PN30. Densitometry was done on individual α3-ir anterior SNR cells and was averaged for each brain. At PN5, females expressed more $GABA_ARs$ α3-ir than males. The asterisks indicate statistically significant differences ($P<0.05$) between the groups linked with bars.

4.2. Baseline sIPSCs

Spontaneous IPSCs were recorded in the presence of CNQX 10 μM and D-AP5 50 μM (Fig. 5 A, B). Under these conditions all sIPSCs were blocked by BIM, which confirms that they were $GABA_A$Rs-mediated (Fig. 5 C).

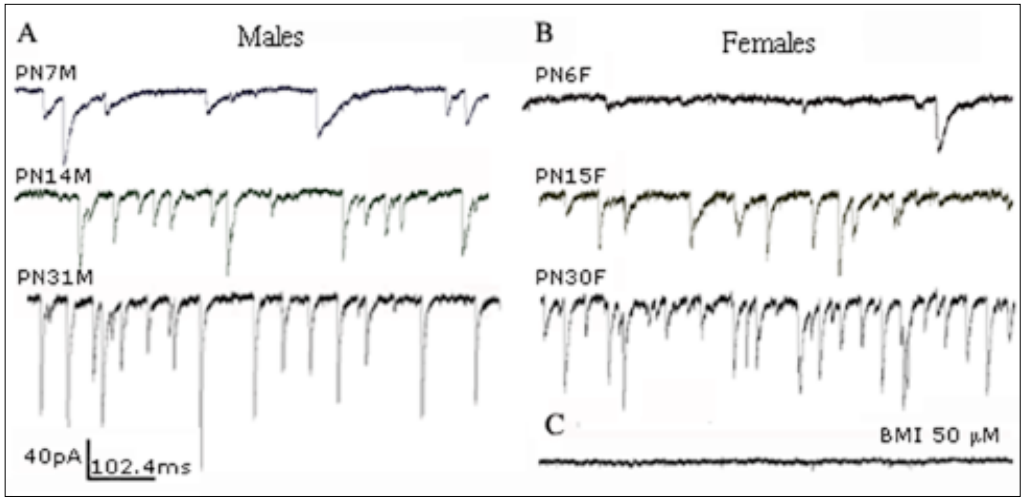

Figure 5. Raw traces of sIPSCs. Raw traces of spontaneous $GABA_A$Rs-mediated IPSCs recorded in voltage-clamp configuration from GABAergic SNR neurons. Whole cell patch clamp recordings were made at a holding potential of -70 mV in the presence of glutamate antagonists CNQX 10 μM and D-AP5 50 μM in males (A) and females (B) of different ages. All sIPSCs were invariably blocked by $GABA_A$Rs antagonist BIM 50 μM in all groups (C).

The sIPSCs could be detected at all studied ages in both sexes. The events reversed close to 0 mV, the theoretical equilibrium potential for Cl^- ions (Fig. 6 A, B).

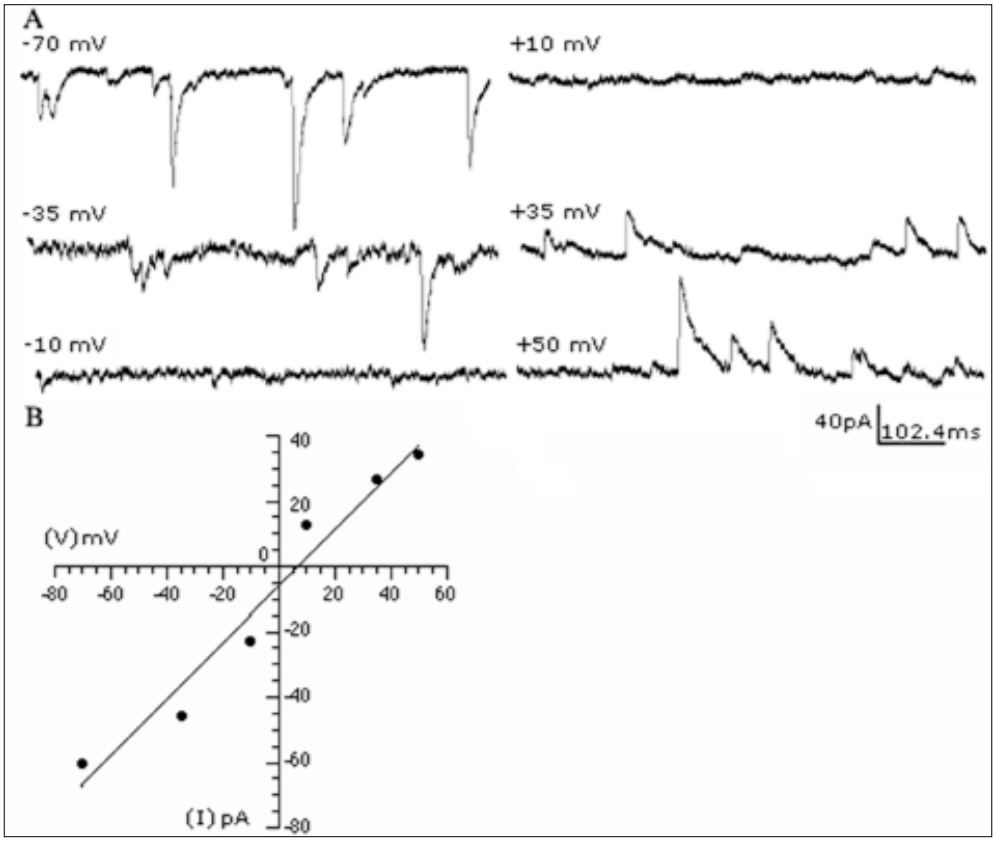

Figure 6. $GABA_A$Rs-mediated sIPSC reversal potential. (A) The recorded sIPSCs were inward at negative holding potentials and outward at positive holding potentials. (B) A current-voltage plot of the average sIPSC amplitude vs. holding potential from the cell in (A) shows that the events reversed close to the theoretical equilibrium potential for Cl^-, as expected with symmetrical intra- and extracellular chloride concentrations, confirming that they were mediated by $GABA_A$Rs. The plot was best fitted with a linear regression.

The results of the two-way ANOVA for the studied sIPSC parameters and inter-group comparisons are presented in Figure 7 and all numeric values are summarized in Table 2.

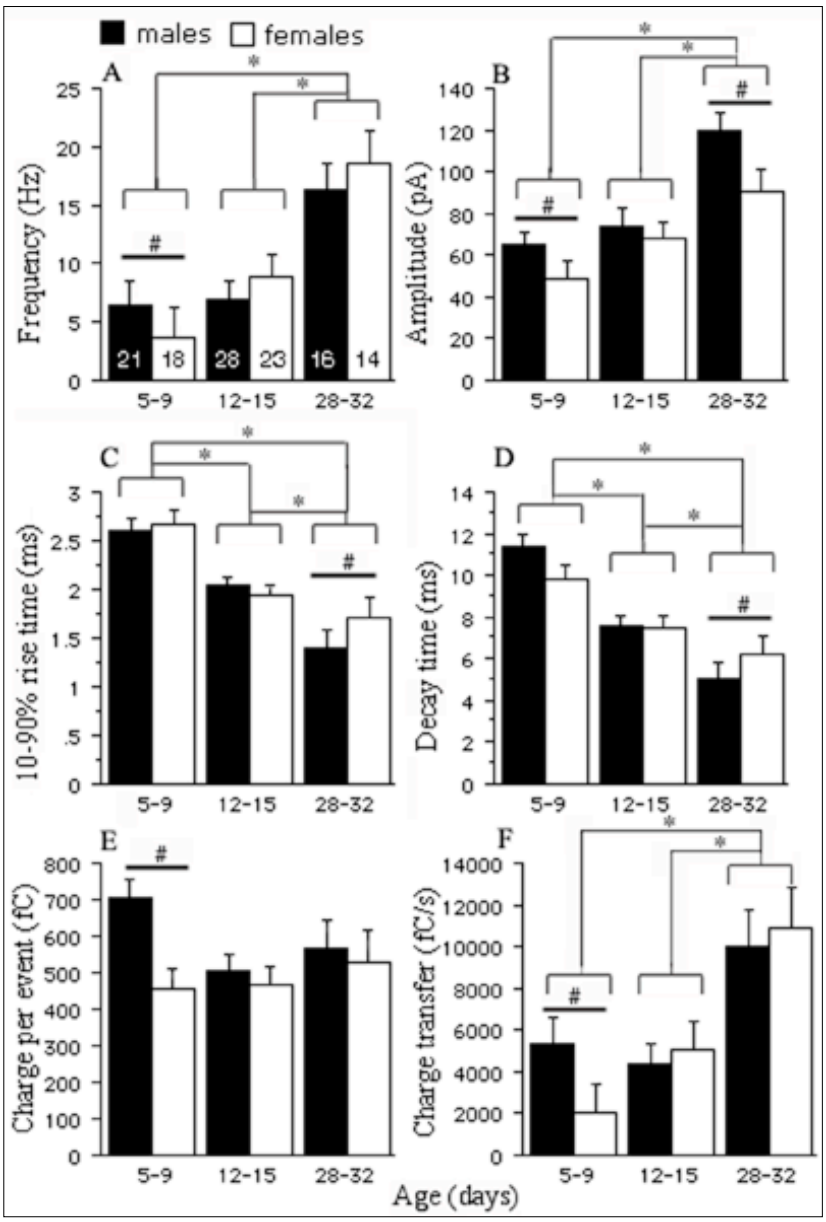

Figure 7. Baseline properties of the spontaneous inhibitory postsynaptic currents during development. *P<0.05 two-way ANOVA; #P<0.05, unpaired t-test; numbers in graph A represent the numbers of cells.

Specifically, the mean sIPSCs frequency significantly rises with age in both sexes.

Although overall sex differences were not found using the two-way ANOVA, separate comparisons of the average sIPSCs frequencies by the unpaired *t*-test in the individual age groups revealed that PN5-9 males have greater frequency than PN5-9 females (Fig. 7A, Table 2).

Table 2. Baseline properties of sIPSCs

Age group/sex	n (cells)	Frequency (Hz)	Amplitude (pA)	10-90% rise time (ms)	Decay time (ms)	Charge per averaged event (fC)	Charge transfer (fC/s)
PN5-9 M	21	6.4±1.9[#]	65.5±7.1[#]	2.61±0.1	11.37±0.5	706±51.9[#]	5326±1553[#]
PN12-15 M	28	7.0±1.7	73.9±6.1	2.03±0.1	7.57±0.4	506±44.9	4400±1345
PN28-32 M	16	16.4±2.2	119.2±8.1[#]	1.4±0.2[#]	5.1±0.6[#]	566±59.4	10010±1779
PN5-9 F	18	3.7±2.1	48.6±7.6	2.67±0.1	9.86±0.5	457±56.0	2061±1678
PN12-15 F	23	8.8±1.8	67.9±6.8	1.94±0.1	7.52±0.5	467±49.6	5025±1484
PN28-32 F	14	18.5±2.4	90.6±8.7	1.72±0.2	6.19±0.6	528±63.4	10894±1902

[#] $P<0.05$ value different from animals of opposite sex in the same age group, unpaired t-test. F=female, M=male.

The mean sIPSCs amplitude also significantly increased with age in both sexes. Furthermore, male SNR neurons had higher sIPSC amplitudes than female ones (Fig. 7B). Although post hoc comparisons with Tukey test did not show any significant sex differences between same age groups, possibly due to the high number of comparisons,

the unpaired *t*-test revealed significantly higher sIPSC amplitudes in males than in females in the PN5-9 and PN28-32 groups (Figs. 7 B, Table 2). Changes in the sIPSCs kinetics seen in our experiments were similar to those found in other studies (Dunning et al., 1999, Okada et al., 2000). Both the 10-90% rise time and decay time progressively accelerated with age (Figs. 7 C, D, 8 A, B, Table 2). The unpaired *t*-test showed that both these parameters were significantly faster in PN28-32 males than females.

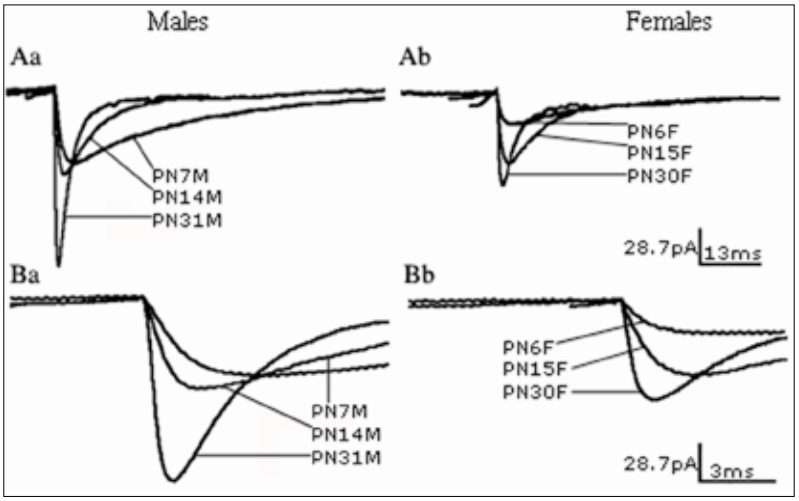

Figure 8. Age-related changes in the averaged $GABA_A$Rs-mediated sIPSC traces of male and female GABAergic SNR neurons. (Aa) and (Ab) show averaged representative sIPSC traces derived from 232-469 events recorded from PN7, PN14, PN31 male and PN6F, PN15, PN30 female GABAergic SNR neurons. The overlapped traces demonstrate acceleration of decay time with age in both genders. The mean amplitude is highest in both PN28-32 groups. (Ba) and (Bb) figures in expanded time scale illustrate shortening of the 10-90% rise time during development.

In the face of significant changes in the amplitude and kinetic parameters, the charge transferred by averaged sIPSC remained quite stable during development. The only exceptions were events in PN5-9 males, which transferred significantly bigger charge than PN5-9 females (Fig. 7E, Table 2). The total charge transfer carried by synaptic

currents gradually grew with maturation in both males and females. PN5-9 males demonstrated higher increase than same age females (Fig. 7F and Table 2).

The occurrence of slow sIPSC kinetics in younger groups and the opposite pattern observed in older animals (Figs. 7, 8) may result from the known maturational subunit changes in $GABA_A$ergic synapses. We performed an additional analysis of the sIPSC 10-90% rise times and decay times in a subset of PN5-9, PN12-15 and PN28-32 male and female cells with almost similar series resistances (Rs ranged from 10.8 to 11.7 MΩ, $P>0.05$) to assess the datapoints distribution. Only single events emerging from the baseline were included to avoid distortion of the kinetic parameters and amplitudes by overlapping events. We analyzed 2223-2309 events from each group and plotted decay times of individual events against their 10-90% rise times. The widespread distribution of events in PN5-9 groups most likely reflected greater subunit heterogeneity of $GABA_A$Rs. (Fig. 9A). In contrast, the scattergrams of PN28-32 groups demonstrated that the occurrence of mainly fast events was confined to a very small area of the plot (Fig. 9B) suggesting that the composition of $GABA_A$Rs was more homogeneous (Moshe et al., 1994).

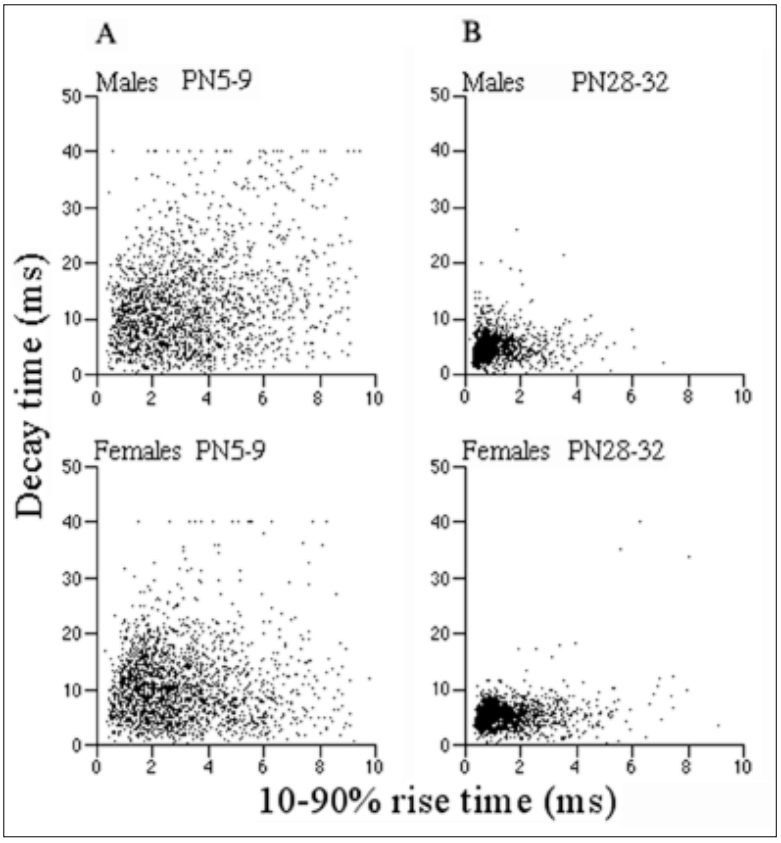

Figure 9. Single event analysis scattegrams. The sIPSCs decay times of single events of SNR GABAergic neurons were plotted against 10-90% rise times in PN5-9 and PN28-32 animals of both genders. The broad dispersion of datapoints in the PN5-9 group plots (column A) reflects heterogeneous $GABA_A$Rs subunit composition. The majority of events have slow activation and deactivation times. The situation is different in PN28-32 groups (column B), where the decay vs. rise time plots show that most events are confined to one limited area of the graph representing fast values. This finding suggests that $GABA_A$Rs subunit composition is more homogenous.

4.3. δ subunit expression in the SNR_A

To study the age- and sex-specific differences in δ $GABA_A$R subunit immunoreactivity (δ-ir) in SNR_A neurons we used immunohistochemistry, because it allows comparisons in protein expression at the cellular level and avoids contamination of readouts by heterogeneous cell populations. The perisomatic δ-ir in the SNR_A changed as a function of age ($F_{age}(2,43)=15.45$; $P<0.001$) but not sex ($F_{sex}(1,43)=0.4$; $P>0.05$). The δ-ir was high at PN5 and PN15 male and female rats and declined significantly at PN30 (Fig. 10

A, B). Results are expressed as "% of δ-ir in PN15 males", which were included as a reference group in each set of immunochemistry. This approach helped minimize interassay variability and allowed comparisons across the different sets of immunochemistry assays. In parallel, a greater than 50% decrease in the total number of δ-ir SNR_A neurons occurred between PN15 and PN30 ($F_{age}(1,19) = 21.99$, $P=0.002$), without any sex differences ($F_{sex}(1, 19) = 0.05$, $P=0.8$) (n=5 rats per group). The percentage of SNR_A neurons expressing δ-ir declined from 79.9 ± 9.8 % at PN15 to only 35.9 ± 4.3 % at PN30 ($F_{age}(1,11) = 13.67$, $P<0.006$, n= 6 rats per age group).

4.4. BIM-sensitive $GABA_AR$ tonic current

Since the δ subunit mediates extrasynaptic tonic $GABA_AR$ responses, we investigated whether the observed age- but not sex-dependent changes in δ-ir functionally correlate with similar changes in BIM-sensitive $GABA_AR$-mediated tonic currents. In order to separate synaptic and tonic currents, whole cell patch clamp recordings were performed using TTX and gabazine, applied prior to BIM. In the presence of glutamatergic inhibitors CNQX and D-AP5, the TTX-induced outward shift of the baseline Ihold in all groups indicated that action potentials contribute to a certain degree to the tonic inward current (Fig. 10 C). We found no age- or sex-related differences in TTX-induced tonic current ($F_{age}(2, 45)=2.84$, $F_{sex}(1, 45)=1.09$, $F_{age*sex}(2, 45)=1.69$, $P>0.05$, two-way ANOVA). These findings suggest that action potential dependent neurotransmitter spillover from the synaptic cleft may contribute to tonic currents (Leao et al., 2000, Jensen et al., 2003). Further addition of gabazine (500 nM) completely blocked residual miniature IPSCs (mIPSCs), but did not alter the Ihold indicating that solely

synaptic receptors were blocked (Fig. 10 C).

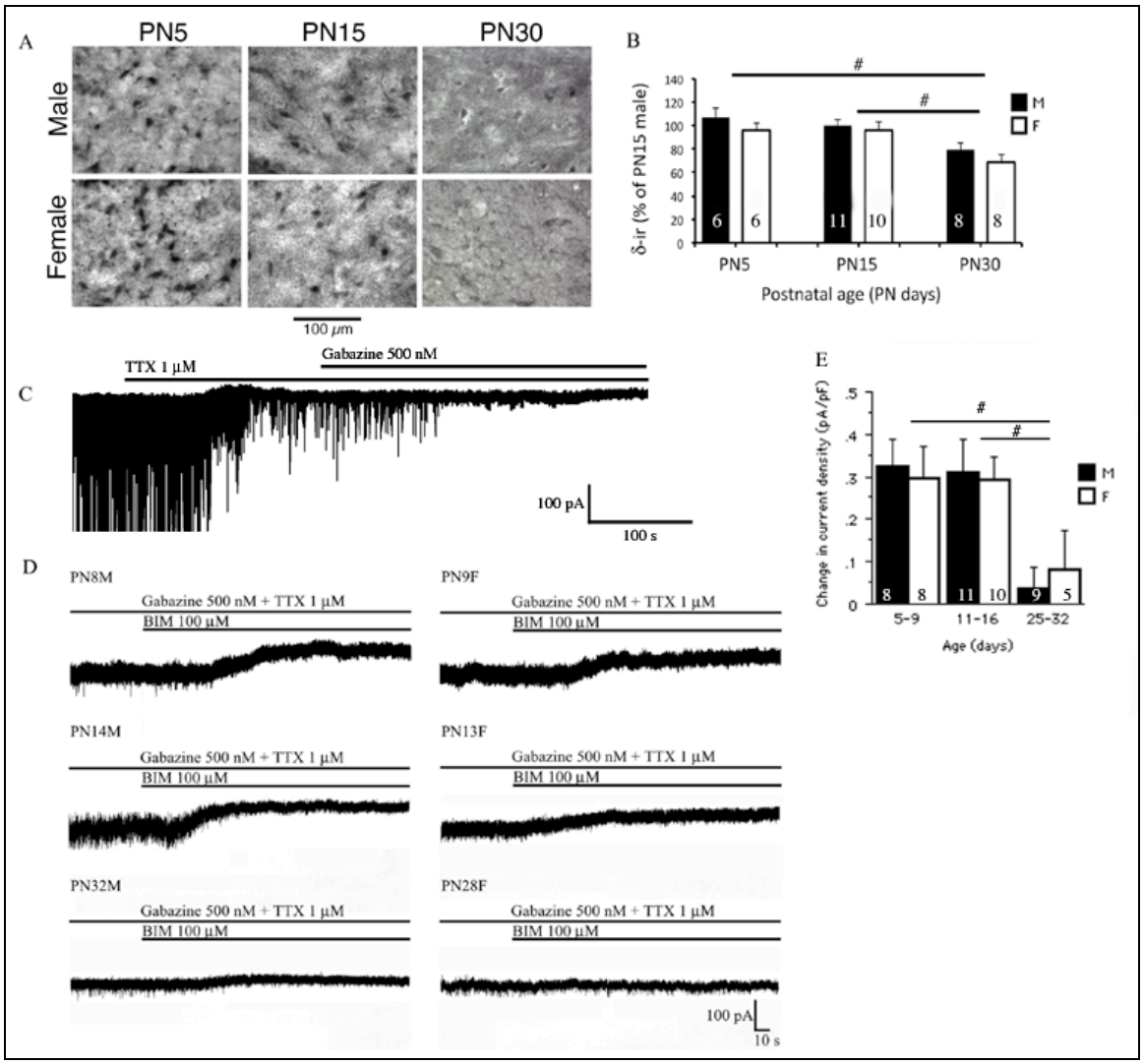

Figure 10. Developmental changes in δ-ir and BIM-sensitive tonic currents in rat SNR_A neurons. The pound keys (#) indicate significant differences ($P<0.05$, post hoc Fisher's test) between linked age groups. No sex differences were noted. The numbers in the column bars indicate the number of cells. All values are expressed as least square mean values ± standard errors (SE). M = male; F = female.

Bath application of BIM (100μM) revealed significant age- but not sex-specific changes in Ihold (Fig. 10 D). When expressed as a tonic current density, the results show that these were not attributable to developmental changes in cell size ($F_{age}(2, 45)=6.77$, $P<0.05$; $F_{sex}(1, 45)=0.003$, $P>0.05$; $F_{age*sex}(2, 45)=0.12$, $P>0.05$, two-way ANOVA) (Fig.10 E). The tonic current density was similar in PN5-9 and PN11-16 groups, without sex differences, but almost disappeared in PN25-32 neurons. The percentage of cells generating tonic current: males PN5-9 88%, PN11-16 82%, PN25-32 33%; females PN5-9 88%, PN11-16 100%, PN25-32 20%. The developmental changes in δ subunit expression and the concurrent differences in the tonic current density unmasked by BIM suggest that the reduction in δ−ir between PN15 and PN30 may underlie the decline in tonic current density in SNR_A GABAergic neurons.

4.5. $GABA_ARs$ subunit composition in SNR_A neurons underlies different pharmacological responses

4.5.1 Zolpidem-induced changes in sIPSCs kinetics and tonic current

The age- and sex-dependent changes in sIPSCs amplitude and kinetics and different α1 subunit expression during maturation led us to investigate whether pharmacological responses to zolpidem would also alter with age and sex.

Zolpidem 0.5 μM, an α1 subunit-selective positive modulator of type I benzodiazepine receptors (Pritchett et al., 1989), was bath applied and changes in the mean sIPSCs decay time and amplitude were analyzed after at least a 5-minute exposure to the drug.

Zolpidem significantly prolonged the decay time and increased the amplitude and

charge per event compared with baseline sIPSCs in most groups (P<0.05, K-S test, Fig. 11).

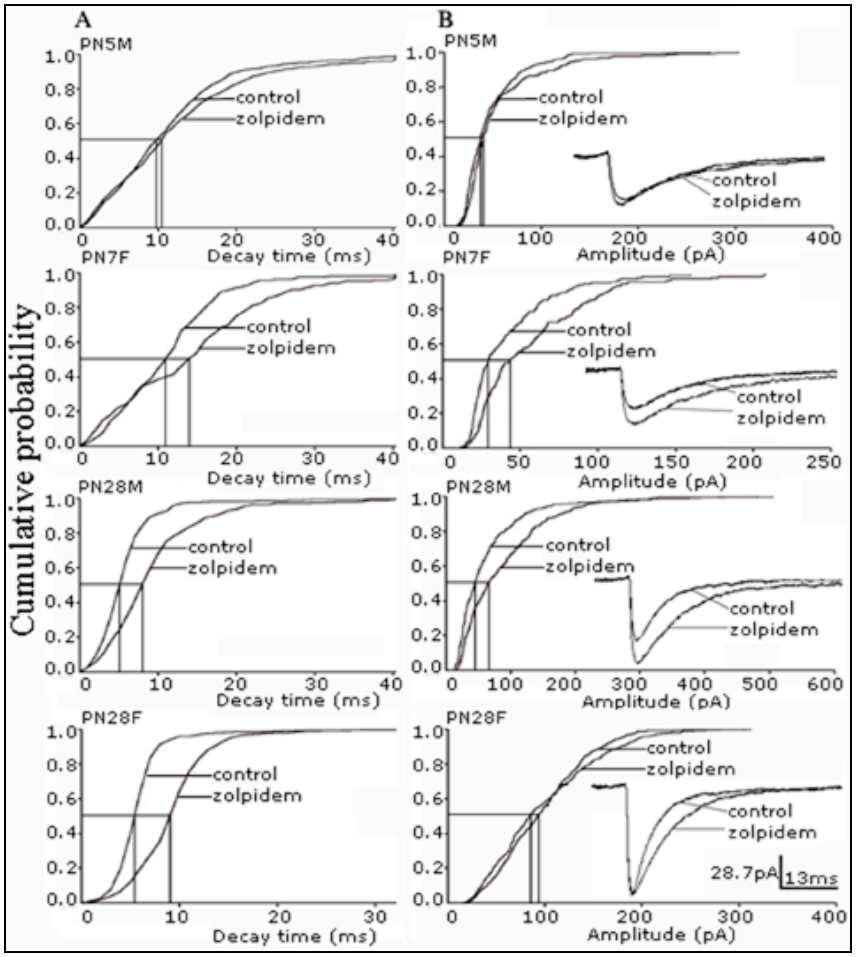

Figure 11. Decay time (column A) and amplitude (column B) cumulative probability plots from representative cells in PN5-9 and 28-32 groups. Zolpidem 0.5 μM significantly changed cumulative amplitude and decay distribution in all cells shown (P<0.05, K-S test) except in the PN5M cell (no change in decay time and amplitude) and PN28F cell (no change in amplitude) (P>0.05 K-S test). While the lack of zolpidem effect both on decay time and amplitude in the PN5M cell can be explained by low α1 subunit expression, the near-to-complete saturation of $GABA_A$Rs may underlie the failure of zolpidem to augment the average sIPSCs amplitude in the PN28F cell. PN12-15 male and female SNR neurons were also responsive to zolpidem (data not shown). The insets in graphs in the column B show overlapped baseline and zolpidem averaged events from the same cells.

PN5-9 males were the only exception where there was no significant increase in any of these variables in response to zolpidem. This may mean that $GABA_A$ receptors in PN5-9 male SNR neurons have less $\alpha 1$ subunits than the other groups. In PN28-32 females, zolpidem significantly increased the decay time and charge per event but not the sIPSC amplitude (Fig. 11). As PN28-32 females had the largest baseline amplitudes compared to the other groups, this may reflect a ceiling effect (baseline amplitude 82.5±12.5 pA, zolpidem amplitude 89.8±11.5 pA, n=6, $p>0.05$, mean±SE, paired *t*-test).

The overall age- and sex-related differences in sensitivity to zolpidem 0.5 μM were determined using the two-way ANOVA by comparing ratios of drug-induced responses to the pre-drug values. The zolpidem-induced percentage increases of the mean decay time and charge per event were greater in older animals than in younger ones ($P<0.05$) but no sex differences were observed (Fig. 12 A, C). No significant overall age and sex specific differences in zolpidem-induced percentage increase of the mean sIPSCs amplitude were observed (Fig. 12 B). These results suggest that SNR_A neurons are sensitive to zolpidem at all studied ages, with a single exception being the PN5-9 males, and that responsiveness to zolpidem increases with age.

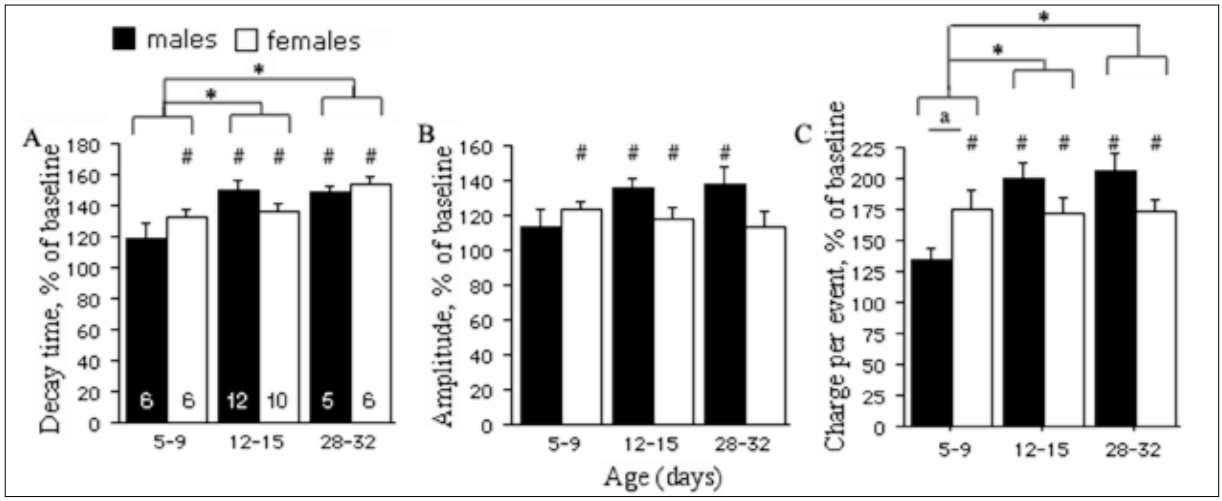

Figure 12. The percentage increase relative to the pre-drug baseline of decay time (panel A), amplitude (panel B), and charge per event (panel C) in response to zolpidem 0.5 μM. The pound keys (#) indicate groups in which zolpidem induced significant changes compared to baseline levels ($P<0.05$, paired t-test, data not shown). The asterisks (*) indicate significant differences from the respective PN5-9 group ($P<0.05$, two-way ANOVA, numbers in the plot A indicate the number of cells).

The next question was whether zolpidem can also affect the baseline tonic current in SNR_A neurons. Indeed, zolpidem (0.5 μM) induced a small inward current in more than 70% of all tested cells. However, there were no significant age and sex differences observed in zolpidem-induced current densities ($F_{age}(2, 27)=0.3$; $F_{sex}(1, 27)=0.09$, $F_{age*sex}(2, 33)=0.17$, $P>0.05$, two-way ANOVA) (Fig. 13).

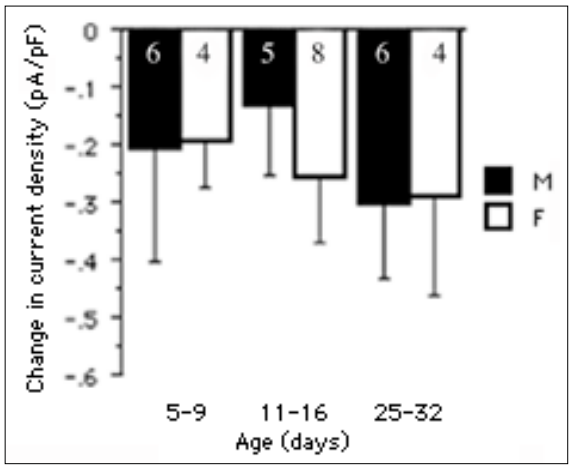

Figure 13. Zolpidem-induced inward current. P>0.05, two-way ANOVA numbers in the plot indicate the number of cells.

4.5.2. THIP-induced changes in tonic current

We then tested whether the decline in δ $GABA_AR$ subunit also parallels the developmental changes in tonic currents induced by THIP, an α4β3δ $GABA_A$Rs agonist and $GABA_CR$ antagonist. THIP (5 μM) application resulted in inward tonic current shifts in all age groups ($F_{age}(2, 44)=3.34$, $F_{sex}(1, 44)=11.85$, $P<0.05$; $F_{age*sex}(2, 44)=0.17$, $P>0.05$; two-way ANOVA) (Fig. 14 A, B). However, THIP induced changes in current density did not follow the same age-specific patterns like the δ-ir and BIM data. Consequently, no age-related differences were found in either sex (P>0.05, unpaired-*t* test). There was an early trend for males to respond with greater THIP-induced tonic current density than females, which was significant in PN5-9 SNR_A (P<0.05, unpaired-*t* test). No other sex differences were observed, although the statistical significance was almost reached in the PN11-16 groups (P=0.055, unpaired-*t* test). The dissociation between the effects of THIP and BIM on the tonic current density suggests that there are sex- but not age-related differences in unoccupied THIP-sensitive receptors which are not due to differences in δ subunit expression in SNR_A neurons.

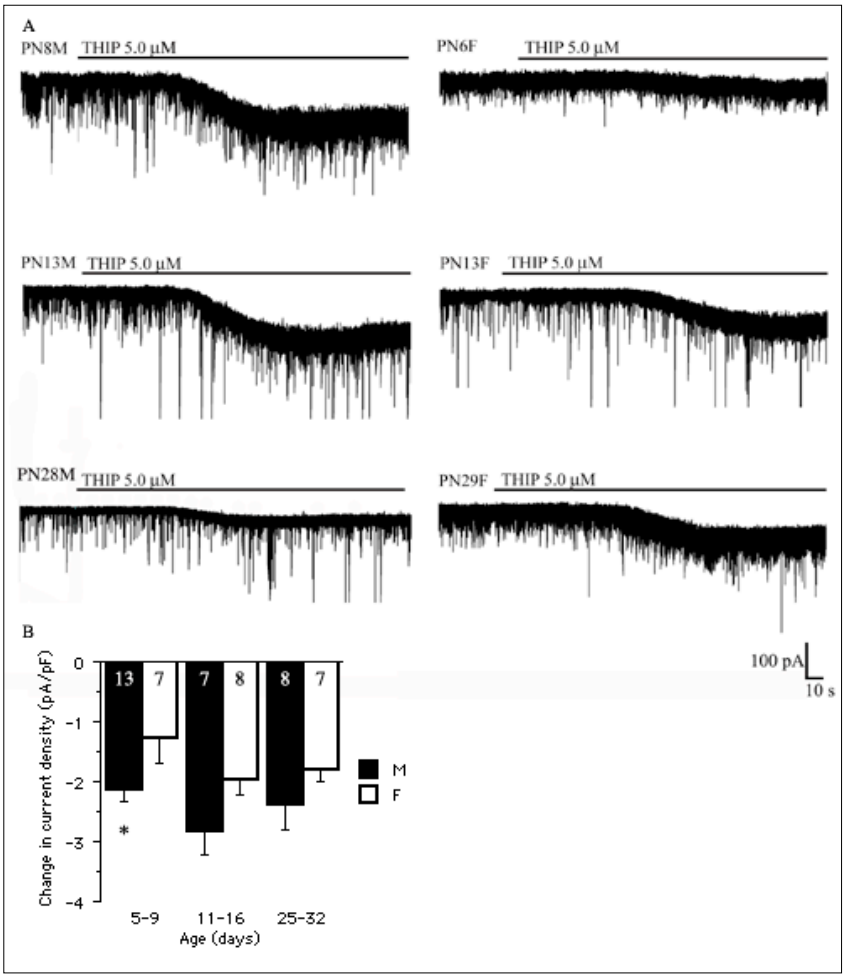

Figure 14. THIP-induced tonic current. The numbers in the column bars indicate the number of cells. All values are expressed as least square mean values ± standard errors (SE). M: male; F: female.

4.5.3. Muscimol-induced changes in tonic current

Knowing the sex- and age-related differences in the α1 subunit expression in the rat SNR_A from our current experiments and previous studies (Wurpel et al., 1988, Veliskova et al., 1998a, Ravizza et al., 2003), we investigated whether a direct α1 agonist muscimol augments inward tonic currents in the similar sex-specific pattern. Muscimol 100 nM induced large changes in current density in all age groups (Fig. 15 A) with age- and sex-specific differences ($F_{age}(2, 41)=9.89$, $P<0.05$; $F_{sex}(1, 41)=9.78$, $P<0.05$; $F_{age*sex}(2, 41)=1.99$, $P>0.05$, two-way ANOVA) (Fig. 15 B). Muscimol-enhanced current density

increased with age in both sexes (PN25-32 and PN11-16 > PN5-9), and was significantly greater in PN11-16 females than in the same age males ($P<0.05$, unpaired *t*-test). These findings indicate a developmental increase in muscimol-induced tonic current in the SNR_A, irrespective of the developmental changes in cell size, and this increase was more pronounced and appeared earlier in females.

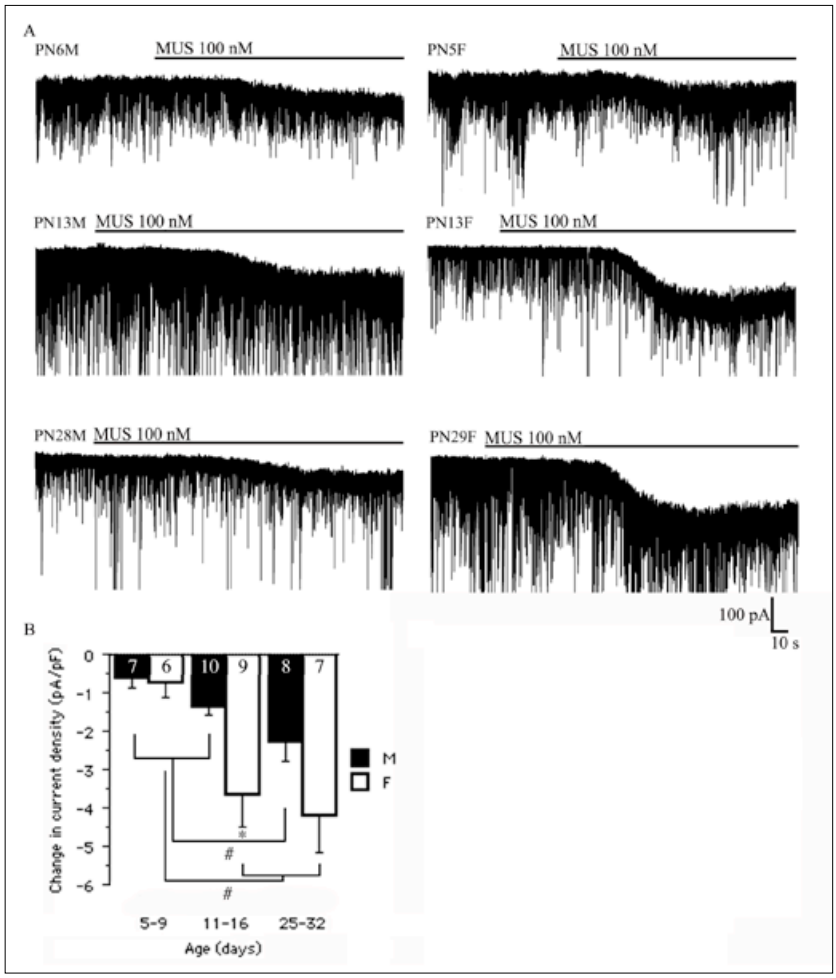

Figure 15. Muscimol-induced tonic current. $^{*}P<0.05$, unpaired t-test, $^{\#}P<0.05$, one-way ANOVA. The numbers in the column bars indicate the number of cells. All values are expressed as least square mean values ± standard errors (SE). M: male; F: female.

5. Discussion

The current study used immunohistochemical methods to describe age- and sex-related differences in the expression of α1, α3 and δ subunits of $GABA_A$ receptors in the rat SNR_A and electrophysiological techniques to measure synaptic and tonic $GABA_A$ receptors-mediated currents. Our results show specific subunit changes in the synaptic and extrasynaptic $GABA_A$ receptors, which underlie differences in both types of GABAergic inhibition in the SNR_A.

5.1. Synaptic GABAergic transmission is linked to the α subunit subtype

5.1.1. sIPSCs frequency and amplitude

Our data show that the mean frequency and amplitude rise in both sexes with age. The developmental increase in $GABA_A$Rs α1-ir and parallel decrease in $GABA_A$Rs α3-ir may partially explain some of these changes. Increased mean sIPSCs frequency and higher amplitude in PN28-32 GABAergic rat SNR neurons in both sexes along with marked acceleration of the sIPSCs kinetics can have several explanations including a) an increased number of $GABA_A$ergic synaptic terminals, b) higher density of postsynaptic $GABA_A$Rs containing mainly α1 subunit and c) decreased expression of postsynaptic $GABA_A$Rs containing α3 subunits.

The higher frequency of sIPSCs in PN28-32 animals can be due to a greater number of synapses formed on nigral GABAergic neurons with age (Phelps and Adinolfi, 1982) (Kraszewski and Grantyn, 1992, Swanwick et al., 2006). However, age-related increases in firing rates of presynaptic striatal and pallidal neurons can also contribute to

higher sIPSCs frequency as we measured both action potential-dependent and -independent events.

The sIPSCs amplitude is determined by the number of open synaptic $GABA_A$Rs and the amount of released GABA (Frerking et al., 1995). The role of an α subunit type in affecting the amplitude of synaptic currents may be limited because, despite the increase in α1 mRNA with age, the mean amplitude decreases in cerebellar granule cells (Brickley et al., 1996) or does not change in dentate gyrus granule cells (Hollrigel and Soltesz, 1997). Furthermore, the reduction of amplitude in $\alpha1^{0/0}$ knockouts most likely relates to a decreased number of $GABA_A$Rs at synapses rather than to a different subunit composition (Vicini et al., 2001, Goldstein et al., 2002). Therefore the increase in the amplitude of sIPSCs after PN15, in both sexes, rather indicates an increased availability of postsynaptic $GABA_A$Rs in GABAergic SNR neurons.

5.1.2. sIPSCs rise time, decay time and charge transfer

The acceleration of the decay and rise times in PN28-32 reflects age-related changes in a variety of pre- and post-synaptic factors that control the kinetics of sIPSCs. An important variable is the type of expressed α subunit (Verdoorn, 1994). Currents produced by recombinant α1 subunit-containing $GABA_A$Rs deactivate faster than those mediated by $GABA_A$Rs composed of α2 or α3 subunits and, as a result, such receptors close more rapidly and their decay time is reduced (Verdoorn, 1994, Gingrich et al., 1995, Lavoie et al., 1997). The amount of charge transferred by an averaged sIPSC is conserved during maturation. This variable is determined to a large extent by the amplitude and decay time of sIPSC. Therefore, older animals, despite shorter decay time, develop equal charge

transfer owing to the higher amplitude. The importance of phasic inhibition during development increases nonetheless as the charge transfer is significantly augmented in PN28-32 males and females. The main reason is increased frequency of sIPSCs due most likely to higher firing rate of presynaptic neurons and/or increased number of active $GABA_A$ergic synapses.

5.1.3. Zolpidem effects on sIPSCs

In order to determine whether the observed changes in the kinetics of sIPSCs can be attributed to changes in α subunit composition, we studied the responsiveness of SNR neurons to the α1-selective agonist zolpidem as well as compared the perisomatic expression of α1-ir across the different groups. Indeed, the zolpidem-induced percentage increase of the decay time in PN28-32 animals was significantly higher than in PN5-9 group in both sexes. This finding is in agreement with the developmental increase in α1 mRNA expression (Moshe et al., 1994, Veliskova et al., 1998a) and α1-ir (this study) in the anterior SNR, as well as with the age-related increase in high-affinity binding sites for the α1 $GABA_A$Rs agonist muscimol in rat SNR (Wurpel et al., 1988).

The zolpidem effect on the sIPSCs amplitude is mediated by increasing the affinity of closed $GABA_A$Rs for GABA rather than by increasing the probability of channel opening or enhancing conductance (Perrais and Ropert, 1999, Hajos et al., 2000, Goldstein et al., 2002). Zolpidem increased the amplitude and charge per event in most age groups, although without any overall age- and sex-related differences. The only group that did not respond to zolpidem was the PN5-9 male rats, which can be explained by the lower expression of α1-ir compared to the same age females. The little change of the mean

sIPSCs amplitude and great enhancement of the decay observed in PN28-32 females reflects most likely near-to-complete saturation of $GABA_A$Rs containing the α1 subunit. Although the age-related changes in the decay time correlated well with the α1-specific assays (zolpidem responses and α1-ir), additional sex-specific factors, not necessarily related to α1 subunit, seem to interfere with the shaping of sIPSC kinetics in PN5-9 and PN28-32 males and females. The absence of significant sex differences in the decay time at PN5-9, despite the enhanced α1-ir expression and zolpidem sensitivity in PN5-9 females, may be partially explained by the higher expression of α3-ir in PN5-9 females. The increased number of α3-containing $GABA_A$Rs in PN5-9 females may therefore create a subpopulation of sIPSCs with slower kinetics, blunting the differences in decay times between males and females.

In the absence of sex differences in zolpidem sensitivity and perisomatic α1-ir or α3-ir, the slower decay and 10-90% rise times, along with the lower amplitude, in PN28-32 females may suggest a more distant site of origin of their sIPSCs compared to the same age males. The significantly slower rise times combined with lower amplitudes in PN28-32 females thus support the hypothesis that their synaptic events may be more subjected to dendritic filtering than those in PN28-32 males. There are no studies to date describing sexual dimorphism in the organization and size of the dendritic tree of GABAergic neurons and $GABA_A$ergic synapses in the SNR of PN28-32 animals but sex differences have been reported in other brain structures, e.g. in the anterior cingulate cortex (Markham and Juraska, 2002), accessory olfactory bulb (Caminero et al., 1991) and subiculum (Andrade et al., 2000).

5.1.4. Discrepancy between α1 mRNA expression, somatic α1-ir and electrophysiology

Based on our *in situ* hybridization data showing higher α1 mRNA expression in PN15 and PN30 females (Ravizza et al., 2003), we expected that the baseline decay time would be faster and zolpidem-induced prolongation of the decay time significantly greater in females than in males. Our current electrophysiological data did not, however, show sex differences between PN12-15 groups and the only significant difference was found between PN28-32 males and females, males having quicker decay time (Fig. 7D, Table 2). The discrepancy between *in situ* α1 mRNA hybridization, somatic α1-ir and expected kinetic data in PN12-15 and PN28-32 rats may have the following explanations: First, despite the higher α1 mRNA in PN15 females, the synaptic α1 protein levels may be similar in males and females as reflected by the same decay time and somatic α1-ir (Fig. 3). This idea is further supported by the same zolpidem-induced prolongation of the decay time. PN12-15 females, however, may have an increased α1 subunit expression in the extrasynaptic compartment as some studies have already suggested that α1-containing $GABA_A$Rs may indeed be present extrasynaptically (and thus do not participate in shaping the decay time) and play an important role in modulating tonic $GABA_A$Rs-mediated currents by benzodiazepines such as zolpidem or lorazepam (Liang et al., 2004, Shen et al., 2005). Indeed, zolpidem, an α1 subunit modulator, in our study induced small but detectable tonic currents. Also muscimol, a direct α1 agonist, generated significantly bigger tonic current in PN11-16 female rats compared with the same age males (see bellow). The dissociation in the magnitude of zolpidem and muscimol sensitive tonic currents may reflect direct agonistic effects of muscimol on α1

subunit containing $GABA_A$Rs.

Second, as discussed earlier, it is possible that the previously reported increased α1 mRNA expression in PN30 females may reflect α1 subunits that are ultimately targeted to $GABA_A$Rs located at distal dendritic synaptic sites.

Additional variable that may influence the sIPSC shape is the vesicular transmitter release. The vesicular GABA release may be more asynchronous in PN5-9 animals than in PN28-32, yielding sIPSCs with slower decay and rise times in the younger age group (Vautrin and Barker, 1995, Williams et al., 1998). Another factor that may contribute to the sex differences in $GABA_A$ receptor function in the SNR are naturally occurring neurosteroids as their site of action was recently identified in the α1 (Ueno et al., 2004, Rahman et al., 2008) and other α subunit subtypes (Hosie et al., 2009). Neurosteroids or their metabolites may also regulate the expression of specific $GABA_A$R subunit (Maguire and Mody, 2007, Peden et al., 2008).

5.2. Characteristics of the tonic $GABA_A$R-mediated inhibition in the SNR_A neurons

5.2.1. The magnitude of the BIM-sensitive tonic current is linked with the δ subunit expression

Our findings demonstrate a pronounced BIM-sensitive $GABA_A$R-mediated tonic current during the first 2 postnatal weeks when $GABA_A$R responses are depolarizing in SNR_A neurons (Kyrozis et al., 2006) and a significant decrease till PN32, probably due to the parallel reduction in the expression of the δ subunit-containing extrasynaptic $GABA_A$Rs in both sexes. $GABA_A$R tonic currents have been proposed to enhance shunting-

mediated inhibition, which prevents neuronal excitation (Song et al., 2011). It is therefore possible that the increased $GABA_AR$ tonic conductance in PN5-16 SNR_A neurons may protect against the appearance of excitatory effects, by augmenting shunting inhibition. Similar age-related decline in δ subunit has been shown in CA1 pyramidal neurons (Shen et al.) but not in cerebellar and cortical neurons (Laurie et al., 1992, Peden et al., 2008). Although the exact subcellular localization of the δ subunit was not explored in this study, the extrasynaptic localization of δ-containing $GABA_ARs$ has been well documented (Nusser et al., 1998, Wei et al., 2003). The age-dependent decline in the tonic current density mediated by δ-containing $GABA_ARs$ is compensated for by an increase of the α1 subunit expression and synaptic $GABA_AR$ inhibition. One can hypothesize that, early in development, activation of tonic $GABA_ARs$ participates in cell differentiation and maturation, filtering out excessive neuronal activation. In contrast, in older ages, subsequent to the establishment of synaptic connectivity, $GABA_AR$-mediated tonic inhibition subsides, yielding to the faster synaptic $GABA_AR$-mediated inhibition that mediates specific functional processes that depend upon inter-neuronal communications (Nguyen et al., 2001, Ben-Ari, 2002).

5.2.2. BIM-sensitive tonic current is AP-dependent

In the presence of glutamatergic inhibitors, TTX inhibited action potential-dependent IPSCs and reduced a tonic inward current shown as an outward shift in the baseline Ihold, without significant age and sex differences. The presence of TTX-sensitive tonic currents suggests that activity-dependent presynaptic neurotransmitter release contributes to the generation of tonic currents controlling GABAergic SNR neurons (Brickley et al.,

1996, Rossi et al., 2003, Glykys and Mody, 2007).

5.2.3 THIP-induced tonic currents do not entirely reflect the δ subunit expression

Interestingly, THIP induced significantly greater tonic responses in PN5-9 males than in females, with no definite age-related differences, when results were adjusted for cell size. Although the α4/δ combination may partially mediate the THIP currents (Brown et al., 2002, Liang et al., 2004, Maguire et al., 2005, Chandra et al., 2006), the sex-specific and absence of significant age-specific THIP responses do not agree with the δ subunit expression patterns in the SNR_A. The α4 mRNA was detected in PN15 and PN30 SNR by RT-PCR at very low levels, but it is not known if it demonstrates sex-specific expression patterns in the SNR_A (Galanopoulou, unpublished observations).

One can speculate that the δ subunit in PN5-9 females may co-assemble in higher proportion with other α subunits than α4 creating $GABA_AR$s less sensitive to THIP (Saxena and Macdonald, 1994, Feng and Macdonald, 2004, Zheleznova et al., 2008). The most likely candidate might be the α1 subunit, the mRNA of which is more abundant in females (Wurpel et al., 1988, Veliskova et al., 1998a, Ravizza et al., 2003).

The possibility that the THIP effect is in part mediated by non-δ $GABA_AR$s is not very probable since currents arising from these receptors would be of negligible significance with the used THIP concentration due to high EC_{50} of these subunit combinations (Storustovu and Ebert, 2006).

The dissociation in the age- and sex-related tonic current shifts induced by THIP (also $GABA_CR$ antagonist) and muscimol ($GABA_CR$ agonist) raises the possibility that

$GABA_C$Rs may contribute to these age and sex-related tonic currents. In preliminary studies we have found very low mRNA levels of the ρ1 subunit of $GABA_C$R in the PN30 SNR (Galanopoulou, unpublished data). In addition, a developmental increase in ρ2 subunit and decrease in ρ3 mRNA has been reported in other brain regions (Ogurusu et al., 1999). Further studies will be useful to identify the specific $GABA_C$R or $GABA_A$R subunit combinations that underlie the observed sex dependent, THIP-induced changes in tonic current in SNR_A neurons.

5.2.4. Muscimol enhancement of the tonic current is associated with α1 subunit expression

In contrast, muscimol-induced $GABA_A$R-mediated responses were more pronounced, in general, in older age groups and in females. Muscimol is a $GABA_A$R agonist which avidly binds at the high affinity site located at the α1 subunit (Wurpel et al., 1988, Baur and Sigel, 2003), but it also binds to α4 (Sur et al., 1999), α5 (Sur et al., 1998), γ or δ subunit-containing $GABA_A$Rs (Mihalek et al., 1999, Storustovu and Ebert, 2006). Muscimol also acts as a partial agonist of ρ1 $GABA_C$ receptors {Chang, 2000 #732; Wang, 1994 #733} and is a weak inhibitor of GABA uptake (Corey et al., 1994). The changes in muscimol-induced tonic currents reported here correlate with the higher expression of α1 subunit mRNA in PN15 females than in males and in PN30 SNR_A (Ravizza et al., 2003) and the developmental increase in high affinity muscimol binding sites between PN16 and adult rat SNR (Wurpel et al., 1988). However, muscimol responses are dissociated from the zolpidem-induced changes in $GABA_A$R tonic current

density. The discordance between the developmental and sex specific patterns between muscimol and zolpidem emphasizes that other mechanisms of action are involved in the generation of these tonic $GABA_A$Rs mediated responses. The much stronger tonic currents elicited by muscimol are most likely due to a direct agonistic effect, as muscimol is a potent direct $GABA_A$R agonist, and – unlike zolpidem - does not depend upon GABA availability. Also, the washout of ambient GABA, under the *in vitro* recording conditions, may diminish the zolpidem effect.

5.2.5. Zolpidem induced tonic current via non-δ-containing $GABA_A$ receptors

Although we did not observe any age- or sex-related differences, despite the significantly higher perisomatic α1 subunit protein expression in PN25-32 than in PN5-9 and PN11-16 groups, zolpidem-induced responses indicate that an α1 subunit selective compound can modulate the tonic current in most GABAergic nigral cells.

The lack of difference can be due to lower levels of ambient GABA in PN25-32 SNR_A than in younger groups, possibly due to enhanced GABA reuptake by GABA transporting mechanisms or washout of external GABA. This would limit the action of zolpidem which does not act as a direct $GABA_A$Rs agonist but solely increases the affinity for GABA. Indeed, inhibition of the GABA uptake by a specific GAT-1 transporter inhibitor NO-711 led to an enhancement of BIM-sensitive tonic current (without zolpidem) especially in the PN25-32 group (data not shown).

The exact $GABA_A$Rs subunit combination making SNR neurons sensitive to zolpidem is unclear. It is very likely that $GABA_A$Rs contain γ and not the δ subunit, as δ-containing receptors are insensitive to zolpidem (Pritchett et al., 1989, Sieghart, 1995,

Saxena and Macdonald, 1996, Nusser and Mody, 2002, Hanson et al., 2008). Even though γ subunit-containing receptors are usually targeted to synapses (Essrich et al., 1998, Alldred et al., 2005), high-resolution immunogold localization showed α1β2/3γ2 $GABA_A$Rs in the extrasynaptic compartment of cerebellar granule cells (Nusser et al., 1995, Nusser et al., 1998). Furthermore, several pharmacological studies have suggested extrasynaptic $GABA_A$Rs sensitive to benzodiazepines (Semyanov et al., 2003, Liang et al., 2004, Maguire et al., 2005, Shen et al., 2005). Nevertheless, one cannot rule out that the summation of prolonged sIPSCs tails in the presence of zolpidem generates the tonic current. This explanation seems less likely, however, because in some cells we see isolated enhancements of sIPSCs decay times but no inward current. Similarly, previously published studies showed that zolpidem, besides enhancing the sIPSCs decay time, produced a significant change in baseline holding current in hippocampal interneurons (Semyanov et al., 2003) or CA1 pyramidal neurons (Liang et al., 2004, Shen et al., 2005). In contrast, it did not result in any shift in dentate gyrus granule cells (Nusser and Mody, 2002) (Mtchedlishvili and Kapur, 2006), cerebellar granule cells (Hamann et al., 2002) and thalamus (Jia et al., 2005), while it significantly prolonged the decay time.

Moreover, zolpidem is not a pure α1 modulator but can also bind to α2 and α3 subunits. The increased expression of α3 subunit in the PN5-9 and PN11-16 SNR_A neurons compared to PN25-32 neurons could therefore compensate for the lower expression of α1 subunits, masking any anticipated age differences in zolpidem-induced tonic currents.

5.3. Possible implications for the SNR_A mediated seizure control

Previous studies from our laboratory have shown age- and sex-specific effects of bilateral SNR_A infusions of $GABA_AR$ agonists and antagonists in the flurothyl model of seizures (Moshe and Albala, 1984, Sperber and Moshe, 1988, Garant et al., 1995, Veliskova and Moshe, 2001). Specifically, intra-SNR_A infusions of muscimol elicit proconvulsant effects in PN15 males and have no effect in PN15 females, whereas the muscimol-sensitive anticonvulsant SNR_A function develops earlier in females (starting at PN25) than in males (starting at PN30). Based on the developmental profile of phasic and tonic $GABA_AR$ responses of SNR_A neurons, there appears to be a developmental shift from an "enhanced tonic $GABA_AR$-mediated SNR_A control" state early in development to a "predominant phasic $GABA_AR$-mediated regulation of SNR_A" in older ages, which is accelerated in females, due to the earlier rise in $\alpha 1$ subunit. We propose that under conditions with prominent δ-dependent tonic $GABA_AR$ activity (i.e. in PN5-16 rats), sustained activation of $GABA_ARs$ leads to inhibition of SNR_A neuronal firing due to shunting inhibition (Staley and Mody, 1992, Galanopoulou et al., 2003) thereby reducing the GABA outflow to downstream target regions (e.g., thalamus, superior colliculus), which are then tonically disinhibited, and precipitating seizures. Of note, silencing of SNR_A neuronal firing by tonic $GABA_AR$ currents (i.e. with muscimol) has been shown in immature SNR_A neurons whether they have depolarizing or hyperpolarizing $GABA_ARs$ (Galanopoulou et al., 2003). The presence of increased $GABA_AR$-mediated tonic current in PN5-9 SNR_A neurons, known to have depolarizing $GABA_AR$ responses, could generate greater shunting inhibition, potentiating this effect (Kyrozis et al., 2006, Song et al., 2011). In contrast, during development, the

incremental control of GABAergic SNR_A neurons by phasic (i.e. α1-containing) $GABA_AR$s, which have faster inactivation kinetics, and the gradual disappearance of tonic $GABA_AR$-mediated control may result in an intermittent, i.e. less persistent, inhibition of the firing of SNR_A neurons. However, nigral neurons can still provide sufficient GABA outflow to the downstream output regions to influence seizure expression.

In support of the differential involvement of tonic and phasic $GABA_AR$s in SNR-mediated seizure control, previous studies in PN15 male rats reported proconvulsant responses following intranigral infusions of GABAergic agonists that elicit prominent tonic GABA responses (i.e. muscimol, THIP) but not with drugs that preferentially activate phasic $GABA_AR$s (i.e. zolpidem) (Moshe and Albala, 1984, Sperber et al., 1987, Xu et al., 1992, Veliskova et al., 1998b). However, both the *in vitro* and *in vivo* studies support that additional sex-specific factors may modify the effects of these GABAergic agonists on the activity of SNR_A neurons and in seizure control during development. These may include the age- and sex-specific developmental profiles of the shift from depolarizing to hyperpolarizing $GABA_AR$ signaling in rat SNR_A (Galanopoulou et al., 2003, Kyrozis et al., 2006), potentially complicated by distinct E_{GABA} maturational patterns in extrasynaptic and postsynaptic $GABA_AR$s (Romo-Parra et al., 2008), age and sex differences in the synaptic organization of the basal ganglia, as well as the complex pharmacological effects of administered GABAergic drugs as shown in this study.

6. Conclusions and main findings

1. Immunohistochemical results support our first hypothesis that the α1 and α3 subunits expression in the SNR_A depends on the sex and age. The α1 subunit is more abundant in older animals and females whereas α3 in younger ones.

The mean sIPSCs frequency, amplitude and charge transfer increase and 10-90% rise time and decay time accelerate in both sexes with age. The developmental increase in $GABA_A$Rs α1-ir and parallel decrease in $GABA_A$Rs α3-ir may partially explain some of these changes. The amount of charge transferred by averaged sIPSC remains practically constant during maturation in both sexes except in PN5-9 males. Sex differences are detected in some of the studied parameters in PN5-9 and PN28-32 groups. The potency of zolpidem 0.5 μM to prolong the decay time is age-dependent in both sexes. However, the responsiveness to zolpidem appears earlier in females (PN5-9 group), and this may be due to the higher expression of $GABA_A$Rs α1-ir in female than in male PN5-9 SNR.

2. PN5-16 GABAergic SNR_A neurons are under the influence of a pronounced BIM-sensitive tonic $GABA_A$R-mediated current, which disappears by PN32, in both sexes. The parallel developmental decline in δ $GABA_A$R subunit expression suggests that the age-related reduction in BIM-sensitive tonic current density could be due to decrease in extrasynaptic δ receptors. However, a zolpidem-induced tonic current enhancement indicates that non-δ $GABA_A$Rs may also contribute to the tonic GABAergic inhibition.

3. The pharmacologically-induced changes in the $GABA_AR$-mediated tonic current follow drug-, age- and sex-specific patterns that cannot be fully explained by the extrasynaptic δ $GABA_ARs$ and probably reflect changes in THIP, muscimol or zolpidem sensitive $GABA_ARs$ and/or GABA availability (synaptic GABA release and uptake).

7. Souhrn

Mnoho experimentálních a klinických prací prokázalo, že podkorové neuronální okruhy včetně těch, jež zahrnují substantia nigra pars reticulata anterior (SNR_A), jsou úzce zapojeny do kontroly propagace a ukončování záchvatů. *In vivo* studie na potkanech vystavených generalizovaným klonickým křečím ve flurothylovém modelu ukázaly, že utlumení GABAergního elektrického výstupu ze SNR_A zvyšuje práh pro vznik záchvatů. Tyto antikonvulzní vlastnosti SNR_A jsou závislé na věku a pohlaví. V naší práci jsme ke studiu GABAergní transmise použili tři věkové skupiny Sprague-Dawley potkanů (PN5-9, PN11-16 a PN25-32 dní, PN = postnatální). Studovali jsme dva typy GABAergní inhibice: a) synaptickou, která vzniká aktivací $GABA_A$ receptorů obsahující $\alpha 1$ a $\alpha 3$ podjednotky a b) tonickou, která je přenášena extrasynaptickými $GABA_A$ receptory, jež obsahují podjednotku δ. Imunohistochemicky jsme prokázali vyšší expresi $\alpha 1$ podjednotky u dospělých zvířat a samiček, kdežto výskyt $\alpha 3$ podjednotky byl nejvyšší v časném stádiu vývoje a postupně klesal do 30. postnatálního dne. Vyšší zastoupení $\alpha 1$ podjednotky se odrazilo ve zrychlení kinetiky postsynaptických proudů (sIPSCs), jejich vyšší amplitudě a frekvenci. Typ α podjednotky podmiňuje citlivost $GABA_A$ receptorů k zolpidemu, který preferenčně působí přes $\alpha 1$ podjednotku. Extrasynaptická δ podjednotka byla zastoupena stejně u obou pohlaví, ale její výskyt byl signifikantně vyšší v raném vývoji a s narůstajícím věkem klesal. V souladu s tímto nálezem byla tonická GABA inhibice největší u mladých zvířat a významně klesala s věkem. Tonický proud měřený po aplikaci THIP (agonista $GABA_A$ receptorů obsahujících δ podjednotku) však nekoreloval s expresí δ podjednotky, protože nebyly překvapivě zjištěny žádné

věkové rozdíly ve velikosti odpovědi. Samci ve skupině PN5-9 byli k THIP citlivější než stejně staré samičky. Tonický proud vzniklý po podání muscimolu (agonista $GABA_A$ receptorů obsahující zejména $\alpha 1$ podjednotku) byl největší u nejstarší věkových skupin a u samic. Nález koreloval s expresí $\alpha 1$ podjednotky, která byla maximální u PN25-32 samiček. Aplikace zolpidemu vyvolala malý tonický proud, což svědčí o tom, že také jiné než $GABA_A$ receptory obsahující δ podjednotku mohou zprostředkovat tonickou GABA inhibici. Znalost pohlavních a věkových rozdílů v GABAergní transmisi v SNR_A je důležitou podmínkou pro vývoj antiepileptik na refrakterní typy záchvatů.

8. Summary

Many experimental as well as clinical studies have shown that subcortical neuronal circuitries including the anterior part of the substantia nigra pars reticulata (SNR) are closely involved in the control of seizures propagation and termination. *In vivo* studies in rats demonstrated that inhibition of the GABAergic SNR_A electrical output increases the seizure threshold in the flurothyl model of generalized clonic seizures. The anticonvulsant properties of the SNR_A are largely age- and sex-depended. In the current experiments were used 3 age groups of Sprague Dawley rats (PN5-9, PN11-16 and PN25-32 days, PN = postnatal) to study GABAergic inhibition. Two types of GABAergic inhibition were studied: a) a synaptic inhibition, which is generated by α1 and α3-containing $GABA_A$Rs and b) a tonic inhibition mediated by extrasynaptic δ subunit-containing $GABA_A$Rs. Immunohistochemistry showed that the α1 subunit expression was generally more abundant in adult rats and females while the α3 subunit dominated in the early development and gradually decreased by the age of PN30. The more α1 subunit was expressed the faster were the kinetics, higher the mean amplitudes and frequencies of spontaneous inhibitory postsynaptic currents (sIPSCs). The α subunit subtype underlies sensitivity to zolpidem, which preferentially acts via the α1 subunit. The extrasynaptic δ subunit was present equally in males and females but its expression was markedly higher in early maturational stages and decreased later on. As a result, the tonic GABA inhibition measured as a BIM-sensitive current was highest in youngest animals and lowest in older ones. However, a THIP-induced tonic current (a $GABA_A$Rs

agonist acting via α4β3δ subunits) did not entirely parallel the δ subunit expression as no age-related differences were found. PN5-9 males were more sensitive to THIP than the same age females. A muscimol-generated tonic current (a $GABA_A$Rs agonist preferentially acting via α1 subunit) was maximal in older rats and prevailed in females, which can be explained by abundance of the α1 subunit at this age and sex. Zolpidem ability to elicit a small inward current in SNR neurons signifies that also non-δ subunit $GABA_A$Rs mediate a tonic current. Knowledge of sex- and age-related differences in GABAergic transmission in the SNR_A is an important precondition to develop more potent antiepileptic drugs for treatment refractory seizures.

9. References

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10. SUPPLEMENT 1. List of publications

1. Articles with IF related to the thesis

Chudomel O, Hasson H, Bojar M, Moshé SL, Galanopoulou AS. Age- and sex-related characteristics of tonic GABA currents in the rat substantia nigra pars reticulata. Neurochem Res. 2015 Apr;40(4):747-57. Epub 2015 Feb 3 **IF in 2015 2,593**

Chudomel O, Herman H, Nair K, Moshé SL, Galanopoulou AS. Age- and gender-related differences in GABA(A) receptor-mediated postsynaptic currents in GABAergic neurons of the substantia nigra reticulata in the rat. *Neuroscience*. 2009 Sep 29;163(1):155-67. Epub 2009 Jun 13. **IF in 2009 3,556**

Giorgi FS, Velíšková J, Chudomel O, Kyrozis A, Moshé SL.The role of substantia nigra pars reticulata in modulating clonic seizures is determined by testosterone levels during the immediate postnatal period. *Neurobiol Dis.*, 2007 Jan;25(1):73-9. **IF in 2007 4,377**

Kyrozis, A, Chudomel, O, Moshé, SL, Galanopoulou AS. Sex-dependent maturation of GABA(A) receptor-mediated synaptic events in rat substantia nigra reticulata. *Neurosci Lett.* 2006 May 1;398(1-2):1-5. **IF in 2006 2,092**

2. Articles with IF not directly related to the content of the thesis

Velíšek L, Velíšková J, Chudomel O, Poon KL, Robeson K, Marshall B, Sharma A, Moshé SL. Metabolic environment in substantia nigra reticulata is critical for the expression and control of hypoglycemia-induced seizures. *J Neurosci.* 2008 Sep 17;28(38):9349-62. **IF in 2008 7,5**

Velíšková, J, Chudomel, O, Poon, KL, Marshall, B, and Velíšek, L. The involvement of the substatnia nigra pars reticulata in hypoglycemic seizures. *Epilepsia* 2007;48 Suppl 5:106-8. **IF in 2007 3,569**

SUPPLEMENT 2. Full versions of articles with IF related to the thesis

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ORIGINAL PAPER

Age- and Sex-Related Characteristics of Tonic Gaba Currents in the Rat Substantia Nigra Pars Reticulata

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Abstract Previous studies have shown that the pharmacologic effects of GABAergic drugs and the postsynaptic phasic $GABA_A$ergic inhibitory responses in the anterior part of the rat substantia nigra pars reticulata (SNR_A) are age- and sex-specific. Here, we investigate whether there are age- and sex-related differences in the expression of the δ $GABA_A$ receptor ($GABA_AR$) subunit and $GABA_AR$ mediated tonic currents. We have used δ-specific immunochemistry and whole cell patch clamp to study $GABA_AR$ mediated tonic currents in the SNR_A of male and female postnatal day (PN) PN5-9, PN11-16, and PN25-32 rats. We observed age-related decline, but no sex-specific changes, in bicuculline (BIM) sensitive $GABA_AR$ tonic current density, which correlated with the decline in δ subunit in the SNR_A between PN15 and 30. Furthermore, we show that the $GABA_AR$ tonic currents can be modified by muscimol ($GABA_AR$ agonist; partial $GABA_CR$ agonist), THIP (4,5,6,7-tetrahydroisoxazolo (5,4-c)pyridin-3-ol: α4β3δ $GABA_ARs$ agonist and $GABA_CR$ antagonist), and zolpidem (α1-subunit selective $GABA_AR$ agonist) in age- and sex-dependent manner specific for each drug. We propose that the emergence of the $GABA_AR$-sensitive anticonvulsant effects of the rat SNR_A during development may depend upon the developmental decline in tonic GABAergic inhibition of the activity of rat SNR_A neurons, although other sex-specific factors are also involved.

Keywords Substantia nigra pars reticulata · Patch clamp · Tonic inhibition · Development · Sex differences · GABA agonists

Abbreviations

SNR	Substantia nigra pars reticulata
SNR_A	Anterior part of the substantia nigra pars reticulata
sIPSCs	Spontaneous inhibitory postsynaptic currents
PN	Postnatal days
-ir	Immunoreactivity
$GABA_ARs$	$GABA_A$ receptors
$GABA_CRs$	$GABA_C$ receptors
aCSF	Artificial cerebrospinal fluid
D-AP5	D-(-)-2-Amino-5-phosphonopentanoic acid
CNQX	6-Cyano-2,3-dihydroxy-7-nitro-quinoxaline
BIM	Bicuculline methobromide
DMSO	Dimethyl sulfoxide
TBS	Tris based saline
THIP	4,5,6,7-Tetrahydroisoxazolo (5,4-c)pyridin-3-ol
TTX	Tetrodotoxin
gabazine	SR 95531 hydrobromide

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RT	Room temperature
NGS	Normal goat serum
SE	Standard error
Rs	Series resistance

Introduction

The substantia nigra reticulata (SNR) is a midbrain structure closely involved in regulation of movement and seizure control [1–5]. Its role in seizure modulation depends on age, sex and is also different in the anterior (SNR_A) versus the posterior SNR region [2, 6]. Specifically, bilateral infusions of muscimol in the SNR_A of PN21 or younger rats have proconvulsant effects in males but have no effect in female rats in the flurothyl model of generalized clonic seizures [7]. During maturation, a muscimol-sensitive anticonvulsant region emerges in the SNR_A of both sexes, but this functional shift occurs earlier in females (first seen at PN25) than in males (first seen at PN30) [2, 6, 7].

We previously described that the properties of spontaneous inhibitory postsynaptic $GABA_A$ receptor ($GABA_AR$) mediated currents (sIPSCs) in GABAergic neurons of the SNR_A are age- and sex-dependent, in part explained by different types of α $GABA_AR$ subunits [8–11].

To further elucidate the molecular and electrophysiological mechanisms underlying the developmental functional changes in the role of GABAergic SNR_A neurons in seizure control, we studied the expression of the δ $GABA_AR$ subunit, a component of extrasynaptic $GABA_ARs$ that mediate tonic $GABA_AR$ inhibition, as well as the age and sex differences of $GABA_AR$ tonic currents in GABAergic SNR_A neurons, using immunohistochemistry and whole cell patch clamp [12–18]. We found that the bicuculline (BIM)-sensitive $GABA_AR$-mediated tonic currents decline between PN15 to PN30 in parallel with the age-related decrease in δ subunit expression, in both sexes. Furthermore, we show that the $GABA_AR$ tonic currents can be modified by muscimol ($GABA_AR$ agonist; partial $GABA_CR$ agonist), THIP (4,5,6,7-Tetrahydroisoxazolo (5,4-c)pyridin-3-ol (gaboxadol): α4β3δ $GABA_ARs$ agonist and $GABA_CR$ antagonist), and zolpidem (α1-subunit selective $GABA_AR$ agonist) in age- and sex-dependent manner.

Materials and Methods

Animals

Male and female Sprague–Dawley rats (Taconic Farms, New York, USA) were divided into 3 different age groups PN5-9, PN11-16 and PN25-32, with the date of birth taken as PN0. Rats were kept at constant temperature (21–23 °C), relative humidity (40–60 %) and a 12 h dark/12 h light cycle (lights on at 7:00 a.m.) with food and water ad libitum in our animal facility accredited by the American Association for the Accreditation of Laboratory Animal Care. Rats younger than 21 days were kept with a dam. After weaning, rats were kept in cages of 3–4 same sex rats with water and food ad libitum. All procedures and experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Institute Committee of our institution.

Immunohistochemistry

For immunochemical detection of the δ subunit immunoreactive cells (δ-ir), PN5, PN15, and PN30 male and female rats were euthanized and transcardially perfused with saline and 10 % neutral buffered formalin (SIGMA-ALDRICH, St Louis, MO), cryoprotected in 30 % sucrose in phosphate buffered saline and stored frozen at −80 °C till further use. Sagittal 40 µm brain sections containing the SNR were immunostained as described previously [8]. We used two sources of rabbit anti-δ antibodies to confirm that the observed expression changes are independent of the δ subunit specific antibody used. Because the results regarding the developmental changes in δ subunit expression were comparable, the data were combined. The first antibody was developed by Dr. Gunther Sperk (1:300; Innsbruck Medical University, Austria) and recognized an epitope consisting of the 44 amino-terminal amino acids of the rat $GABA_AR$ δ subunit [19]. The second was a commercial polyclonal rabbit anti-δ antibody, also recognizing the amino-terminus of the rat $GABA_AR$ δ subunit (Catalog number AB9752, 1:300; Millipore, Billerica Massachusetts). Cellular densitometry of δ-ir SNR sections was performed as described previously [8, 20]. A mean value for the cellular δ-ir signal density was obtained per rat and was used in the statistics. In the cell count experiments, the number of total δ-ir SNR_A neurons was counted from 1 section per brain in sagittal SNR sections at the level of the subthalamic nucleus. Similar cell counts were done on adjacent Nissl-stained SNR_A sections. Statistics were performed on the "total numbers of δ-ir SNR_A neurons per section" as well as the percent of δ-ir neurons among the total Nissl-stained SNR_A neurons expressed as "[(total numbers of δ-ir SNR_A neurons)/(total numbers of Nissl-stained SNR_A neurons) * 100]".

Drugs

BIM, SR 95531 hydrobromide (gabazine), TTX, THIP, muscimol, and D-AP5 were dissolved in distilled water

whereas CNQX and zolpidem were dissolved in DMSO (final dilution 1:1,000). Bicuculline (and its water soluble preparations such as bicuculline methobromide - BIM) is a competitive $GABA_A$Rs antagonist. Gabazine is a selective high-affinity antagonist binding at low-affinity $GABA_A$Rs [21, 22]. TTX is a highly selective neuronal Na^+ channel blocker [23], which completely inhibits firing action potentials [24]. D-AP5 blocks glutamatergic NMDA receptors-mediated currents whereas CNQX inhibits AMPA receptors [25]. Muscimol is a $GABA_A$R agonist and partial $GABA_C$R agonist. THIP is an agonist for α4δ-containing $GABA_A$Rs and $GABA_C$R antagonist. All drugs were diluted to the desired concentration after bath applied in aCSF and washed in the recording chamber at a flow rate of 4 ml/min. BIM, and zolpidem were purchased from Sigma-Aldrich, St. Louis, MO; gabazine, TTX, THIP, muscimol, D-AP5 and CNQX from Tocris Bioscience, Ellisville, MO.

Slice Preparation

Sagittal slices containing SNR were prepared from animals at PN5-9, 11–16 and 25–32. Rats were deeply anesthetized with isoflurane and decapitated. The brain was quickly removed and placed in oxygenated (95 O_2, 5 % CO_2) ice-cold sucrose slicing solution containing (in mM): 187 sucrose, 3 KCl, 2 $CaCl_2$, 1.9 $MgCl_2$, 1.2 NaH_2PO_4, 26 $NaHCO_3$ and 20 D-glucose, pH 7.4, 300–310 mOsm. 300 μM thick sagittal slices were cut using a vibratome (Leica, VT1000S). Slices were transferred into oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl; 2.5 KCl; 1 NaH_2PO_4; 26 $NaHCO_3$; 2 $CaCl_2$; 1.3 $MgSO_4$ and 20 glucose, pH 7.3–7.4, 290–300 mOsm, and allowed to recover at room temperature for at least 1 h before recording.

Electrophysiology

Cells were visualized with an upright Eclipse E600-FN microscope (Nikon) in the SNR_A [7, 8]. Whole-cell patch clamp recordings were made from electrophysiologically identified GABAergic neurons using an Axopatch 200B amplifier (Molecular Devices, Union City, CA). Patch pipettes were pulled using Flaming/Brown micropipette puller (Sutter Instruments Co, Novato, CA) from thin-wall borosilicate glass tubing (1.5 mm OD; World Precision Instruments, Sarasota, FL) and had open tip resistance 2–3 MΩ when filled with an intracellular solution containing (in mM): 140 CsCl, 4 NaCl, 1 $MgCl_2$, 10 HEPES, 10 EGTA, 2 Mg-ATP, 290 mOsm, pH 7.3 adjusted with CsOH. No correction was made for the liquid junction potential of +4.3 mV. Slices were continuously perfused at a rate of 4 ml/min with oxygenated aCSF solution. All recordings were performed at room temperature.

Neurons were voltage-clamped at a holding potential of −70 mV, therefore all GABAergic events were observed as inward currents. Series resistance was estimated by measuring the transient current in response to either −1 or −5 mV, 200 ms-long hyperpolarizing voltage steps. Cells were accepted for further analysis provided that the series resistance after 40–60 % compensation did not exceed 15 MΩ and/or did not change by more than 15 % during data acquisition. The input resistance could not be exactly measured due to the high intracellular Cs^+ concentration, which blocks K^+ channels [26]. Synaptic currents were recorded in the presence of glutamate antagonists D-AP5 (50 μM) and CNQX (10 μM) to block excitatory amino acid-mediated transmission. Recorded data were filtered at 2 kHz (low-pass Bessel filter) and sampled at 10 kHz. The bandwidth was sufficient enough to include all fast frequencies of interest [8]. All data were recorded with pClamp 8 analysis software (Molecular Devices Co, Sunnyvale, CA) through a Digidata 1322A digitizer (Molecular Devices Co, Sunnyvale, CA).

Baseline and post-drug holding currents (Ihold) were measured by averaging the Ihold from 20 epochs (50–100 ms each), 1 epoch per second, over a 20 s period. For baseline Ihold, the 20 s period immediately prior to the time of drug application was used. For post-drug Iholds, 20 s periods during the time of peak or trough drug responses were used, which was usually 80–100 s from the time of the drug administration. Gaussian all-point histograms were constructed from these epochs using 0.5 pA bins. The data-points not contaminated by IPSCs were fitted according to the Levenberg–Marquardt method to obtain the mean Ihold amplitudes. The difference between the baseline and post-drug Iholds expressed the magnitude of the tonic current. In order to eliminate the cell size as a confounding factor in measurements, all drug-induced changes in Ihold were related to the cell capacitance and expressed as a tonic current density (pA/pF) and this value was eventually used for definite comparison of age- and sex-related differences. The cell capacitance was calculated from current transients recorded in response to 5 mV hyperpolarizing voltage steps.

In the first set of experiments, bicuculline methobromide (BIM, 100 μM) was used to reveal the tonic current measured as the change in the Ihold. TTX (1 μM) and gabazine (500 nM) were used to eliminate IPSCs prior to BIM application to determine baseline Ihold as used in other papers to separate synaptic and extrasynaptic responses [15, 27].

In the remaining pharmacological studies (muscimol, THIP, zolpidem), TTX and gabazine were not used to simulate our previous in vivo studies [2, 6, 7]. In order to obtain the mean Ihold and tonic current density changes, only all point histograms of episodes uncontaminated by IPSCs were used.

Statistics

Two-way ANOVA followed by Fisher's post hoc *t* test was used to compare age and sex differences in tonic current changes. Because the sensitivity of the two-way ANOVA comparisons decreases as the number of inter-group comparisons increases, we utilized unpaired *t* test to explore whether significant differences in the studied variables existed in specific same age groups that demonstrated visible gender-related differences. All values are expressed as least square mean values ± SE. F values for each variable are given as $F_{variable}$ (degrees of freedom, residuals).

Results

δ Subunit Expression

To study the age- and sex-specific differences in δ $GABA_AR$ subunit immunoreactivity (δ-ir) in SNR_A neurons we used immunohistochemistry, because it allows comparisons in protein expression at the cellular level and avoids contamination of readouts by heterogeneous cell populations. The perisomatic δ-ir in the SNR_A changed as a function of age [$F_{age}(2,43) = 15.45$; $P < 0.0001$) but not sex ($F_{sex}(1,43) = 0.4$; $P > 0.05$]. The δ-ir was high at PN5 and PN15 male and female rats and declined significantly at PN30 (Fig. 1a, b). In parallel, a greater than 50 % decrease in the total number of δ-ir SNR_A neurons occurred between PN15 and PN30 [$F_{age}(1,19) = 21.99$, $P = 0.0002$], without any sex differences [$F_{sex}(1, 19) = 0.049$, $P = 0.8$) (n = 5 rats per group]. The percentage of SNR_A neurons expressing δ-ir declined from 79.9 ± 9.8 % at PN15 to only 35.9 ± 4.3 % at PN30 [$F_{age}(1,11) = 13.67$, $P < 0.0061$, n = 6 rats/age group].

BIM-*Sensitive $GABA_AR$ Tonic Current*

Since δ subunit mediates extrasynaptic tonic $GABA_AR$ responses, we investigated whether the observed age but not sex-dependent changes in δ-ir functionally correlate with similar changes in BIM-sensitive $GABA_AR$-mediated tonic currents also occurs in SNR_A neurons. In order to separate synaptic and tonic currents, whole cell patch clamp recordings were performed using TTX and gabazine, applied prior to BIM. In the presence of glutamatergic inhibitors CNQX and D-AP5, the TTX -induced outward shift of the baseline Ihold in all groups indicated that action potentials contribute to a certain degree to the tonic inward current (Fig. 1c). We found no age or sex differences in TTX-induced tonic current [$F_{age}(2, 45) = 2.84$, $F_{sex}(1, 45) = 1.09$, $F_{age*sex}(2, 45) = 1.69$, $P > 0.05$, two-way ANOVA]. These findings suggest that action potential dependent neurotransmitter spillover from the synaptic cleft or depolarizing shifts caused by ionic concentration disturbances due to fast-firing post-synaptic sodium channel activation may contribute to tonic currents [28, 29]. Further addition of gabazine (500 nM) completely blocked residual miniature IPSCs (mIPSCs), but did not alter the Ihold indicating that solely synaptic receptors were blocked (Fig. 1c).

Bath application of BIM (100 μM) revealed significant age- but not sex-specific changes in Ihold (Fig. 1d). When expressed as a tonic current density, the results show that these were not attributable to developmental changes in cell size [$F_{age}(2, 45) = 6.77$, $P < 0.05$; $F_{sex}(1, 45) = 0.0003$, $P > 0.05$; $F_{age*sex}(2, 45) = 0.12$, $P > 0.05$, two-way ANOVA] (Fig. 1e). The tonic current density was similar in PN5-9 and PN11-16 groups, without sex differences, but almost disappeared in PN25-32 neurons. The percentage of cells generating tonic current: males PN5-9 88 %, PN11-16 82 %, PN25-32 33 %; females PN5-9 88 %, PN11-16 100 %, PN25-32 20 %, paired *t* test, $P < 0.05$. The developmental changes in δ subunit expression and the concurrent differences in the tonic current density unmasked by BIM suggest that the reduction in δ-ir between PN15 and PN30 may underlie the decline in tonic current density in SNR_A GABAergic neurons.

THIP Induced Changes in Tonic Current Density

We then tested whether the decline in δ $GABA_AR$ subunit also parallels the developmental changes in tonic currents induced by THIP, an α4β3δ $GABA_ARs$ agonist and $GABA_CR$ antagonist. THIP (5 μM) application resulted in inward tonic current shifts in all age groups [$F_{age}(2, 44) = 3.34$, $F_{sex}(1, 44) = 11.85$, $P < 0.05$; $F_{age*sex}(2, 44) = 0.17$, $P > 0.05$; two-way ANOVA] (Fig. 2a). However, THIP induced changes in current density did not follow the same age-specific patterns as the δ-ir and BIM data. Consequently, no age-related differences were found in either sex ($P > 0.05$, unpaired-*t* test). There was an early trend for males to respond with greater THIP-induced tonic current density than females, which was significant in PN5-9 SNR_A ($P < 0.05$, unpaired-*t* test). No other sex differences were observed, although the statistical significance was almost reached in the PN11-16 groups ($P = 0.055$, unpaired-*t* test). The dissociation between the effects of THIP and BIM on tonic current density suggests that there are sex-, but not age-, related differences in unoccupied THIP-sensitive receptors which are not due to differences in δ subunit expression in SNR_A neurons.

Muscimol-Induced Tonic Current Density

Because our prior studies indicated sex- and age-related differences in α1 subunit expression in rat SNR_A [8, 9, 11,

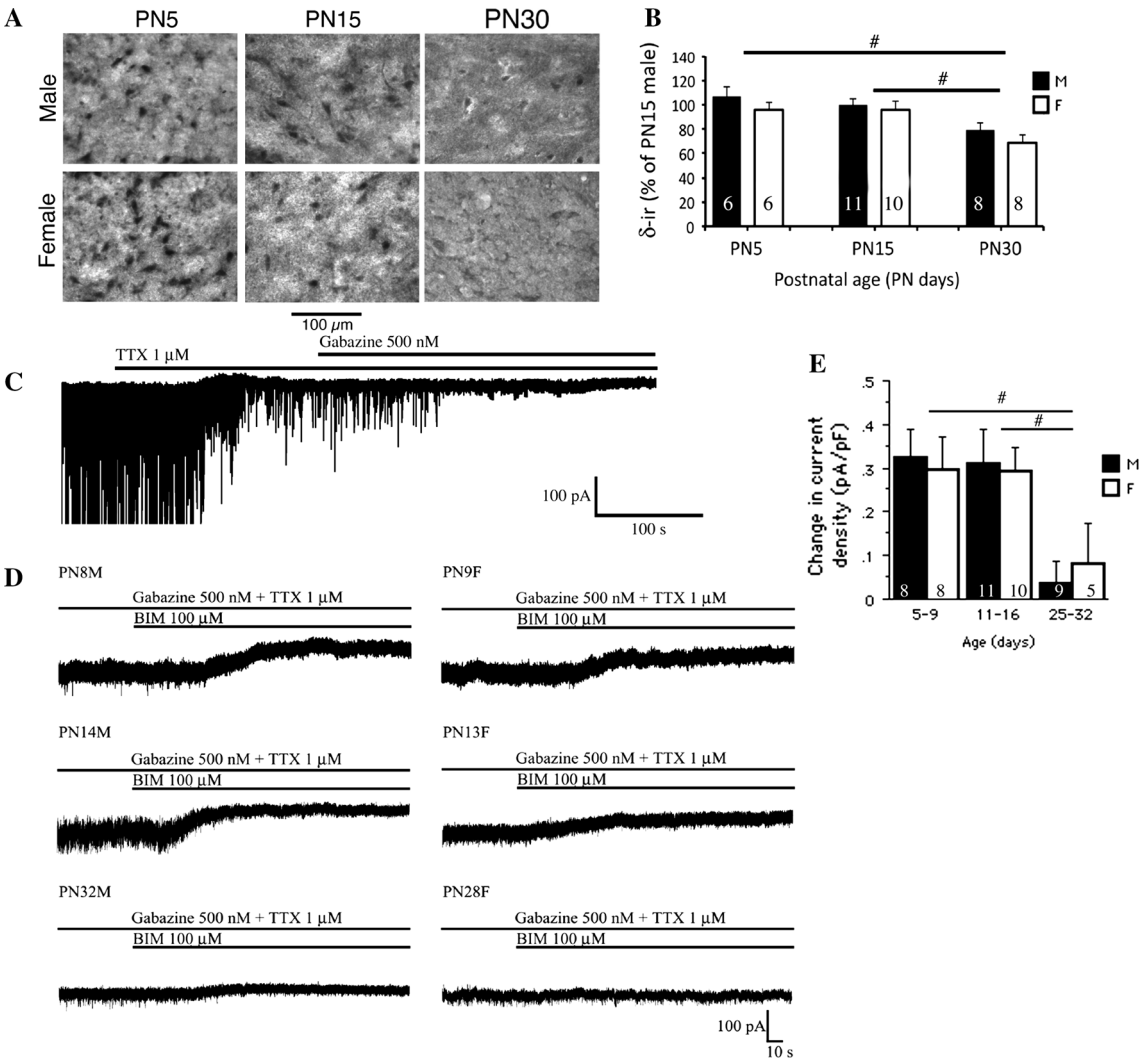

Fig. 1 Developmental changes in δ-ir and BIM-sensitive tonic currents in rat SNR_A neurons. **a** Representative photographs from SNR_A sections from male and female rats show strong δ-ir expression at PN5 and PN15 and significant reduction at PN30, without any sex differences. Significant reduction in the numbers of δ-ir SNR neurons was also seen at PN30 compared to PN15 SNR sections, as discussed in the results section. **b** Schematic depiction of cellular δ-ir densitometric results obtained from SNR_A sections confirms the decrease in cellular δ-ir expression between PN5 and PN30 as well as between PN15 and PN30, without sex differences. Results are expressed as " % of δ-ir in PN15 males", which were included as a reference group in each set of immunochemistry. This approach helped minimize interassay variability and allowed comparisons across the different sets of immunochemistry assays. Please note that the PN30 δ-ir reflects the mean intensity of the few remaining δ-ir cells, which were already significantly reduced in number (see Fig. 1a). The pound keys (hash) indicate significant differences ($P < 0.05$, post hoc Fisher's test) between linked age groups. No sex differences were noted. **c** A representative recording in the presence of glutamatergic antagonists D-AP5 and CNQX shows an outward shift of Ihold following TTX 1 μM application indicating reduction of tonic inward current. At the same time, action potential dependent IPSCs are blocked. No further Ihold shifts were observed after gabazine 500 nM was washed in while all residual miniature IPSCs disappeared. **d** Representative recordings from GABAergic nigral neurons demonstrate the $GABA_A$Rs-mediated tonic current as an outward shift of baseline Ihold after BIM 100 μM application. All IPSCs were previously eliminated by means of TTX 1 μM and gabazine 500 nM. **e** The BIM-induced changes in tonic current density significantly decrease with age while no sex differences were noted ($F_{age}(2, 45) = 6.77$, $P < 0.05$; $F_{sex}(1, 45) = 0.0003$, $P > 0.05$; $F_{age*sex}(2, 45) = 0.12$, $P > 0.05$, two-way ANOVA). The current density was smaller between PN25-32 and PN5-9 as well as PN25-32 and PN11-16 while no differences were observed between PN5-9 and PN11-16 groups ($^{\#}P < 0.05$, post hoc Fisher's test). The numbers in the *column bars* indicate the number of cells. All values are expressed as least square mean values ± SE. *M* male, *F* female. Number of animals per group: males PN5-9 n = 5, PN11-16 n = 6, PN28-32 n = 7; females PN5-9 n = 6, PN11-16 n = 7, PN28-32 n = 5

30], we investigated whether the direct α1 agonist muscimol augments inward tonic currents in SNR_A neurons in the similar sex-specific pattern. Muscimol 100 nM induced large changes in current density in all age groups (Fig. 3a) with age- and sex-specific differences [$F_{age}(2, 41) = 9.89$, $P < 0.05$; $F_{sex}(1, 41) = 9.78$, $P < 0.05$; $F_{age*sex}(2,$

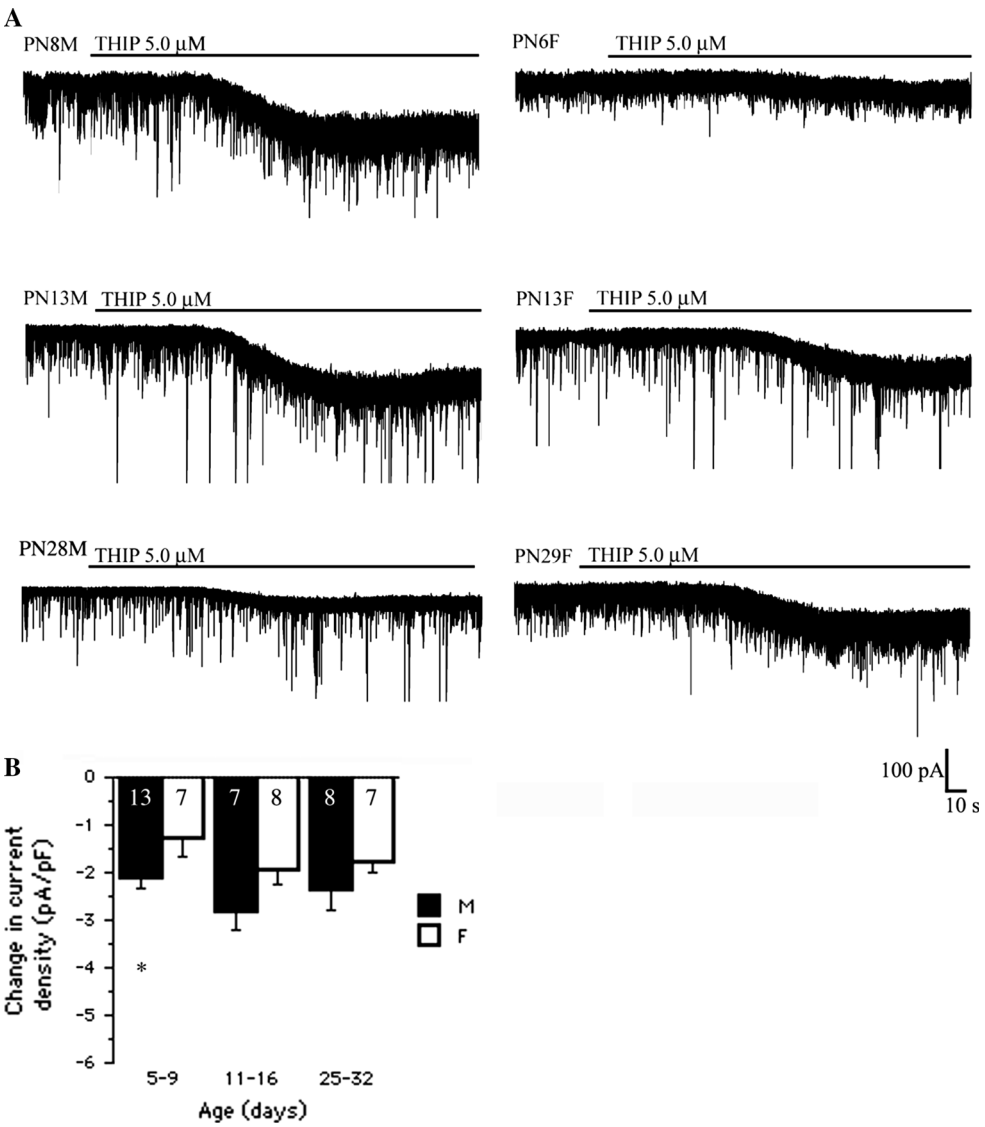

Fig. 2 Sex-specific changes in THIP-induced tonic current in rat SNR_A neurons. **a** Representative recordings demonstrating THIP (5 μM) induced changes in the tonic current in SNR_A GABAergic neurons in the presence of D-AP5 50 μM and CNQX 10 μM. **b** When the cell size was taken into consideration, the THIP-induced changes in current density measurements demonstrate significant age- and sex-related differences ($F_{age}(2, 44) = 3.34$, $F_{sex}(1, 44) = 11.85$, $P < 0.05$, $F_{age*sex}(2, 44) = 0.17$, $P > 0.05$, two-way ANOVA). However, intergroup comparisons showed bigger current density shifts in PN5-9 males than same age females (*$P < 0.05$, unpaired *t* test) and borderline in PN11-16 group ($P = 0.055$, unpaired *t* test). No age-related differences between same-sex groups were found. The numbers in the *column bars* indicate the number of cells. All values are expressed as least square mean values ± SE. *M* male, *F* female. Number of animals per group: males PN5-9 n = 11, PN11-16 n = 6, PN28-32 n = 6; females PN5-9 n = 5, PN11-16 n = 6, PN28-32 n = 7

41) = 1.99, $P > 0.05$, two-way ANOVA] (Fig. 3b). Muscimol-enhanced current density increased with age in both sexes (PN25-32 and PN11-16 > PN5-9), and was significantly greater in PN11-16 females than in same age males ($P < 0.05$, unpaired *t* test). These findings indicate a developmental increase in muscimol-induced tonic current in the SNR_A, irrespective of the developmental changes in cell size, and this increase was more pronounced and appeared earlier in females.

Zolpidem Effects

To determine whether age- and sex-related changes in α1-containing $GABA_AR$ mediated tonic responses may explain the age and sex differences in muscimol effects, we examined the effects of zolpidem, a selective agonist of α1 subunit containing $GABA_ARs$ [31]. Zolpidem (0.5 μM) induced a small inward current in more than 70 % of all tested cells (Fig. 4a). There were no significant age and sex differences observed in zolpidem-induced current densities [$F_{age}(2, 27) = 0.3$; $F_{sex}(1, 27) = 0.09$, $F_{age*sex}(2, 33) = 0.167$, $P > 0.05$, two-way ANOVA] (Fig. 4c).

The dissociation in the magnitude of zolpidem and muscimol sensitive tonic currents may therefore reflect direct agonistic effects of muscimol on α1-subunit containing $GABA_ARs$ (extra- or post-synaptic).

Discussion

Our study demonstrates that PN5-16 GABAergic SNR_A neurons are under the influence of a pronounced BIM-sensitive tonic $GABA_AR$-mediated current, which disappears by PN32, in both sexes. The parallel developmental

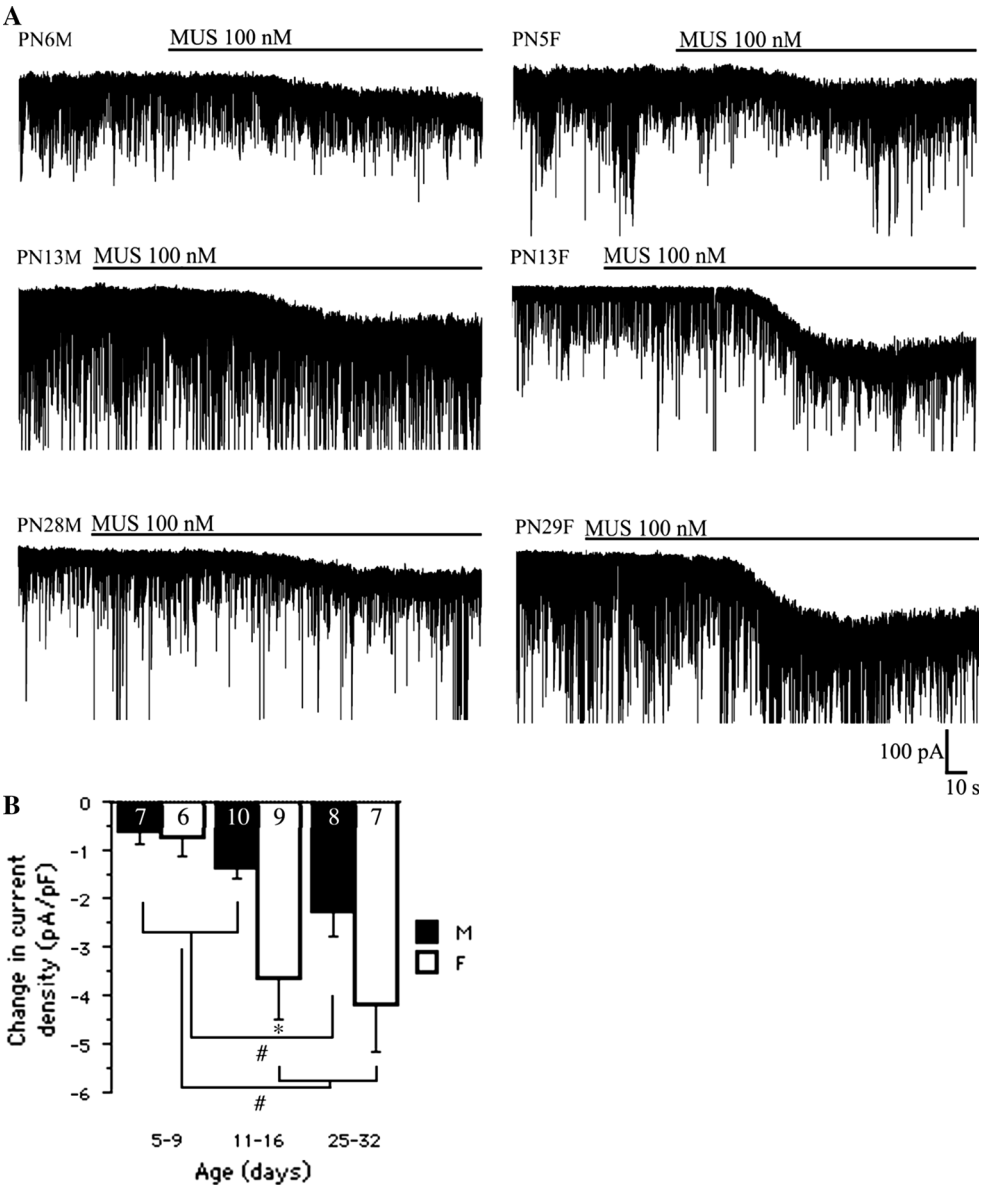

Fig. 3 Sex and age-specific changes in muscimol-induced tonic currents in rat SNR_A. **a** Representative traces demonstrating that muscimol 100 nM induced an inward current in all groups. Recordings were performed in the presence of D-AP5 50 μM and CNQX 10 μM. Note the larger deflection in PN13 and PN29 female cells compared with their male counterparts. **b** The current density shifts induced by muscimol changed as a function of age and sex ($F_{age}(2, 41) = 9.89$, $P < 0.05$; $F_{sex}(1, 41) = 9.78$, $P < 0.05$; $F_{age*sex}(2, 41) = 1.99$, $P > 0.05$, two-way ANOVA). Significant sex differences were found only in the PN11-16 group (*$P < 0.05$, unpaired *t* test). In males, the current density in PN25-32 was significantly higher than in the two younger age groups (#$P < 0.05$, one-way ANOVA). In females, the current density in PN5-9 group was significantly lower than in PN11-16 and PN25-32 groups (#$P < 0.05$, one-way ANOVA). The numbers in the *column bars* indicate the number of cells. All values are expressed as least square mean values ± SE. *M* male, *F* female. Number of animals per group: males PN5-9 n = 3, PN11-16 n = 3, PN28-32 n = 5; females PN5-9 n = 3, PN11-16 n = 4, PN28-32 n = 5

decline in δ $GABA_AR$ subunit expression suggests that the age-related reduction in BIM-sensitive tonic current density could be due to decrease in extrasynaptic δ receptors. In contrast, the pharmacologically-induced changes in $GABA_AR$-mediated tonic current density follow drug-, age- and sex-specific patterns that cannot be fully explained by the extrasynaptic δ $GABA_ARs$ and probably reflect changes in THIP, muscimol or zolpidem sensitive $GABA_ARs$ and/or GABA availability (synaptic GABA release and uptake).

Our findings demonstrate a pronounced BIM-sensitive $GABA_AR$-mediated tonic current during the first two postnatal weeks when $GABA_AR$ responses are depolarizing in SNR_A neurons [32] and a significant decrease till PN32, probably due to the parallel reduction in the expression of the δ subunit-containing extrasynaptic $GABA_ARs$ in both sexes. $GABA_AR$ tonic currents have been proposed to enhance shunting-mediated inhibition, which prevents neuronal excitation [33]. It is therefore possible that the increased $GABA_AR$ tonic conductance in PN5-16 SNR_A neurons may protect against the appearance of excitatory effects, by augmenting shunting inhibition. Similar age-related decline in δ subunit has been shown in CA1 pyramidal neurons [34] but not in cerebellar and cortical neurons [35, 36]. Although the exact subcellular localization of the δ subunit was not explored in this study, the extrasynaptic localization of δ-containing $GABA_ARs$ has been well documented [12, 13]. The age-dependent decline in the tonic current density mediated by δ-containing $GABA_ARs$ is compensated for by an increase of the α1

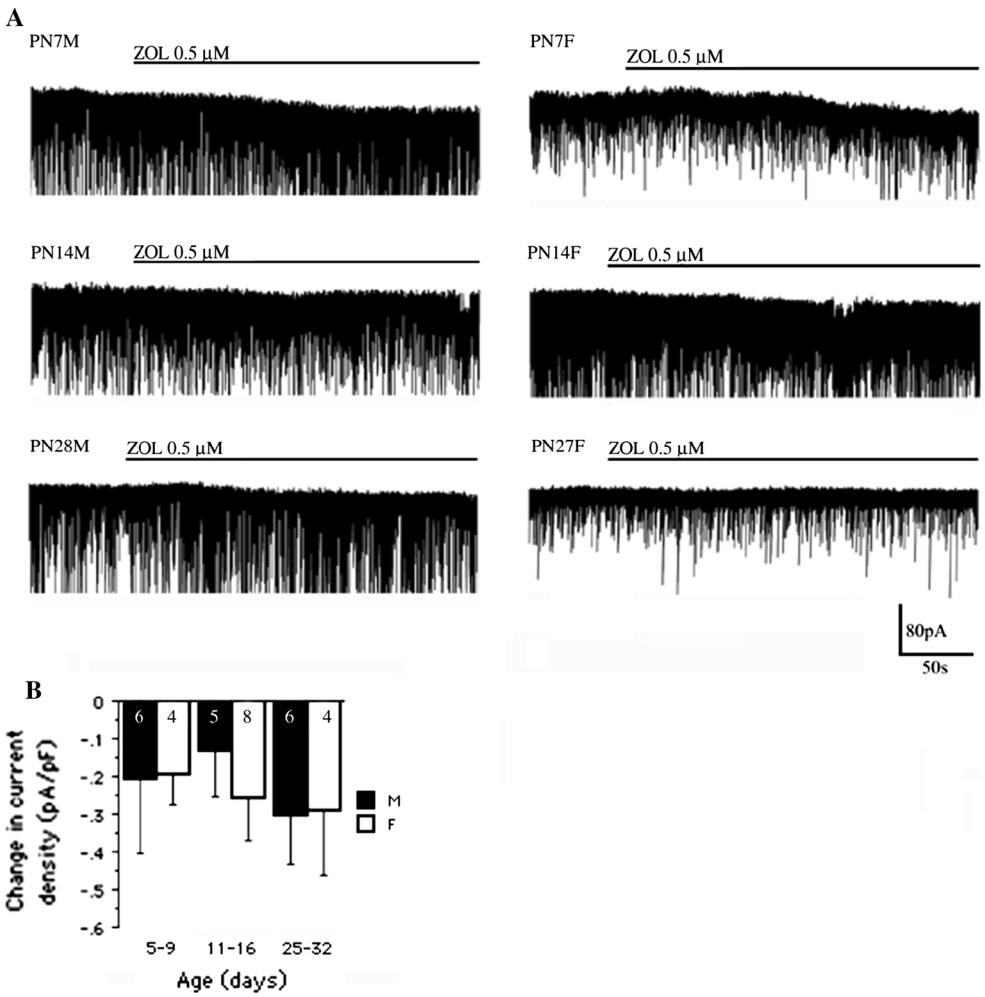

Fig. 4 Zolpidem-induced tonic responses in rat SNR_A neurons. **a** Representative traces show that zolpidem 0.5 μM gave rise to a small inward current in SNR_A neurons across all age groups in the presence of D-AP5 50 μM and CNQX 10 μM. **b** The zolpidem-induced changes in current density measurements did not, however, demonstrate any significant sex- or age-dependent differences ($F_{age}(2, 27) = 0.3$; $F_{sex}(1, 27) = 0.09$, $F_{age*sex}(2, 33) = 0.167$, $P > 0.05$, two-way ANOVA). The numbers in the *column bars* indicate the number of cells. All values are expressed as least square mean values ± SE. *M* male, *F* female. Number of animals per group: males PN5-9 n = 5, PN11-16 n = 2, PN28-32 n = 3; females PN5-9 n = 4, PN11-16 n = 3, PN28-32 n = 4

subunit expression and synaptic $GABA_AR$ inhibition [8]. One can hypothesize that, early in development, activation of tonic $GABA_ARs$ participates in cell differentiation and maturation, filtering out excessive neuronal activation. In contrast, in older ages, subsequent to the establishment of synaptic connectivity, $GABA_AR$-mediated tonic inhibition subsides, yielding to the faster synaptic $GABA_AR$-mediated inhibition that mediates specific functional processes that depend upon inter-neuronal communications [37, 38].

In the presence of glutamatergic inhibitors, TTX inhibited action potential-dependent IPSCs and reduced a tonic inward current shown as an outward shift in the baseline Ihold, without significant age and sex differences. The presence of TTX-sensitive tonic currents suggests that activity-dependent presynaptic neurotransmitter release contributes to the generation of tonic currents controlling GABAergic SNR neurons [39–41].

Interestingly, THIP induced significantly greater tonic responses in PN5-9 males than in females, with no definite age-related differences, when results were adjusted for cell size. Although the α4/δ combination may partially mediate the THIP currents [27, 42–44], the sex-specific and absence of significant age-specific THIP responses do not agree with the δ subunit expression patterns in the SNR_A. The α4 mRNA was detected in PN15 and PN30 SNR by RT-PCR at very low levels, but it is not known if it demonstrates sex-specific expression patterns in the SNR_A (A.S. Galanopoulou, unpublished observations). The dissociation in the age- and sex-related tonic current shifts induced by THIP (also $GABA_CR$ antagonist) and muscimol ($GABA_CR$ agonist) raises the possibility that $GABA_CRs$ may contribute to these age and sex-related tonic currents. In preliminary studies we have found very low mRNA levels of the ρ1 subunit of $GABA_CR$ in the PN30 SNR (Galanopoulou AS, unpublished). In addition, a developmental increase in ρ2 subunit and decrease in ρ3 mRNA has been reported in other brain regions [45] Further studies will be useful to identify the specific $GABA_CR$ or $GABA_AR$ subunit combinations that underlie the observed sex dependent, THIP-induced changes in tonic current in SNR_A neurons.

In contrast, muscimol-induced $GABA_AR$-mediated responses were more pronounced, in general, in older age

groups and in females. Muscimol is a $GABA_AR$ agonist which avidly binds at the high affinity site located at the α1 subunit [30, 46], but it also binds to α4 [47], α5 [48], γ or δ subunit-containing $GABA_ARs$ [49, 50]. Muscimol also acts as a partial agonist of ρ1 $GABA_C$ receptors {Chang, 2000 #732; Wang, 1994 #733} and is a weak inhibitor of GABA uptake [51]. The changes in muscimol-induced tonic currents reported here correlate with the higher expression of α1 subunit mRNA in PN15 females than in males and in PN30 SNR_A [9] and the developmental increase in high affinity muscimol binding sites between PN16 and adult rat SNR [30]. However, muscimol responses are dissociated from the zolpidem-induced changes in $GABA_AR$ tonic current density. The discordance between the developmental and sex specific patterns between muscimol and zolpidem emphasizes that other mechanisms of action are involved in the generation of these tonic $GABA_ARs$ mediated responses. The much stronger tonic currents elicited by muscimol are most likely due to a direct agonistic effect, as muscimol is a potent direct $GABA_AR$ agonist, and—unlike zolpidem—does not depend upon GABA availability. Also, the washout of ambient GABA, under the in vitro recording conditions, may diminish the zolpidem effect. Muscimol-induced activation of α5 $GABA_ARs$ or of $GABA_CRs$ would also be worth testing in the future as to their role in underlying the age- and sex-specific muscimol effects in the SNR_A [52, 53].

We did not observe any age- or sex-related differences in zolpidem induced tonic responses, despite the significantly higher perisomatic α1 subunit protein expression in PN25-32 than in PN5-9 and PN11-16 groups [8]. This can be due to lower levels of ambient GABA in PN25-32 SNR_A than in younger groups, possibly due to enhanced GABA reuptake by GABA transporting mechanisms or washout of external GABA. This would limit the action of zolpidem which solely increases the affinity for GABA. Moreover, zolpidem is not a pure α1 modulator but can also bind to α2 and α3 subunits. The increased expression of α2 and α3 subunits in the PN5-9 and PN11-16 SNR_A neurons compared to PN25-32 neurons could therefore compensate for the lower expression of α1 subunits, masking any anticipated age differences in zolpidem-induced tonic currents [8].

Possible Implications for the SNR_A Mediated Seizure Control

Previous studies from our laboratory have shown age- and sex-specific effects of bilateral SNR_A infusions of $GABA_AR$ agonists and antagonists in the flurothyl model of seizures [2, 6, 7, 54]. Specifically, intra-SNR_A infusions of muscimol elicit proconvulsant effects in PN15 males and have no effect in PN15 females, whereas the muscimol-sensitive anticonvulsant SNR_A function develops earlier in females (starting at PN25) than in males (starting at PN30). Based on the developmental profile of phasic and tonic $GABA_AR$ responses of SNR_A neurons ([8] and this study), there appears to be a developmental shift from an "enhanced tonic $GABA_AR$-mediated SNR_A control" state early in development to a "predominant phasic $GABA_AR$-mediated regulation of SNR_A" in older ages, which is accelerated in females, due to the earlier rise in α1 subunit [8]. We propose that under conditions with prominent δ-dependent tonic $GABA_AR$ activity (i.e. in PN5-16 rats), sustained activation of $GABA_ARs$ leads to inhibition of SNR_A neuronal firing due to shunting inhibition [55, 56] thereby reducing the GABA outflow to downstream target regions (e.g., thalamus, superior colliculus), which are then tonically disinhibited, and precipitating seizures. Of note, silencing of SNR_A neuronal firing by tonic $GABA_AR$ currents (i.e. with muscimol) has been shown in immature SNR_A neurons whether they have depolarizing or hyperpolarizing $GABA_ARs$ [56]. The presence of increased $GABA_AR$-mediated tonic current in PN5-9 SNR_A neurons, known to have depolarizing $GABA_AR$ responses, could generate greater shunting inhibition, potentiating this effect [32, 33]. In contrast, during development, the incremental control of GABAergic SNR_A neurons by phasic (i.e. α1-containing) $GABA_ARs$, which have faster inactivation kinetics, and the gradual disappearance of tonic $GABA_AR$-mediated control may result in an intermittent, i.e. less persistent, inhibition of the firing of SNR_A neurons. However, nigral neurons can still provide sufficient GABA outflow to the downstream output regions to influence seizure expression.

In support of the differential involvement of tonic and phasic $GABA_ARs$ in SNR-mediated seizure control, previous studies in PN15 male rats reported proconvulsant responses following intranigral infusions of GABAergic agonists that elicit prominent tonic GABA responses (i.e. muscimol, THIP) but not with drugs that preferentially activate phasic $GABA_ARs$ (i.e. zolpidem) [2, 57–59]. However, both the in vitro and in vivo studies support that additional sex-specific factors may modify the effects of these GABAergic agonists on the activity of SNR_A neurons and in seizure control during development. These may include the age- and sex-specific developmental profiles of the shift from depolarizing to hyperpolarizing $GABA_AR$ signaling in rat SNR_A [32, 56], potentially complicated by distinct E_{GABA} maturational patterns in extrasynaptic and postsynaptic $GABA_ARs$ [60], age and sex differences in the synaptic organization of the basal ganglia, as well as the complex pharmacological effects of administered GABAergic drugs as shown in this study.

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Conflict of interest The authors declare that they have no conflict of interest.

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Age- and gender-related differences in $GABA_A$ receptor-mediated postsynaptic currents in GABAergic neurons of the substantia nigra reticulata in the rat

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Abstract

The responsiveness of the rat anterior substantia nigra pars reticulata (SNR) GABAergic neurons to $GABA_A$ergic drugs changes with age and gender, altering its role in seizure control. To determine whether maturational and gender-specific differences in the properties of spontaneous $GABA_A$Rs-mediated inhibitory postsynaptic currents (sIPSCs) underlie these events, we studied sIPSCs at baseline and after application of the α1 $GABA_A$Rs subunit selective agonist zolpidem, at postnatal days (PN) 5-9, PN12-15, and PN28-32. Results were correlated with the α1 and α3 $GABA_A$Rs subunit immunoreactivity (-ir) at PN5, PN15, and PN30, using immunochemistry. The mean frequency, amplitude and charge transfer increased whereas the 10–90% rise time and decay time accelerated with age in both genderes. The faster sIPSC kinetics in older rats were paralleled by increased α1-ir and decreased α3-ir. At PN5-9, males had more robust sIPSCs (frequency, amplitude, charge carried per event and charge transfer) than females. At PN28-32, males exhibited higher amplitudes and faster kinetics than females. The zolpidem-induced increase of decay times, amplitude and charge transfer and α1-ir expression were the lowest in PN5-9 males but increased with age, in both genders. Our findings demonstrate that alterations in $GABA_A$Rs subunit expression partially underlie age- and gender-specific sIPSC changes in SNR neurons. However, the observation of gender differences in sIPSC kinetics that cannot be attributed to changes in perisomatic α1 expression suggests the existence of additional gender-specific factors that control the sIPSC kinetics in rat SNR.

Keywords

patch clamp; seizures; development; synaptic inhibition; immunohistochemistry; zolpidem

The substantia nigra reticulata (SNR) is a midbrain structure involved in the regulation of movement and seizure control (Iadarola and Gale, 1982, Moshe and Albala, 1984, Gale, 1985, Deransart et al., 1998, Veliskova and Moshe, 2006). The majority of cells in the SNR are fast spiking GABAergic neurons (Richards et al., 1997), which receive inhibitory input via

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postsynaptic $GABA_A$Rs from the striatum but also from the globus pallidus (Smith and Bolam, 1989, Smith and Bolam, 1991, Bolam et al., 2000, Misgeld, 2004). $GABA_A$Rs are heteropentamers composed of homologous subunits (α1-6, β1-3, γ1-3, δ, ε, π, θ) (Levitan et al., 1988, Olsen et al., 1991, Barnard et al., 1998, Benarroch, 2007). Many studies have shown that in particular the α subunit subtypes undergo significant changes in expression during brain development. In most studied brain regions, including the SNR, the α2 and α3 subunits are highly expressed in the early stages of postnatal maturation and their levels gradually decrease with age while the α1 subunit mRNA and protein levels rise (Laurie et al., 1992, Fritschy et al., 1994, Veliskova et al., 1998). The type of α subunit in $GABA_A$Rs changes their pharmacological and kinetic properties. The decay time of the inhibitory postsynaptic currents (IPSCs) is faster when the α1 subunit is present, whereas it slows down if $GABA_A$Rs contain α2 or α3 subunits instead (Verdoorn, 1994, Gingrich et al., 1995, Lavoie et al., 1997). Our previous studies on the α1 mRNA expression in the SNR showed higher levels in PN30 than in PN15 rats (Moshe et al., 1994) and in female rats than age-matched male rats (Veliskova et al., 1998, Ravizza et al., 2003).

In this study, we seek to determine whether there are age- and gender-related changes in $GABA_A$Rs-mediated sIPSCs and zolpidem sensitivity in the GABAergic SNR neurons of PN5-32 rats.

Experimental procedures

We used Sprague-Dawley rats of both genders divided into 3 different age groups PN5-9, PN12-15 and PN28-32, with the date of birth taken as PN0 (Taconic Farms, New York, USA). Rats were kept at constant temperature (21 – 23°C), relative humidity (40 – 60%) and a 12 h dark/12 h light cycle (lights on at 7:00am) with food and water *ad libitum* in our animal facility accredited by the American Association for the Accreditation of Laboratory Animal Care. Rats younger than 21 days were kept with a dam. All procedures and experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Slices containing SNR were prepared from rats of either gender at PN5-9, 12-15 and 28-32. Rats were deeply anesthetized with isoflurane and decapitated. The brain was quickly removed and placed in oxygenated (95% O_2/5% CO_2) ice-cold sucrose slicing solution containing (in mM): 187 sucrose, 3 KCl, 2 $CaCl_2$, 1.9 $MgCl_2$, 1.2 NaH_2PO_4, 26 $NaHCO_3$ and 20 D-glucose, pH 7.4, 300–310 mOsm. 300 μM thick sagittal slices were cut using a vibratome (Leica, VT1000S). Slices were transferred into oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl; 2.5 KCl; 1 NaH_2PO_4; 26 $NaHCO_3$; 2 $CaCl_2$; 1.3 $MgSO_4$ and 20 glucose, pH 7.3-7.4, 290–300 mOsm, and allowed to recover at room temperature for at least 1 hour before recording.

Cells were visualized with an upright Eclipse E600-FN microscope (Nikon) in the anterior part of the SNR (Veliskova and Moshe, 2001). Whole-cell patch clamp recordings were made from electrophysiologically identified GABAergic neurons using an Axopatch 200B amplifier (Molecular Devices, Union City, CA). Patch pipettes were pulled using Flaming/Brown micropipette puller (Sutter Instruments Co, Novato, CA) from thin-wall borosilicate glass tubing (1.5 mm OD; World Precision Instruments, Sarasota, FL) and had open tip resistance 2–3 MΩ when filled with an intracellular solution containing (in mM): 140 CsCl, 4 NaCl, 1 $MgCl_2$, 10 HEPES, 10 EGTA, 2 Mg-ATP, 290 mOsm, pH 7.3 adjusted with CsOH. No correction was made for the liquid junction potential of +4.3 mV. Slices were continuously perfused at a rate of 4 ml/min with oxygenated aCSF solution. All recordings were performed at room temperature. Neurons were voltage-clamped at a holding potential of −70 mV and

broken in to establish whole cell configuration recordings. We waited 3–5 minutes after breaking-in until the holding current stabilized.

The SNR consists predominantly of GABAergic neurons but it also contains a small portion of dopaminergic cells. The two populations can be distinguished by their electrophysiological responses to hyperpolarizing current. To determine whether neurons were GABAergic, they were stepwise hyperpolarized in current clamp configuration by injection of negative current (from −70 mV to −130 mV) and the decisive parameter for accepting a cell as GABAergic was lack of hyperpolarization-induced inward rectification or sag (Richards et al., 1997, Radnikow and Misgeld, 1998). After determining the cell phenotype, we switched back to the voltage clamp mode and held the cell at −70 mV. All GABAergic events were thus observed as inward currents. Series resistance was estimated by measuring the transient current in response to 1- to 5-mV 200 ms-long hyperpolarizing voltage steps. Cells were accepted for further analysis provided that the series resistance after 40–60% compensation did not exceed 15 MΩ and/or did not change by more than 15% during data acquisition. The input resistance could not be exactly measured due to the high intracellular Cs^+ concentration, which blocks K^+ channels (Shao and Dudek, 2005). Synaptic currents were recorded in the presence of glutamate antagonists D-(−)-2-Amino-5-phosphonopentanoic acid (D-AP5, 50 μM) and 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline (CNQX, 10 μM) to block excitatory amino acid-mediated transmission.

Bicuculline methobromide (BIM) and D-AP5 were dissolved in distilled water whereas CNQX and zolpidem were dissolved in dimethyl sulfoxide (DMSO, final dilution 0.001%). All drugs were bath applied. BIM and zolpidem were purchased from Sigma-Aldrich, St. Louis, MO; D-AP5 and CNQX from Tocris Bioscience, Ellisville, MO. Sprague –Dawley male and female rats were transcardially perfused with saline and then formalin at PN5, PN15 and PN30. Their brains were collected, fixed overnight in formalin, immersed in 30% sucrose and when they sank they were frozen and kept at −80°C till use. Sagittal 40μm sections were cut in a MICROM cryostat (MICROM International, Walldorf, Germany) and were stained with rabbit antibodies specific for the α1 or α3 $GABA_A$Rs subunits (Millipore, Billerica MA). The anti-α1 antibody recognizes the aminoacid sequence 1–16 of the rat α1 subunit protein, whereas the anti-α3 antibody recognizes the aminoacid sequence 1–15 of the rat α3 subunit protein. Both were used at a dilution of 1:800. Immunochemistries were done in free-floating sections. The steps included incubation with 1% H_2O_2 in Tris based saline (TBS) for 30 minutes at room temperature (RT); blocking in TBS with 10% normal goat serum (NGS) and 0.4% Triton-X-100 (90 minutes, RT); incubation with the primary antibody in TBS with Triton X100 0.4% and 3% NGS (2–3 days, 4°C with shaking); incubation with secondary biotinylated anti-rabbit antibody (Vector Labs, Burlingame, CA) (1:200 dilution) in TBS with 0.4% Triton-X-100 and 3% NGS, RT; and further peroxidase based staining as per manufacturer's protocols (Vector Labs; ABC Elite kit and 3,3′-diaminobenzidine/nickel substrate kit). In every assay a representative brain from all groups was included to minimize inter-assay variability. Brains were coded to permit blinded assessment of the values.

Because the SNR is sparsely populated, densitometric analysis of cellular perisomatic immunostaining was done by sampling representative stained cells from 4–5 anterior SNR sections per rat, using the Image J software (Wayne Rasband, Research Services Branch, NIMH, Bethesda Maryland, USA). The densitometry values of these cells were averaged for each brain and results were used in the statistical analysis. Details of this densitometric analysis are as described in (Galanopoulou, 2006). Similar densitometric analysis of immunochemically stained sections has been used extensively to provide semi-quantitative assessment of the level of protein expression in the stained cells (Rieux et al., 2002, Galanopoulou, 2006, Galanopoulou, 2008).

Synaptic currents were filtered at 2 kHz (low-pass Bessel filter), sampled at 10 kHz and recorded with pClamp 8 analysis software (Molecular Devices Co, Sunnyvale, CA) through a Digidata 1322A digitizer (Molecular Devices Co, Sunnyvale, CA). Spontaneous inhibitory postsynaptic currents (sIPSCs) were analyzed offline using Mini Analysis Program (Synaptosoft, Decatur, GA). Individual events were automatically selected if their amplitude and area under curve were 5-fold higher than the set threshold detection parameters. All recordings were subsequently visually checked to remove artifacts. Both single and multiple peaked events were included into the analysis. A minimum of 50 accepted events per cell was analyzed (on average 450 events) and averaged to obtain mean values. The amplitude was measured from the baseline to the peak of the synaptic current. We further analyzed the 10–90% rise time, the 37 % decay time (measured as a time required for the current to decay to 37% of its peak amplitude) and the charge transferred by a single sIPSC (calculated by the software as an integrated area under curve). The charge transfer was calculated as a product of the mean sIPSC frequency and charge transferred by averaged sIPSC ($q = f_{mean} \times q_{averaged\ sIPSC}$). Baseline sIPSCs were analyzed before zolpidem 0.5 μM was applied and compared with events when the drug was present in the slice for at least 5 minutes. Two-way ANOVA followed by post hoc *t*-test (Tukey) was used to compare age and gender differences in sIPSCs properties. Because the sensitivity of the two-way ANOVA and Tukey post hoc decreases as the number of inter-group comparisons increases, we utilized unpaired *t*-test to explore whether significant differences in the studied variables existed in specific same age groups that demonstrated visible gender-related differences. The paired *t*-test was used to assess zolpidem effects. The Kolmogorov-Smirnov (K-S) two-sample, two-tailed test was used to compare cumulative amplitude, 10–90% rise time and decay time distributions. Statistics on the densitometry measurements were carried on with repeated measures multiple factor ANOVA and Tukey post hoc comparisons, using Statview and JMP softwares (SAS Institute, Cary, NC, USA). All values are expressed as least square mean value ± standard error (SE).

Results

Baseline sIPSCs

Spontaneous IPSCs were recorded in the presence of CNQX 10 μM and D-AP5 50 μM. Under these conditions all sIPSCs were blocked by BIM, which confirms that they were $GABA_A$Rs-mediated (Fig. 1A,B,C). The sIPSCs could be detected at all studied ages in both genders. The events reversed close to 0 mV, the theoretical equilibrium potential for Cl^- ions (Fig. 2A,B). All numeric values are summarized in Table 1.

The results of the two-way ANOVA for the studied sIPSC parameters and inter-group comparisons are presented in Figure 3. Specifically, the mean sIPSCs frequency significantly rises with age in both genders. Although overall gender differences were not found using the two-way ANOVA, separate comparisons of the average sIPSCs frequencies by the unpaired *t*-test in the individual age groups revealed that PN5-9 males have greater frequency than PN5-9 females (Fig. 3A, Table 1). The mean sIPSCs amplitude also significantly increased with age in both genders. Furthermore, male SNR neurons had higher sIPSC amplitudes than female ones (Fig. 3B). Although, post hoc comparisons with Tukey test did not show any significant gender differences between same age groups, possibly due to the high number of comparisons, the unpaired *t*-test revealed significantly higher sIPSC amplitudes in males than in females in the PN5-9 and PN28-32 groups (Figs. 3B, 4Aa,b, Table 1). Changes in the sIPSCs kinetics seen in our experiments were similar to those found in other studies (Dunning et al., 1999,Okada et al., 2000). Both the 10–90% rise time and decay time progressively accelerated with age (Figs. 3C,D, 4A,B, Table 1). The unpaired *t*-test showed that both these parameters are significantly faster in PN28-32 males than females. In the face of significant changes in the amplitude and kinetic parameters, the charge transferred by averaged sIPSC remained quite

stable during development. The only exceptions were events in PN5-9 males, which transferred significantly bigger charge than PN5-9 females (Fig. 3E, Table 1, Tukey post hoc and unpaired *t*-test). The total charge transfer carried by synaptic currents gradually grew with maturation in both males and females. PN5-9 males demonstrated higher increase than same age females (Fig. 3F and Table 1, unpaired *t*-test only).

The occurrence of slow sIPSC kinetics in younger groups and the opposite pattern observed in older animals (Figs. 3,4) may result from the known maturational subunit changes in $GABA_A$ergic synapses. We performed an additional analysis of the sIPSC 10–90% rise times and decay times in a subset of PN5-9, PN12-15 and PN28-32 male and female cells with almost similar series resistances (Rs ranged from 10.8 to 11.7 MΩ, P>0.05) to assess the datapoints distribution. Only single events emerging from the baseline were included to avoid distortion of the kinetic parameters and amplitudes by overlapping events. We analyzed 2223–2309 events from each group and plotted decay times of individual events against their 10–90% rise times. The widespread distribution of events in PN5-9 groups most likely reflected greater subunit heterogeneity of $GABA_A$Rs. (Fig. 5A). In contrast, the scattergrams of PN28-32 groups demonstrated that the occurrence of mainly fast events was confined to a very small area of the plot (Fig. 5B) suggesting that the composition of $GABA_A$Rs was more homogeneous (Moshe et al., 1994).

Zolpidem

The age- and gender-dependent changes in sIPSCs amplitude and kinetics and different α1 mRNA subunit expression during maturation led us to investigate whether pharmacological responses to zolpidem would also alter with age and gender.

Zolpidem 0.5 μM, an α1 subunit-selective positive modulator of type I benzodiazepine receptors (Pritchett et al., 1989), was bath applied and changes in the mean sIPSCs decay time and amplitude were analyzed after at least a 5-minute exposure to the drug.

Zolpidem significantly prolonged the decay time and increased the amplitude and charge per event compared with baseline sIPSCs in most groups (P<0.05, K-S test, Fig. 6 P<0.05, paired t-test, Fig. 7). The only exception was PN5-9 males where there was no significant increase in any of these variables in response to zolpidem. This may mean that $GABA_A$ receptors in PN5-9 male SNR neurons have less α1 subunits than the other groups. In PN28-32 females, zolpidem significantly increased the decay time and charge per event but not the sIPSC amplitude (Fig. 6 and 7). As PN28-32 females had the largest baseline amplitudes compared to the other groups, this may reflect a ceiling effect (baseline amplitude 82.5±12.5 pA, zolpidem amplitude 89.8 ±11.5 pA, n=6, p>0.05, mean±SE, paired *t*-test).

The overall age- and gender-related differences in sensitivity to zolpidem 0.5 μM were determined using the two-way ANOVA by comparing ratios of drug-induced responses to the pre-drug values. The zolpidem-induced percentage increases of the mean decay time and charge per event were greater in older animals than in younger ones (P<0.05) but no gender differences were observed (Fig. 7). No significant overall age and gender specific differences in zolpidem-induced percentage increase of the mean sIPSCs amplitude were observed. (Fig. 7).

These results suggest that SNR neurons are sensitive to zolpidem at all studied ages, with single exception the PN5-9 males, and that responsiveness to zolpidem increases with age.

Age- and gender-related differences in the expression of α1 and α3 $GABA_A$Rs subunits in the anterior SNR

To correlate the described age- and gender-specific changes in the electrophysiological properties of sIPSCs and zolpidem effects, we compared the expression of the α1 and α3 $GABA_A$Rs subunits in the anterior SNR of PN5, PN15, and PN30 male and female rats, using specific immunochemistries. Representative photos and the results of the statistical comparisons of the densitometric comparison of perisomatic α1-immunoreactivity (-ir) and α3-ir are presented in Table 2 and Figs. 8 and 9. The $GABA_A$Rs α1-ir increased between PN15 and PN30 in both genders (Table 2, Fig. 8). Gender had a significant overall effect, with females expressing more $GABA_A$Rs α1-ir than males (Table 2, Fig. 8). However, in inter-group comparisons of same age groups, only the PN5 females had statistically higher expression of $GABA_A$Rs α1-ir than males (Table 2, Fig. 8). In contrast, the $GABA_A$Rs α3-ir decreased between PN5 and PN30 in both genders, without any significant gender differences (Table 2, Fig. 9). The decrease was steeper in females, with significant statistical differences among all 3 age groups. In males, significant differences were observed only between PN5 and PN30 rats (Table 2, Fig. 9). Once again, gender differences were only observed at PN5, when females expressed more $GABA_A$Rs α3-ir than males (Table 2, Fig. 9).

Discussion

The current study describes age- and gender-related differences in the properties of sIPSCs in GABAergic neurons of the anterior SNR, a region that undergoes developmental changes in terms of its ability to modify seizure thresholds (Veliskova and Moshe, 2001). Our data show that the mean frequency, amplitude and charge transfer increase and 10–90% rise time and decay time accelerate in both genders with age. The developmental increase in $GABA_A$Rs α1-ir and parallel decrease in $GABA_A$Rs α3-ir may partially explain some of these changes. The amount of charge transferred by averaged sIPSC remains practically constant during maturation in both genders except in PN5-9 males. Gender differences are detected in some of the studied parameters in PN5-9 and PN28-32 groups. The potency of zolpidem 0.5 μM to prolong the decay time is age-dependent in both genders. However, the responsiveness to zolpidem appears earlier in females (PN5-9 group), and this may be due to the higher expression of $GABA_A$Rs α1-ir in female than in male PN5-9 SNR.

Increased mean sIPSCs frequency and higher amplitude in PN28-32 GABAergic rat SNR neurons in both genders along with marked acceleration of the sIPSCs kinetics can have several explanations including a) an increased number of $GABA_A$ergic synaptic terminals, b) higher density of postsynaptic $GABA_A$Rs containing mainly α1 subunit and c) decreased expression of postsynaptic $GABA_A$Rs containing α3 subunits.

The higher frequency of sIPSCs in PN28-32 animals can be due to a greater number of synapses formed on nigral GABAergic neurons with age (Phelps and Adinolfi, 1982) (Kraszewski and Grantyn, 1992, Swanwick et al., 2006). However, age-related increases in firing rates of presynaptic striatal and pallidal neurons can also contribute to higher sIPSCs frequency as we measured both action potential-dependent and -independent events.

The sIPSCs amplitude is determined by the number of open synaptic $GABA_A$Rs and the amount of released GABA (Frerking et al., 1995). The role of an α subunit type in affecting the amplitude of sIPSCs may be limited because, despite the increase in α1 mRNA with age, the mean amplitude decreases in cerebellar granule cells (Brickley et al., 1996) or does not change in dentate gyrus granule cells (Hollrigel and Soltesz, 1997). Furthermore, the reduction of amplitude in $\alpha 1^{0/0}$ knockouts most likely relates to a decreased number of $GABA_A$Rs at synapses rather than to a different subunit composition (Vicini et al., 2001, Goldstein et al.,

2002). Therefore the increase in the amplitude of sIPSCs after PN15, in both genders, indicates an increased availability of postsynaptic $GABA_A$Rs in GABAergic SNR neurons.

The acceleration of the decay and rise times in PN28-32 reflects age-related changes in a variety of pre- and post-synaptic factors that control the kinetics of sIPSCs. An important variable is the type of expressed α subunit (Verdoorn, 1994). Currents produced by recombinant α1 subunit-containing $GABA_A$Rs deactivate faster than those mediated by $GABA_A$Rs composed of α2 or α3 subunits and, as a result, such receptors close more rapidly and their decay time is reduced (Verdoorn, 1994, Gingrich et al., 1995, Lavoie et al., 1997). To determine whether the observed changes in the kinetics of sIPSCs can be attributed to changes in α subunit composition, we utilized more α1 subunit-specific functional and expression assays. We studied the responsiveness of SNR neurons to the α1-selective agonist zolpidem as well as compared the perisomatic expression of α1-ir across the different groups, using semi-quantitative densitometric immunochemical analysis. Indeed, the zolpidem-induced percentage increase of the decay time in PN28-32 animals was significantly higher than in PN5-9 group in both genders. This finding is in agreement with the developmental increase in α1 mRNA expression (Moshe et al., 1994, Veliskova et al., 1998) and α1-ir (Fig. 8) in the anterior SNR, as well as with the age-related increase in high-affinity binding sites for the α1 $GABA_A$Rs agonist muscimol in rat SNR (Wurpel et al., 1988).

The zolpidem effect on the sIPSCs amplitude is mediated by increasing the affinity of closed $GABA_A$Rs for GABA rather than by increasing the probability of channel opening or enhancing conductance (Perrais and Ropert, 1999, Hajos et al., 2000, Goldstein et al., 2002). Zolpidem increased the amplitude and charge per event in most age groups, although without any overall age- and gender-related differences. The only group that did not respond to zolpidem was the PN5-9 male rats, which can be explained by the lower expression of α1-ir in the anterior SNR of PN5-9 males compared to same age females. The little change of the mean sIPSCs amplitude and great enhancement of the decay observed in PN28-32 females reflects most likely near-to-complete saturation of $GABA_A$Rs containing the α1 subunit.

Although the age-related changes in decay time correlated well with the α1-specific assays (zolpidem responses and α1-ir), additional gender-specific factors, not necessarily related to α1 subunit, seem to interfere with the shaping of sIPSC kinetics in PN5-9 and PN28-32 males and females. The absence of significant gender differences in decay time at PN5-9, despite the enhanced α1-ir expression and zolpidem sensitivity in PN5-9 females, may be partially explained by the higher expression of α3-ir in PN5-9 females. The increased number of α3-containing $GABA_A$Rs in PN5-9 females may therefore create a subpopulation of sIPSCs with slower kinetics, blunting the differences in decay times between males and females.

In the absence of gender differences in zolpidem sensitivity and perisomatic α1-ir or α3-ir, the slower decay and 10–90% rise times of sIPSCs, along with the lower amplitude, in PN28-32 females may suggest a more distant site of origin of their sIPSCs compared to same age males. The significantly slower rise times combined with lower amplitudes in PN28-32 females thus support the hypothesis that their synaptic events may be more subjected to dendritic filtering than those in PN28-32 males. There are no studies to date describing sexual dimorphism in the organization and size of the dendritic tree of GABAergic neurons and $GABA_A$ergic synapses in the SNR of PN28-32 animals but gender differences have been reported in other brain structures, e.g. in the anterior cingulate cortex (Markham and Juraska, 2002), accessory olfactory bulb (Caminero et al., 1991) and subiculum (Andrade et al., 2000).

Based on our *in situ* hybridization data showing higher α1 mRNA expression in PN15 and PN30 females (Ravizza et al., 2003), we expected that the baseline decay time would be faster and zolpidem-induced prolongation of the decay time significantly greater in females than in

males. Our current electrophysiological data did not, however, show gender differences between PN12-15 groups and the only significant difference was found between PN28-32 males and females, males having quicker decay time (Fig. 3D, Table 1). The discrepancy between *in situ* α1 mRNA hybridization, somatic α1-ir and expected kinetic data in PN12-15 and PN28-32 rats may have the following explanations: First, despite the higher α1 mRNA in PN15 females, the synaptic α1 protein levels may be similar in males and females as reflected by the same decay time and somatic α1-ir (Fig. 8). This thought is further supported by the same zolpidem-induced prolongation of the decay time. PN12-15 females, however, may have an increased α1 subunit expression in the extrasynaptic compartment. Such α1-containing $GABA_A$Rs do not participate in shaping the decay time. This hypothesis is currently being a subject of our next experiments as some studies have already suggested that α1-containing $GABA_A$Rs may indeed be present extrasynaptically and play an important role in modulating tonic $GABA_A$Rs-mediated currents by benzodiazepines such as zolpidem or lorazepam (Liang et al., 2004, Shen et al., 2005). Second, as discussed earlier, it is possible that the previously reported increased α1 mRNA expression in PN30 females may reflect α1 subunit that is ultimately targeted to $GABA_A$Rs located at distal dendritic synaptic sites. Additional variable that may influence the sIPSC shape is the vesicular transmitter release. The vesicular GABA release may be more asynchronous in PN5-9 animals than in PN28-32, yielding sIPSCs with slower decay and rise times in the younger age group (Vautrin and Barker, 1995, Williams et al., 1998). Another factor that may contribute to the gender differences in $GABA_A$ receptor function in the SNR are naturally occurring neurosteroids as their site of action was recently identified in the α1 (Ueno et al., 2004, Rahman et al., 2008) and other α subunit subtypes (Hosie et al., 2009). Neurosteroids or their metabolites may also regulate the expression of specific $GABA_A$R subunit (Maguire and Mody, 2007, Peden et al., 2007). However, more studies will be necessary to confirm whether they could underlie observed age and gender sIPSCs differences in the SNR. The amount of charge transferred by averaged sIPSC is conserved during maturation. The amount of charge is determined to a large extent by the amplitude and decay time of sIPSC. Therefore, older animals, despite shorter decay time, develop equal charge transfer owing to the higher amplitude. The importance of phasic inhibition during development increases nonetheless as the charge transfer is significantly augmented in PN28-32 males and females. The main reason is increased frequency of sIPSCs due most likely to higher firing rate of presynaptic neurons and/or increased number of active $GABA_A$ergic synapses.

The above findings support that during development, PN5-32 GABAergic anterior SNR neurons acquire more active $GABA_A$ergic synapses per neuron, more activated synaptic $GABA_A$Rs per sIPSC, their sIPSCs have faster kinetics and increased zolpidem-sensitivity, which is partially attributed to the increase in α1-ir and decrease in α3-ir. This developmental transformation may contribute to the late appearance of the $GABA_A$-sensitive anticonvulsant region of the anterior SNR, which has been linked to an increase in high affinity binding sites for muscimol, an agonist for α1-containing $GABA_A$Rs (Wurpel et al., 1988).

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List of abbreviations

SNR

substantia nigra pars reticulata

sIPSCs
spontaneous inhibitory postsynaptic currents

PN
postnatal days

-ir
immunoreactivity

$GABA_ARs$
$GABA_A$ receptors

aCSF
artificial cerebrospinal fluid

D-AP5
D-(−)-2-Amino-5-phosphonopentanoic acid

CNQX
6-cyano-2,3-dihydroxy-7-nitro-quinoxaline

BIM
bicuculline methobromide

DMSO
dimethyl sulfoxide

TBS
Tris based saline

RT
room temperature

NGS
normal goat serum

K-S test
Kolmogorov-Smirnov test

SE
standard error

Rs
series resistance

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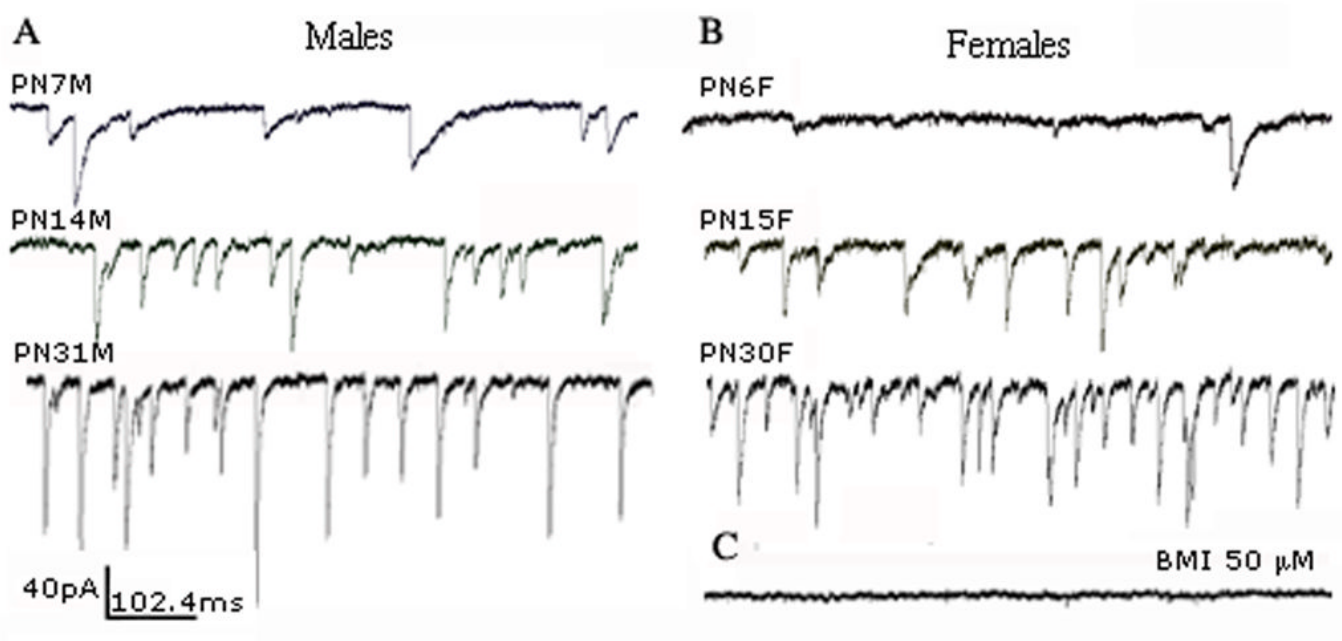

Fig. 1.

Raw traces of spontaneous $GABA_A$Rs-mediated inhibitory postsynaptic currents (sIPSCs) recorded in voltage-clamp configuration from GABAergic SNR neurons. Whole cell patch clamp recordings were made at a holding potential of −70 mV in the presence of glutamate antagonists CNQX 10 μM and D-AP5 50 μM in males (A) and females (B) of different ages. All sIPSCs were invariably blocked by $GABA_A$Rs antagonist bicuculline methobromide (BIM) 50 μM in all groups (C).

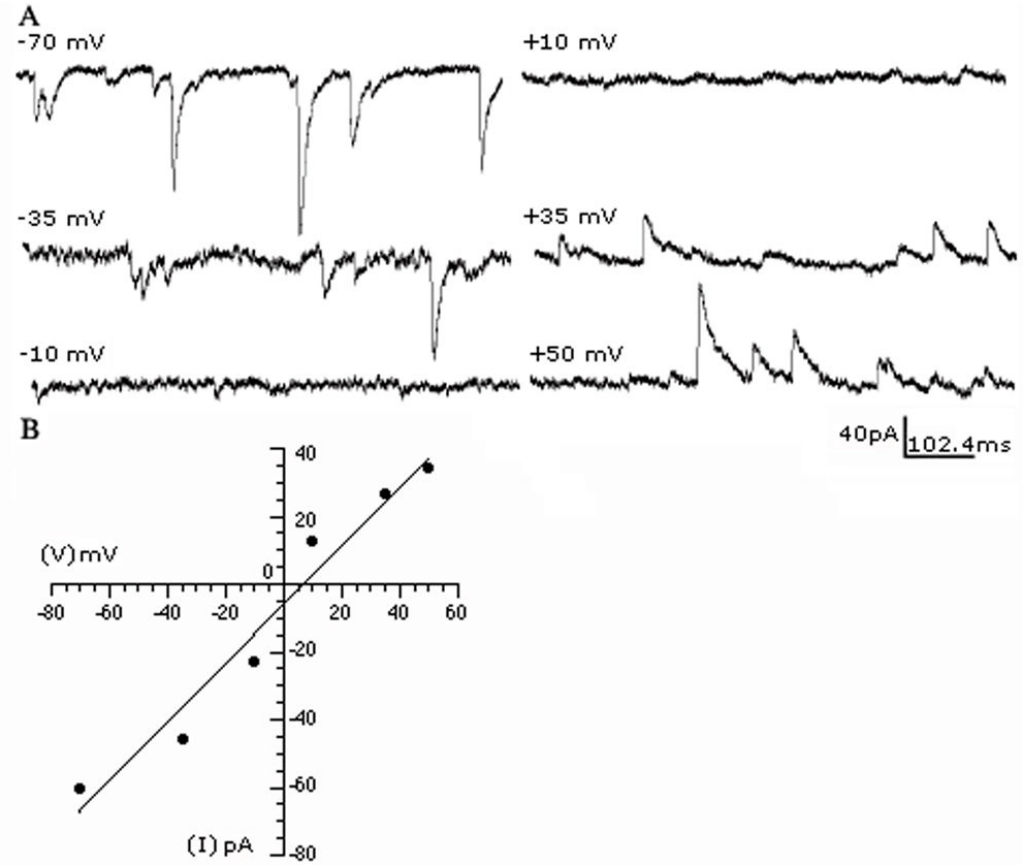

Fig. 2.

$GABA_A$Rs-mediated sIPSC reversal potential. (A) The recorded sIPSCs were inward at negative holding potentials and outward at positive holding potentials. (B) A current-voltage plot of the average sIPSC amplitude vs. holding potential from the cell in (A) shows that the events reversed close to the theoretical equilibrium potential for Cl^-, as expected with symmetrical intra- and extracellular chloride concentrations, confirming that they were mediated by $GABA_A$Rs. The plot was best fitted with a linear regression.

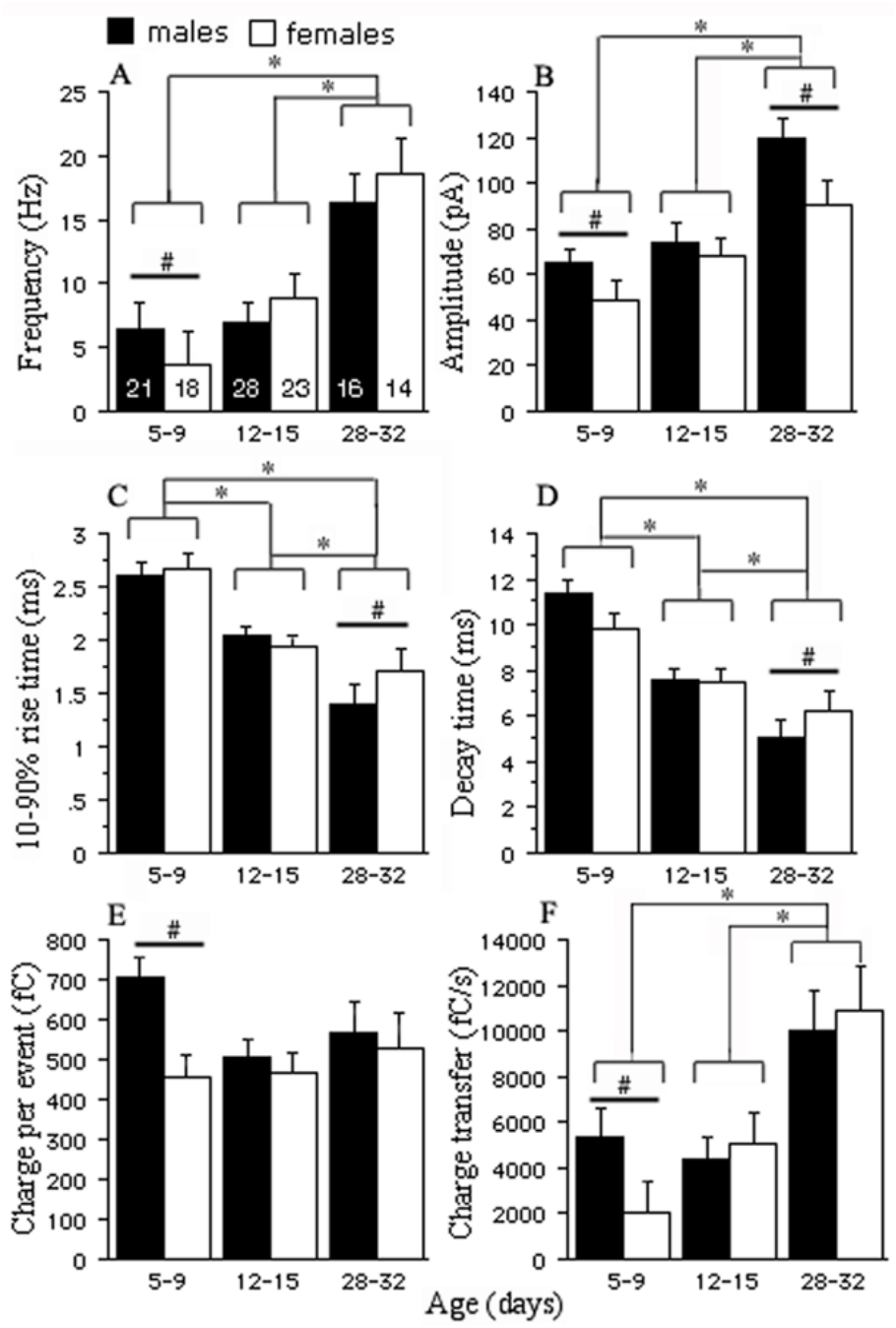

Fig. 3.

Baseline properties of the sIPSCs during development. (A) The mean sIPSCs frequency in PN28-32 groups is significantly higher than in the two remaining groups in both genders ($P<0.05$, two-way ANOVA, see Table 1 for numerical values). An additional inter-group analysis using the unpaired *t*-test (see Methods) showed that the mean sIPSCs frequency is higher in PN5-9 males than in females ($P<0.05$, unpaired *t*-test). (B) The mean sIPSCs amplitude is greatest in the oldest group compared with PN5-9 and PN12-15 animals and males have higher amplitudes than females ($P<0.05$, two-way ANOVA). The unpaired *t*-test comparisons demonstrate that PN5-9 and PN28-32 males exhibit higher amplitudes than their female counterparts ($P<0.05$). The mean 10–90% rise time (C) and decay time (D) shorten

with age in both genders and significant differences were observed between each age group ($P<0.05$, two-way ANOVA). The only sex differences were noted in PN28-32 animals, males having faster decay and 10–90% rise time than females ($P<0.05$, unpaired *t*-test). (E) The mean charge transferred by averaged sIPSC remains constant during development in both males and females. PN5-9 males have significantly greater charge than PN5-9 females ($P<0.05$, unpaired *t*-test). (F) The importance of synaptic $GABA_A$Rs-mediated inhibition rises with age as the synaptic charge transfer considerably grows in both genders ($P<0.05$, two-way ANOVA). No differences were found between PN5-9 and 12–15 animals. The unpaired *t*-test revealed that the PN5-9 males have bigger charge transfer than PN5-9 females ($P<0.05$). ($^{*}P<0.05$ two-way ANOVA; $^{\#}P<0.05$, unpaired *t*-test; numbers in graph A represent the numbers of cells).

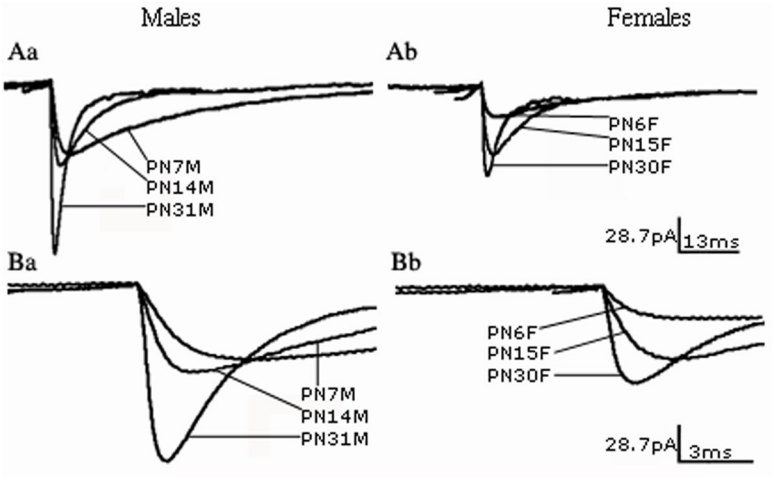

Fig. 4.

Age-related changes in the averaged $GABA_A$Rs-mediated sIPSC traces of male and female GABAergic SNR neurons. (Aa) and (Ab) show averaged representative sIPSC traces derived from 232–469 events recorded from PN7, PN14, PN31 male and PN6F, PN15, PN30 female GABAergic SNR neurons. The overlapped traces demonstrate acceleration of decay time with age in both genders. The mean amplitude is highest in both PN28-32 groups. (Ba) and (Bb) figures in expanded time scale illustrate shortening of the 10–90% rise time during development.

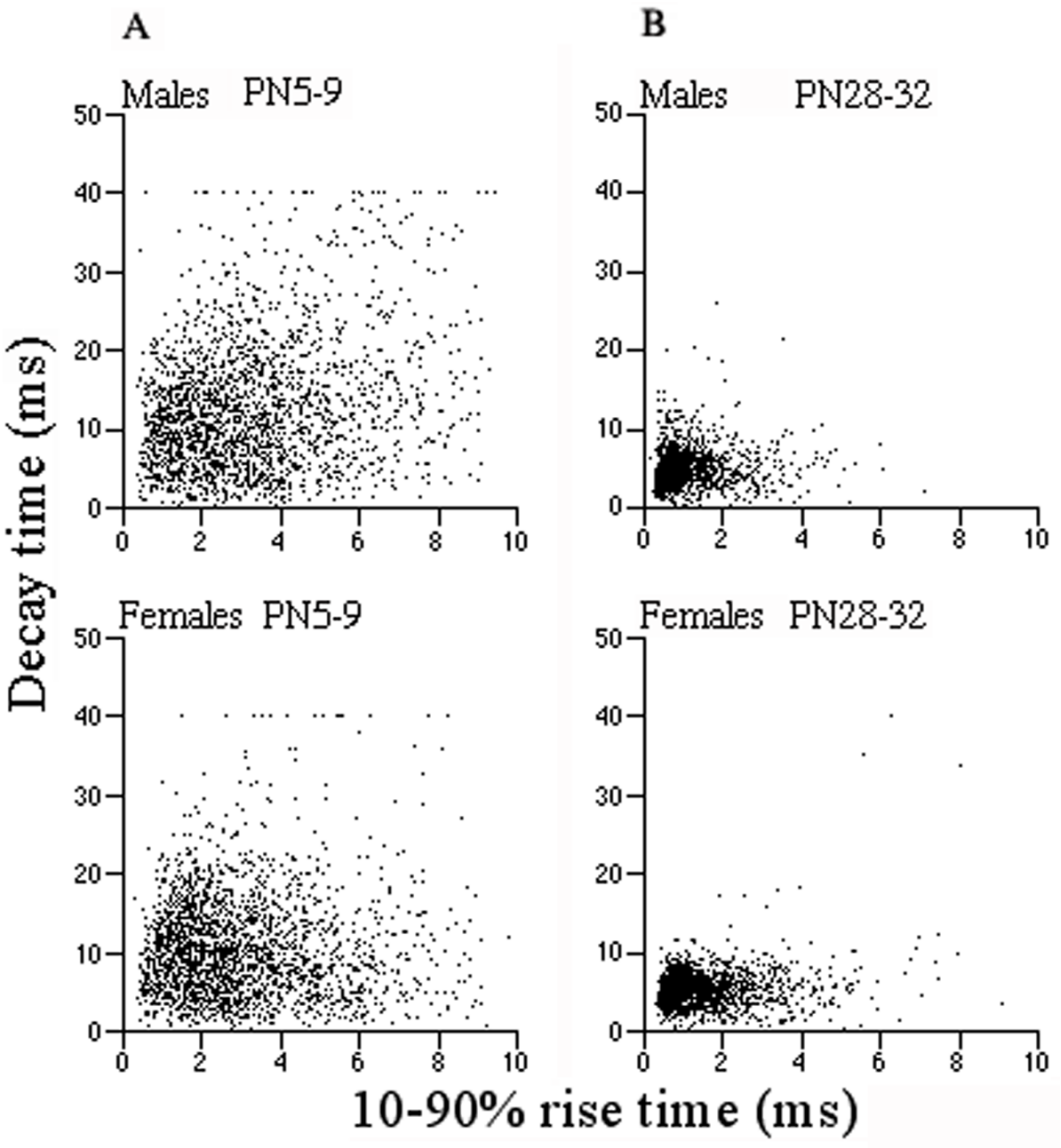

Fig. 5.

The sIPSCs decay times of single events of SNR GABAergic neurons were plotted against 10–90% rise times in PN5-9 and PN28-32 animals of both genders. The broad dispersion of datapoints in the PN5-9 group plots (column A) reflects heterogeneous $GABA_A$Rs subunit composition. The majority of events have slow activation and deactivation times. The situation is different in PN28-32 groups (column B), where the decay vs. rise time plots show that most events are confined to one limited area of the graph representing fast values. This finding suggests that $GABA_A$Rs subunit composition is more homogenous.

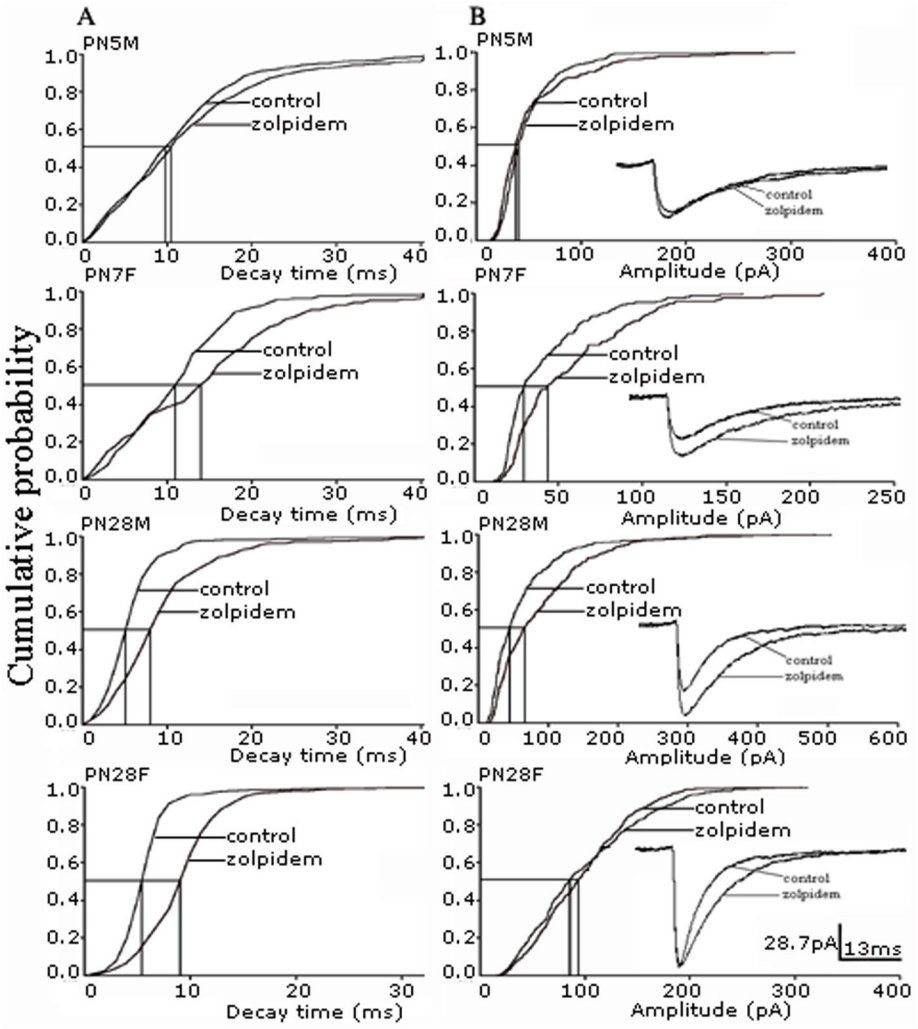

Fig. 6.

Decay time (column A) and amplitude (column B) cumulative probability plots were constructed from representative cells in PN5-9 (male cell 450 events, female 164) and 28–32 groups (male cell 373 events, female 450). Zolpidem 0.5 μM significantly changed cumulative amplitude and decay distribution in all cells shown ($P<0.05$, K-S test) except in the PN5M cell (no change in decay time and amplitude) and PN28F cell (no change in amplitude) ($P>0.05$ K-S test). While the lack of zolpidem effect both on decay time and amplitude in the PN5M cell can be explained by low α1 subunit expression, the near-to-complete saturation of $GABA_AR$s may underlie the failure of zolpidem to augment the average sIPSCs amplitude in the PN28F cell. Please note that PN12-15 male and female SNR neurons were also responsive to zolpidem (data not shown). The insets in graphs in the column B show overlapped baseline and zolpidem averaged events from the same cells.

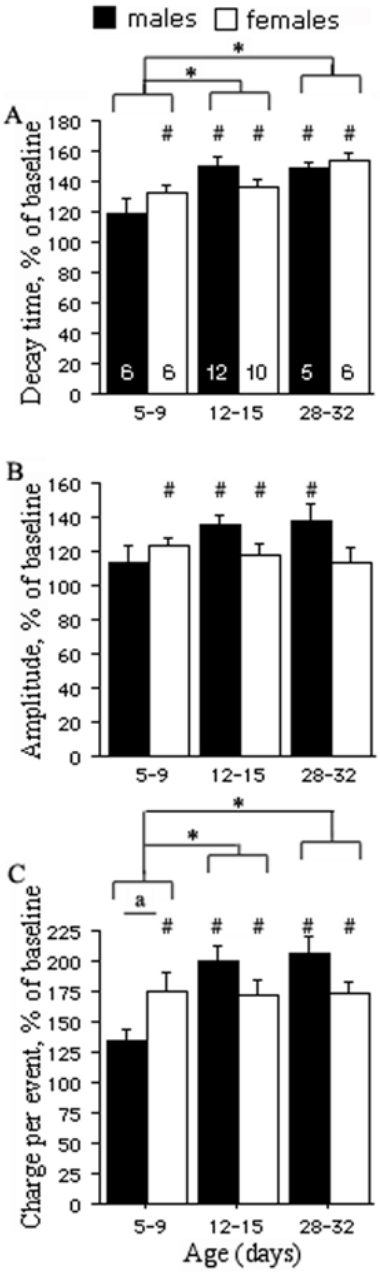

Fig. 7.

The percentage increase relative to the pre-drug baseline of decay time (panel A), amplitude (panel B), and charge per event (panel C) was used to compare age- and sex-related differences in response to zolpidem 0.5 μM. In most groups, zolpidem increased decay time, amplitude and charge per event compared to their baseline values, as indicated by the pound key (#) marks ($P<0.05$, paired *t*-test, data no shown). However, the only group that showed no response to zolpidem was the PN5-9 males. In PN28-32 females, zolpidem increased decay time and charge per event but not the amplitude of sIPSCs. This probably reflects a ceiling effect, as the baseline amplitudes in this group were already the highest (baseline amplitude 82.5±12.5 pA, zolpidem amplitude 89.8±11.5 pA, n=6, $p>0.05$, mean±SE, paired *t*-test).

(A) The graph shows a significantly greater prolongation of decay time in PN12-15 and PN28-32 groups compared with PN5-9 groups. No sex-related differences were detected (unpaired *t*-test). (B) In contrast, the increase of sIPSCs amplitude by zolpidem does not differ significantly across ages and genders. (C) The overall increase of charge transferred per event is greater in PN12-15 and PN28-32 animals compared with PN5-9 groups. There is a significant effect of the age*sex interaction, which indicates sex-specific patterns in the developmental changes in zolpidem sensitivity. Specifically, PN5-9 females show significantly bigger enhancement in zolpidem compared with PN5-9 males ("a" indicates $P \leq 0.05$, unpaired *t*-test), and this zolpidem sensitivity remains stable till PN28-32. In contrast, there is a significant increase in zolpidem sensitivity in males between PN5-9 and PN12-15. The pound keys (#) indicate groups in which zolpidem induced significant changes compared to baseline levels ($P<0.05$, paired *t*-test, data no shown). The asterisks (*) indicate significant differences from the respective PN5-9 group ($P<0.05$, two-way ANOVA, numbers in the plot A indicate the number of cells).

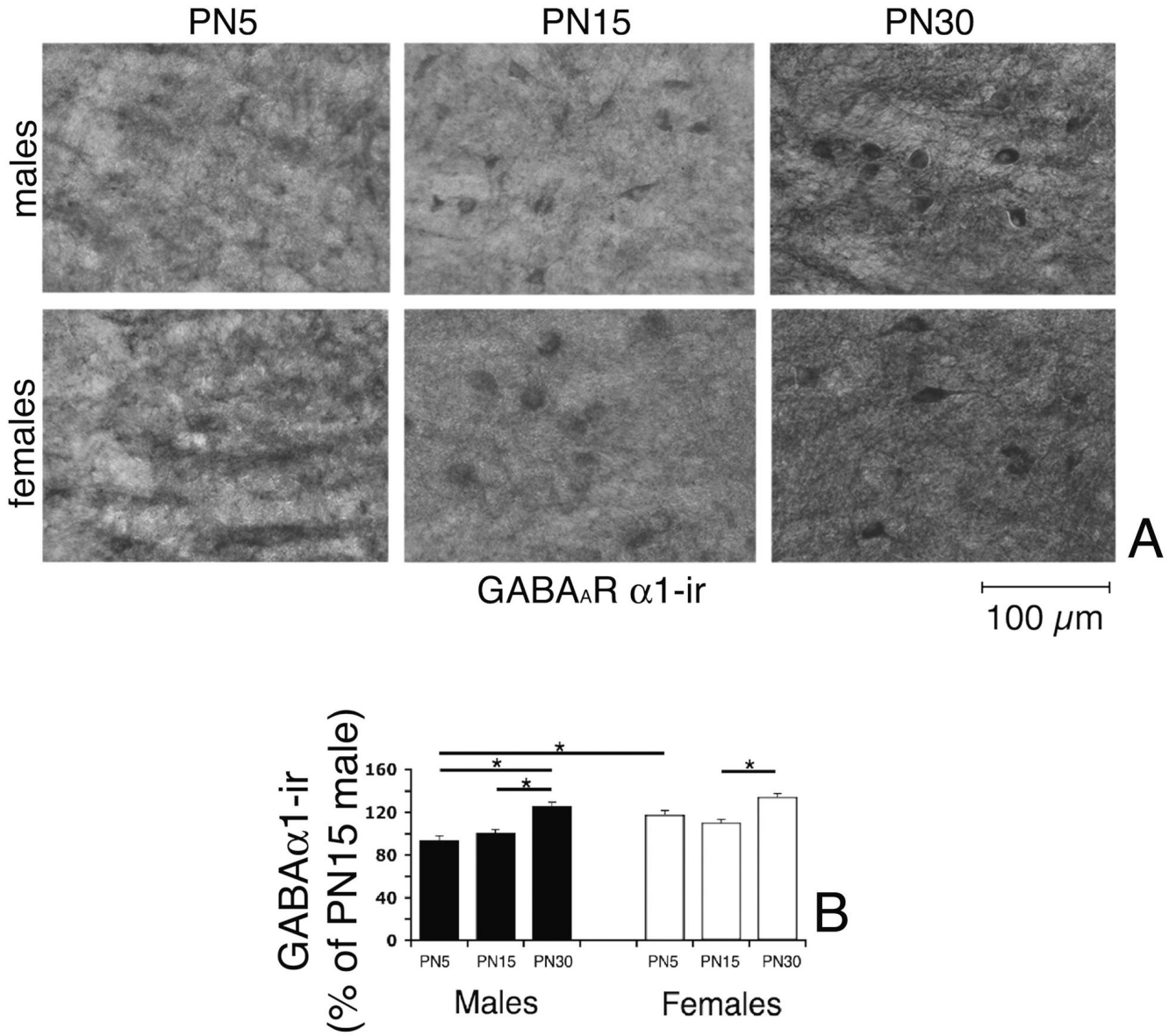

Fig. 8.

Expression of $GABA_A$Rs α1-ir in the anterior SNR of PN5, PN15, and PN30 male and female rats.

Panel A: Representative photographs of anterior SNR neurons stained with anti-$GABA_A$Rs α1 specific antibody. The α1-ir increases with age in both genders, in both the somata and the dendritic processes. The scale bar indicates 100μm distance.

Panels B: Densitometric comparisons of perisomatic α1-ir confirmed the developmental increase in $GABA_A$Rs α1-ir in both male and female rats between PN5 and PN30. As the SNR is sparsely populated, densitometry was done on individual α1-ir anterior SNR cells and was averaged for each brain. At PN5, females expressed more $GABA_A$Rs α1-ir than PN5 males. The asterisks indicate statistically significant differences ($P<0.05$) between the groups linked with bars.

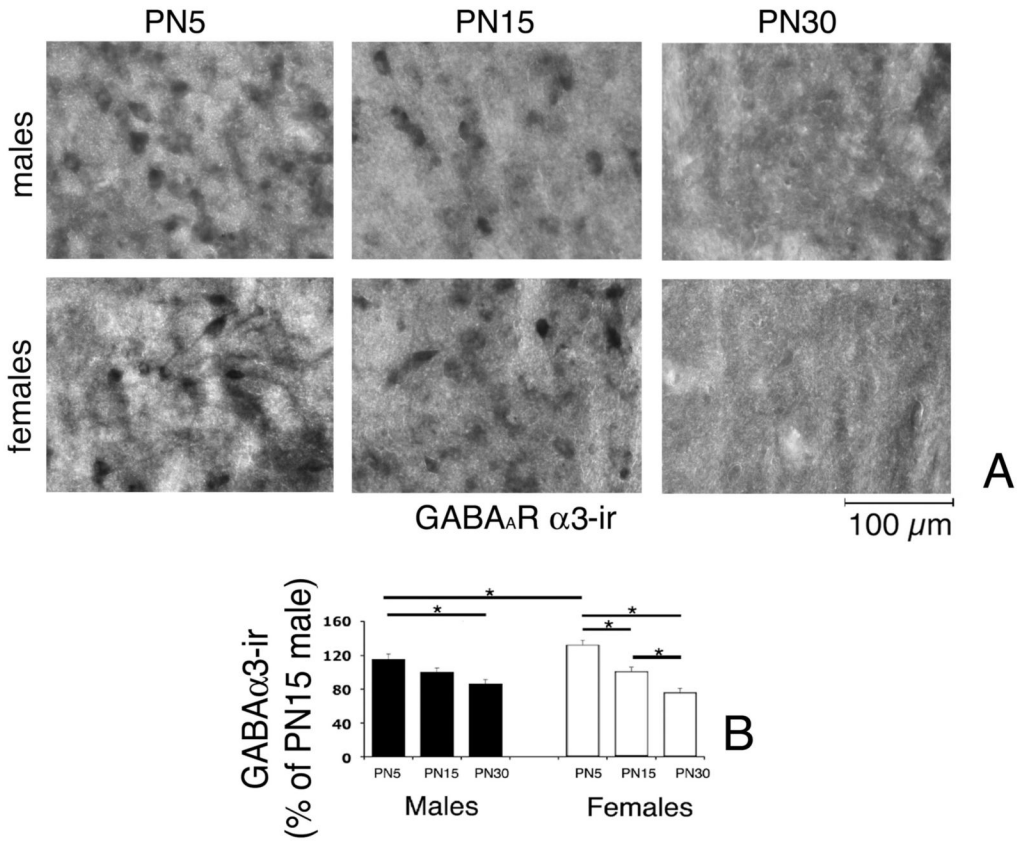

Fig. 9.

Expression of $GABA_AR$ α3-ir in the anterior SNR of PN5, PN15, and PN30 male and female rats.

Panel A: Representative photographs of anterior SNR neurons stained with anti-$GABA_ARs$ α3 specific antibody. The α3-ir decreases with age. The scale bar indicates 100μm distance.

Panels B: Densitometric comparisons of perisomatic α3-ir confirmed the developmental decrease in $GABA_ARs$ α3-ir in both male and female rats between PN5 and PN30. Densitometry was done on individual α3-ir anterior SNR cells and was averaged for each brain. At PN5, females expressed more $GABA_ARs$ α3-ir than males. The asterisks indicate statistically significant differences ($P<0.05$) between the groups linked with bars.

Table 1

Baseline properties of sIPSCs

Age group/sex	n (cells)	Frequency (Hz)	Amplitude (pA)	10–90% rise time (ms)	Decay time (ms)	Charge per averaged event (fC)	Charge transfer (fC/s)
PN5-9 M	21	6.4±1.9#	65.5±7.1#	2.61±0.1	11.37±0.5	706±51.9#	5326±1553#
PN12-15 M	28	7.0±1.7	73.9±6.1	2.03±0.1	7.57±0.4	506±44.9	4400±1345
PN28-32 M	16	16.4±2.2	119.2±8.1#	1.4±0.2#	5.1±0.6#	566±59.4	10010±1779
PN5-9 F	18	3.7±2.1	48.6±7.6	2.67±0.1	9.86±0.5	457±56.0	2061±1678
PN12-15 F	23	8.8±1.8	67.9±6.8	1.94±0.1	7.52±0.5	467±49.6	5025±1484
PN28-32 F	14	18.5±2.4	90.6±8.7	1.72±0.2	6.19±0.6	528±63.4	10894±1902

#P<0.05 value different from animals of opposite sex in the same age group, unpaired *t*-test. F=female, M=male.

Table 2

Age and sex specific effects on the expression of α1 and α3 $GABA_A$R-ir in rat anterior SNR.

A. Main effects, two-way ANOVA, repeated measures.

Groups	n (rats/group)	Age (F-value)	Sex (F-value)	Age*Sex (F-value)
α1 $GABA_A$R-ir	5	8.4*	6.5*	0.6
α3 $GABA_A$R-ir	4	12.2*	0.2	1.8

B. Least squares means tables.

Groups	α1 $GABA_A$R-ir somatic expression		α3 $GABA_A$R-ir somatic expression	
	n (rats)	Least Squares Means (% of PN15 male group)	n (rats)	Least Squares Means (% of PN15 male group)
PN5 M	5	93.1±4.7#	4	115.4±6.1#
PN15 M	5	100±4.3#	4	100±5.5
PN30 M	5	124.8±4.5	4	86±5.5
PN5 F	5	116.7±4.8§	4	131.9±6#
PN15 F	5	109.4±4.3#	4	100.9±5.6 #¶
PN30 F	5	133.2±4.6	4	75.56±5.5

*P<0.05

#P<0.05 vs PN30 same sex group;

§P<0.05 vs PN5 males;

¶P<0.05 vs PN5 females; Tukey post hoc comparisons.

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The Role of Substantia Nigra Pars Reticulata in Modulating Clonic Seizures is Determined by Testosterone Levels During the Immediate Postnatal Period

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Abstract

GABAergic activation of substantia nigra pars reticulata (SNR) at postnatal day (PN) 15 has sex-specific features on seizure control *in vivo* and electrophysiological responses *in vitro*. In males, the $GABA_A$-receptor agonist muscimol has proconvulsant effects and induces depolarizing responses. In females, muscimol has no effect on seizures and evokes hyperpolarizing responses. We determined the time period during which sex hormones must be present to produce the sex-specific muscimol effects on seizures and their influence on SNR $GABA_A$ receptor-mediated postsynaptic currents. Exposure to testosterone or its metabolites (estrogen or dihydrotestosterone) during PN0-2 in females or males castrated at PN0 was sufficient to produce proconvulsant muscimol effects but did not affect the *in vitro* GABA responses, which remained hyperpolarizing. The data suggest that the PN0-2 period is critical for the development of the seizure-controlling SNR system; the hormonal effect on seizure control is independent from their effect on GABA conductance.

Keywords

substantia nigra; sex hormones; muscimol infusions; seizure; GABA; patch clamp

During development, circulating sex hormones have organizational effects leading to permanent differences between males and females in distinct brain regions (Gorski, 1984). The presence or absence of testosterone, acting via its metabolites, dihydrotestosterone and estrogen, determines the future “male” or “female” brain phenotype (McEwen, 1992). In humans, the incidence of epilepsy is higher in the youngest age groups and population studies have shown that males have a higher incidence of seizures than females (Hauser, 1997). These epidemiological data strengthen the need to understand mechanisms underlying the sex-dependent differentiation of the CNS, especially in relation to structures involved in seizure

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control. Understanding of how the sex hormones can modulate seizure-controlling substrates during development is important for the identification of possible targets for prevention or treatments of seizure disorders.

The substantia nigra pars reticulata (SNR), a midbrain structure populated largely by GABAergic neurons, serves as a main output of the basal ganglia. The SNR is involved in the control of seizures in an age- and sex-specific fashion (Velíšková and Moshé, 2001; Velíšková and Moshé, 2006). In immature rats [postnatal day (PN) 15], localized bilateral SNR microinfusions of muscimol, an agonist at $GABA_A$ receptors, have proconvulsant effects on flurothyl-induced clonic seizures in males but not in females (Moshé et al., 1994; Velíšková and Moshé, 2001). Additionally, *in vitro* gramicidin patch clamp recordings in slices from PN14-17 males and females show sex-specific responses of the SNR GABAergic neurons to bath application of muscimol (Galanopoulou et al., 2003b) and to synaptically released GABA (Kyrozis et al., 2006). In males, both have depolarizing effects on the membrane potential of the SNR GABAergic neurons, while in females the response is hyperpolarizing.

The majority of sex differences in the brain develop under the influence of perinatal testosterone (Arnold and Gorski, 1984). In rats during postnatal development, the brain is especially sensitive to the organizational effects of testosterone during the first six days of life (Goy and McEwen, 1980). We have previously shown that the presence of postnatal testosterone is responsible for the male proconvulsant phenotype of SNR muscimol effects on clonic seizures (Velíšková and Moshé, 2001). Testosterone depletion by castration of males at PN0 leads to female-like responses of SNR muscimol on clonic flurothyl-induced seizures at PN15. Daily administration of testosterone (from PN0 to PN14) to PN0 castrated males or to intact female rats results in the male proconvulsant phenotype (Velíšková and Moshé, 2001). These data show that postnatal testosterone has pivotal organizing effects on the SNR seizure-controlling network but do not define the critical period during which the presence of testosterone leads to the formation of the muscimol-sensitive proconvulsant SNR region in PN15 rats. Further, as the testosterone affects the brain via its metabolites (Arnold and Gorski, 1984), the question remains, whether estrogen or dihydrotestosterone mediates the sexual differentiation of the SNR.

The main aims of this study were to determine *in vivo*, 1) the critical postnatal period necessary for the testosterone exposure to induce the proconvulsant phenotype of SNR muscimol effects on clonic seizures and 2) whether exposure to one of testosterone metabolites (dihydrotestosterone or estrogen) or both is necessary to achieve this effect. We also tested, using *in vitro* gramicidin perforated patch clamp recordings, whether the SNR $GABA_A$ receptor-mediated postsynaptic currents (PSCs) are changing as the result of the hormonal exposure during the critical period.

Methods

Sprague-Dawley rats (Taconic Farms, New York) were housed under standard conditions in our animal facility, which is accredited by the American Association for Accreditation of Laboratory Animal Care. The pups were kept with their dams. The day of birth was considered PN0.

In vivo studies

Neonatal castration—Castration in males was performed as described previously (Velíšková and Moshé, 2001). Briefly, a sufficient level of general anesthesia was reached by lowering the body temperature with crushed ice. Under aseptic conditions, two small incisions were performed on the lower abdomen between the hindlimb insertion point and genital prominentia and the testes were carefully extracted. The wounds were sutured and treated with

disinfectant. The pups were warmed for 2 h at 30°C and returned to their respective dams. The rats were castrated at PN0 to prevent any postnatal testosterone surge (Pfeiffer, 1936). In additional rats, the natural testosterone surge was permitted during the early neonatal period by performing the castration at a later date. In a pilot experiment we found that in males castrated at PN5, SNR muscimol infusions at PN15 had proconvulsant effects compared to saline controls. This effect is similar to that observed in intact male rats with undisturbed testosterone surge (Velíšková and Moshé, 2001). Thus, to further narrow the early postnatal period of testosterone exposure, necessary to induce the muscimol-sensitive proconvulsant SNR region, we used males castrated at PN3 (n=15).

Administration of testosterone—Female rats (n=26) and males castrated at PN0 (prior to their natural testosterone surge; n=14) were injected daily with testosterone propionate (TP; 0.1 mg/0.02ml s.c.) from PN0 to PN2. This dose of TP, when administered daily for 15 days, induced the proconvulsant male type SNR muscimol effect on the clonic seizure threshold in females and in PN0 castrated males (Velíšková and Moshé, 2001).

Administration of testosterone metabolites—Female rats (n=14) or males castrated at PN0 (n=17) were injected with diethylstilbestrol (DES; 2 μg/0.02ml s.c.) or 5α-androstan-17b-ol-3-one (5α-dihydrotestosterone, DHT; 100 μg/0.02 ml s.c.; females: n=12; PN0 castrated males: n=12) from PN0 to PN2. DES is a synthetic estrogen, which in contrast to β-estradiol does not bind to α-fetoprotein and thus produces stable estrogen blood levels (Savu et al., 1979). At this daily dose, DES produces sexual differentiating effects in other brain regions (McAbee and Doncarlos, 1999). DHT is a non-aromatizable androgen, which has been shown to exert sexual differentiating effects (van der Schoot, 1980).

Stereotaxic surgery and seizure testing—Bilateral cannulae (Plastics One, Roanoke, VA, USA) were implanted in the SNR at PN13 under ketamine (70 mg/kg)-xylazine (10 mg/kg) anesthesia as described previously (Velíšková and Moshé, 2001). With the incisor bar positioned 3.5 mm below the interaural bar, the stereotaxic coordinates for the SNR were: anterior-posterior = 5.3 mm from bregma, medial-lateral = 3.5 mm from midline, dorsal-ventral = 6.0 mm below the skull. The cannulae were inclined at an angle of 15° from the sagittal plane. After surgery, rats were allowed to recover for 2 days with their dams.

At PN15, 30 min prior to seizure testing, muscimol (100 ng/0.25 μl saline) or saline (0.25 μl) were injected in the SNR bilaterally (Velíšková and Moshé, 2001).

For seizure testing, rats were exposed to flurothyl as described previously (Velíšková and Moshé, 2001). The flurothyl seizure threshold was determined as the amount of flurothyl (in μl) necessary to elicit the first clonic seizure characterized by facial and forelimb clonus while the righting reflex is preserved.

After completion of the seizure testing, the animals were sacrificed, their brains were removed and frozen in 2-methylbutane at -35°C. Coronal sections were stained with thionin for histological examination of the cannulae placements. Only rats with bilateral symmetrical placements of both cannulae in the SNR were used. We have found previously that drug infusions above the SNR have no effect on flurothyl seizure threshold (Xu et al., 1991; Velíšková et al., 1998).

For clarity of the comparisons in the graphic presentation of the data, we added in Figure 2, an inset showing the original, already published, data (Velíšková and Moshé, 2001) of the effects of SNR muscimol infusions on flurothyl seizure threshold at PN15 males, PN15 females, and PN15 neonatally castrated males.

In vitro studies—For the *in vitro* gramicidin perforated patch clamp studies we used the following groups of rats: intact males and females, males castrated at PN0, females treated with TP, DHT or DES between PN0-PN2, and males castrated at PN3 (which were thus exposed to natural testosterone for three days).

Coronal 300 μm thick slices including the SNR were cut from ketamine-anesthetized PN13-16 rats with a vibratome (OTS 4000, Electron Microscopy Sciences, Hatfield, PA). Dissection solution contained (in mM): sucrose 210, KCl 3.5, $CaCl_2$ 1, $MgCl_2$ 4, $NaHCO_3$ 26, NaH_2PO_4 1.25, D-glucose 10.

Slices were incubated for at least one hour in a chamber with artificial cerebrospinal fluid, which contained (in mM): NaCl 126, KCl 4, NaH_2PO_4 1.25, $MgCl_2$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, D-glucose 10. Perforated patch clamp recordings from SNR neurons were performed with the cation-permeable ionophore gramicidin (0.5 μg/ml; DMSO 0.1% v/v), to avoid alteration of intracellular chloride. The pipette solution contained (in mM): K_2SO_4 77, NaCl 5, $MgCl_2$ 2, $CaCl_2$ 0.5, EGTA 5, HEPES 10, pH=7.3, 295 mOsm. The junction potential (measured -10 mV) was electronically compensated. All bath solutions were bubbled with 95% O_2/5% CO_2 gas mixture and supplemented with ascorbic acid (100 μM), and pyruvate (500 μM); pH=7.3-7.4; 300-310 mOsm. Recordings were performed at room temperature.

Only presumed SNR GABAergic neurons were included in the study. Their distinction from the infrequently encountered SNR dopaminergic cells was made on the basis of their narrow action potentials (<1ms halfwidth), biphasic after hyper polarization, firing rate and lack of the voltage sag in response to hyperpolarizing currents (Richards et al., 1997).

To obtain PSCs generated by $GABA_A$ receptor activation, we delivered rectangular electrical pulses of 100 μsec duration using a stainless steel bipolar stimulating electrode (Frederick Haer Co., Bowdoingham, ME) inserted in the slice at a distance of 200-400 μm from the recorded neuron. 6-cyano-7-nitroquinoxalene-2,3,-dione (CNQX; 10 μM) and kynurenic acid (1mM) were present in the bath solution to block PSCs generated by synaptic glutamate release.

The resting membrane potential was determined as an average of the action potential threshold and the potential of the through of the afterhyperpolarization as described previously (Kyrozis et al., 2006). To assess the reversal potential (EGABA) of GABAergic PSCs, 10 mV voltage steps between -110 and -30 mV were applied from a holding potential of -70 mV and stimulations were delivered at each voltage. Peak current of every PSC was measured. A straight line was fitted to the points of the current-voltage curve and its intersection with zero current was taken as the reversal potential. The electrical driving force was defined as EGABA and its difference from resting membrane potential (EGABA-Vr).

Table 1 summarizes the experimental design and hormonal treatments for both the *in vivo* and *in vitro* experiments.

Data Analysis

We compared the flurothyl seizure threshold between the SNR muscimol- and saline-infused rats at PN15 using the Student's t-test. We used one-way ANOVA followed by a post-hoc Fisher Protected Least Significant Difference Test to determine whether the individual hormone treatments had equal effects in both sexes.

In the electrophysiological evaluation, GABAergic responses were compared by one-way ANOVA, followed by a Tukey-Kramer Multiple Comparison Test.

The significance level for all statistical comparisons was preset to $P<0.05$. The data are presented as mean ± SEM.

Results

In vivo SNR muscimol effects on clonic seizure threshold in PN15 rats

Determination of the critical period in males: In our previous study we found that the lack of postnatal testosterone by neonatal castration in males at PN0 (Pfeiffer, 1936) resulted in loss of the proconvulsant SNR muscimol effects on seizures (Velíšková and Moshé, 2001). In the present study, rats were castrated at PN3 to allow for exposure to testosterone for the first three days of life. In these PN3 castrated rats (Figure 1), muscimol (n= 7) infusions into the SNR had a proconvulsant effect compared to respective saline-infused (n=8) controls (Student's t-test $t=2.6$; $P<0.05$).

Effects of exogenous testosterone administration during PN0-2: In intact females, administration of testosterone at PN0-2 resulted in the proconvulsant effects of SNR muscimol (n=13) on flurothyl seizures compared to their respective saline-infused (n=13) controls (Student's t-test: $t=4.9$; $P<0.05$; Figure 2). Similarly, in males castrated at PN0 with exogenous testosterone for three days (PN0-2), SNR muscimol (n=6) had a proconvulsant effect on flurothyl seizures compared to saline-infused (n=8) controls (Student's t-test: $t=4.9$; $P<0.05$; Figure 2).

Effects of administration of testosterone metabolites during PN0-2: We determined whether estrogenic or androgenic metabolites of testosterone are responsible for the occurrence of the proconvulsant effects of SNR muscimol infusions (Figure 2). In females, administration of either DES or DHT resulted in proconvulsant SNR muscimol effects on seizures compared to SNR saline-infused controls (DES: muscimol, n=7; saline, n=7; Student's t-test: $t=4.6$; DHT: muscimol, n=6; saline, n=6; Student's t-test: $t=4.0$; $P<0.05$). In castrated males at PN0, administration of both testosterone metabolites also resulted in proconvulsant effects of SNR muscimol infusions on flurothyl seizures compared to saline-infused controls (DES: muscimol, n=9; saline, n=8; Student's t-test: $t=4.9$; DHT: muscimol, n=6; saline, n=6; Student's t-test: $t=4.7$; $P<0.05$).

In vitro perforated-patch clamp recording—Only GABAergic neurons were studied. The resting membrane potential (Vr) did not differ significantly among the groups (males castrated PN0, n=6: -63.2 ± 1.0 mV; intact females, n=7: -66.7 ± 1.3 mV; intact males, n=4: -61.0 ± 3.1 mV; females TP, n=4: -63.0 ± 2.4 mV; females DES, n=17: -64.6 ± 1.1 mV; females DHT, n=14: -66.9 ± 1.6 mV; males castrated PN3, n=8: -61.0 ± 2.2 mV $P=0.18$).

Effects associated with castration in males at PN0—We first tested the GABA responses of the SNR GABAergic neurons in male rats castrated at PN0 (Figure 3B) since this had not been determined in our previous studies. In neurons from neonatally castrated males at PN0, the driving force was hyperpolarizing, a -6.3 ± 3.2 mV change from resting potential (EGABA was -69.5 ± 3.1 mV). A similar effect was observed in intact females: the driving force was also hyperpolarizing, a -5.4 ± 1.9 mV change from resting potential (EGABA was -72.1 ± 2.2 mV). On the other hand in intact males, we found a depolarizing effect: The driving force was a +6.5 ± 2.3 mV change from resting potential (EGABA was -55.8 ± 2.2 mV).

Effects of exogenous testosterone administration during PN0-2—In females, administration of testosterone at PN0-2 did not change the female-like hyperpolarizing GABA responses. Figure 3C shows that the driving force was a -8.3 ± 5.1 mV change from resting potential (EGABA was 71.3 ± 6.0 mV).

Effects of administration of testosterone metabolites during PN0-2—In females, administration of either testosterone metabolite did not affect the hyperpolarizing GABA responses (Figure 3C): DES: the driving force was a -6.6 ± 0.7 mV change from resting potential (EGABA was -71.2 ± 1.3 mV); DHT: the driving force was a -7.0 ± 1.4 mV change from resting potential (EGABA was -73.9 ± 1.6 mV).

Effect of endogenous testosterone during the first three days of life—In males, castration at PN3 allowed exposure to natural testosterone for three days of life. This short exposure to natural testosterone was not sufficient to induce depolarizing effects and in contrast to intact males, the GABA responses were hyperpolarizing. The driving force was a -10.8 ± 1.9 mV change from resting potential (EGABA was -71.8 ± 1.5 mV).

The driving force and the EGABA in intact males were significantly different from any other group tested as revealed by ANOVA with Tukey-Kramer Multiple Comparisons Test. There were no other statistically significant differences among the hormone-treated groups or between the intact females and castrated males at PN0 or PN3 in any parameter tested.

Discussion

In vivo studies—The SNR controls seizures in a sex-specific manner. In PN15 rats, bilateral muscimol infusions in the SNR have a proconvulsant effect in male rats but no effect in female rats on clonic seizures induced by flurothyl (Velíšková and Moshé, 2001). The proconvulsant muscimol effect is lost in males by castration at PN0 but not in sham operated males. These data imply a role of postnatal testosterone exposure in the formation of the proconvulsant effects but not a role of perinatal stress (Velíšková and Moshé, 2001). In the current study, we identified a critical period necessary for testosterone exposure to induce the proconvulsant type of SNR muscimol effects on seizures in either sex. The data show that the testosterone surge in males during the first three postnatal days (Weisz et al., 1980) or administration of exogenous testosterone during PN0-2 in females and neonatally castrated males (at PN0) is sufficient to imprint the proconvulsant phenotype at PN15.

We further show that the sex differences in SNR responses to muscimol at PN15 can be induced by administration of both testosterone metabolites, DES and DHT from PN0-2. Our study corroborates data regarding the important role of both estrogens and androgens in sexual differentiation of distinct brain regions (Cooke et al., 1998). It was previously thought that the masculinization of the brain is mainly mediated by the action of estrogen (Kelly, 1991), while the role of androgens was restricted to spinal cord structures, such as nucleus bulbocavernosus (Breedlove et al., 1982). However, recent reports suggest that androgens are also important for the sexual differentiation in the CNS. Studies using male rats with testicular feminization mutation (*tfm*) revealed the significant role of androgen receptors in the masculinization of several forebrain regions, including amygdala, hippocampus, and locus coeruleus (Garcia-Falgueras et al., 2005; Jones and Watson, 2005; Morris et al., 2005). The important role of androgen receptor function in sexual behavior has been also shown using androgen receptor knockout mice, stressing a role for both androgen and estrogen receptors (Sato et al., 2004). Thus, both androgen and estrogen receptor signaling seems to be important for masculinizing effects (Shughrue and Dorsa, 1994; Breedlove, 1997; Han and De Vries, 2003).

In vitro studies—The sex difference in the effects of SNR muscimol infusions on seizures at PN15 could potentially be, at least in part, due to sex differences in the local responses of SNR neurons to $GABA_A$ receptor activation. In previous studies, we found that the male PN15 SNR expressed lower mRNA levels of the $GABA_A$ receptor α1 subunit and neuronal specific potassium chloride cotransporter KCC2 compared to the female SNR (Galanopoulou et al., 2003b; Ravizza et al., 2003). The *in vitro* recordings showed that male SNR neurons were

depolarized in response to muscimol application or synaptically released GABA, while female neurons were hyperpolarized (Galanopoulou et al., 2003b; Kyrozis et al., 2006). In the present study, we examined whether perinatal hormonal manipulations that affected the SNR-mediated seizure control had an analogous effect on the electrical direction of $GABA_A$ receptor responses (Galanopoulou et al., 2003b; Kyrozis et al., 2006). Similarly to the demasculinizing effect observed *in vivo* (Velíšková and Moshé, 2001), the present data show that castration in males at PN0 is associated with hyperpolarizing GABA responses, which are characteristic for females at this age. A three-day exogenous administration of testosterone or its metabolites between PN0-2 in female rats or the natural presence of testosterone and its metabolites for 3 days in male rats prior to castration at PN3 is not sufficient to produce the depolarizing GABA responses characteristic for males around PN14. It should be pointed out that we did not perform any studies following the administration of testosterone or its metabolites in male rats castrated at PN0. This is because male rats castrated at PN3 are exposed to natural testosterone (and its metabolites) for 3 days, a condition that is modeled in females treated with testosterone PN0-2.

The data suggest that the SNR GABA responses may require longer exposure period than 3 days or continuous testosterone administration to masculinize male rats and defeminize female rats in term of local SNR GABA responses at PN15. Alternatively, exposure at later time points may be more capable of altering the responses. One of the main regulators of the direction of GABA responses is the neuronal specific potassium chloride co-transporter KCC2, which is essential for lowering the intracellular Cl^- (Rivera et al., 1999). The depolarizing GABA responses in the SNR of male rats are associated with low expression of the KCC2, while the hyperpolarizing GABA responses in females correspond to higher expression of KCC2 in the SNR (Galanopoulou et al., 2003b). The SNR expression of KCC2 can be acutely modulated by exogenous administration of testosterone or its metabolites (Galanopoulou and Moshé, 2003a) at a later age (PN13-15). Galanopoulou showed that the estrogenic derivatives of testosterone maintain KCC2 expression low, facilitating therefore the appearance of depolarizing $GABA_A$ receptor responses. Nevertheless, preliminary data from this laboratory show that early postnatal administration of testosterone or its metabolites (PN0-2) does not affect the SNR KCC2 expression in females (Galanopoulou, unpublished data), again suggesting that longer periods of administration of these hormones or actual hormonal milieu are needed to alter the KCC2 levels and accordingly the *in vitro* GABA responses. Indeed, Cooke et al. (Cooke et al., 1999) reported that the continuous presence of circulating testosterone is required for the maintenance of sex differences in the volume of medial amygdala in adult rats. Similarly, the sexually dimorphic nucleus of the preoptic area has also been shown to require the continuous presence of circulating testosterone to maintain its volume (Bloch and Gorski, 1988).

The dissociation between the in vivo and in vitro sex hormone-induced effects on SNR function—The data concerning EGABA should be contrasted with the data showing that the SNR-based muscimol-sensitive network involved in seizure control can be modified long-tern by a relative short period of exposure to testosterone and its metabolites.

One of the reasons for the discrepancy between the *in vivo* and *in vitro* effects may be the distinct maturational pattern resulting from the early postnatal hormonal treatment. We previously demonstrated that in intact rats, the switch from SNR muscimol proconvulsant to anticonvulsant effects *in vivo* (Velíšková and Moshé, 2001) follows the switch from the depolarizing to the hyperpolarizing GABA responses in the SNR neurons *in vitro* (Kyrozis et al., 2006). In males, the anticonvulsant muscimol effects occur after PN25 but the hyperpolarizing GABA responses are already present at PN17. The maturation in females is faster than in males: the *in vivo* muscimol anticonvulsant effects occur after PN15, while the *in vitro* GABA effects are hyperpolarizing around PN10. The results from the present study in

PN15 rats suggest that the natural testosterone exposure in males castrated at PN3 or the perinatal hormonal administration in females may lead to a maturational pattern similar to that observed in PN 21 intact male rats. At this age, muscimol infusions still have proconvulsant effects (Velíšková and Moshé, 2001), while the EGABA responses are hyperpolarizing (Kyrozis et al., 2006).

Other factors beyond the local SNR GABAergic neuron effects may help explain the dissociation between the *in vivo* and *in vitro* data. Although it has been suggested that the nigral dopaminergic system may not be directly involved in seizure control (Albala et al., 1986; Gale, 1988), it may play a supporting role during seizures and thus hormone-induced changes in this system may affect the muscimol-induced *in vivo* effects. Sex differences in the SN dopaminergic system have been described including sex-dependent responses to muscimol (Robinson et al., 1981; Miller, 1983; Reisert et al., 1987; Dluzen and McDermott, 2000; Galanopoulou, 2006). Fin ally, the SNR input/output structures may be differentially affected by the hormonal treatments. The natural or exogenously administered circulating sex hormones influence the whole brain and the organizational effects of the sex hormones are not restricted to the SNR. Previously, using the 2-deoxyglucose mapping technique, we found that SNR muscimol infusions induce sex-dependent uptake pattern differing in several input/output SNR structures (Velisek et al., 2005). Thus, the *in vivo* effects of SNR muscimol on seizures may be dependent by other structures within the SNR seizure-controlling network and may not simply reflect only the local SNR properties. Ongoing studies in our laboratory are exploring this possibility.

Conclusions

Clinical evidence shows gender- and age-related expression in many seizure syndromes (Hauser, 1994; Christensen et al., 2005). The incidence of epilepsy is generally higher in males than in females (Hauser, 1997). As the SNR is part of a seizure controlling system, understanding the sex differences and the role of sex hormones in modulation of this system should offer significant insights in the pathophysiology and treatment of seizures.

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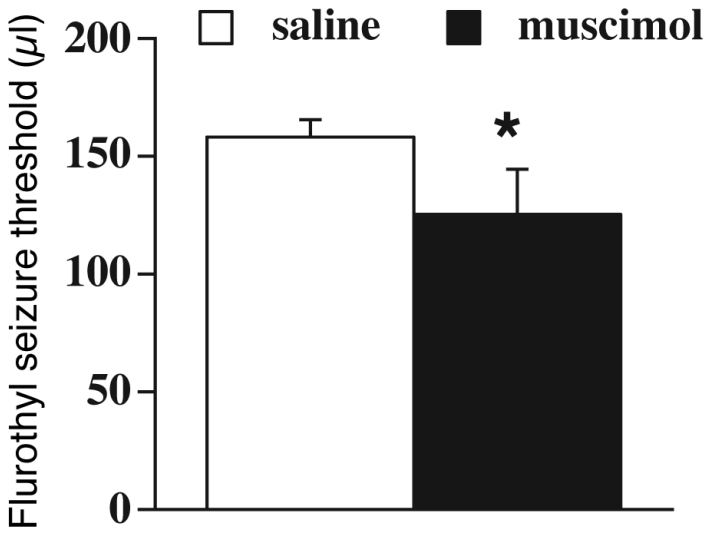

Figure 1.

Determination of the critical period in males. The flurothyl seizure threshold for the first clonic seizure was determined at PN15 in male rats castrated at PN3. The rats were thus exposed to natural testosterone for three days. Muscimol infusions in the SNR had proconvulsant effects compared to saline-infused controls suggesting that exposure to natural testosterone during the early postnatal period is responsible for the male proconvulsant phenotype of seizure-controlling SNR effects. The asterisk indicates a significant difference ($P < 0.05$) from saline-infused control.

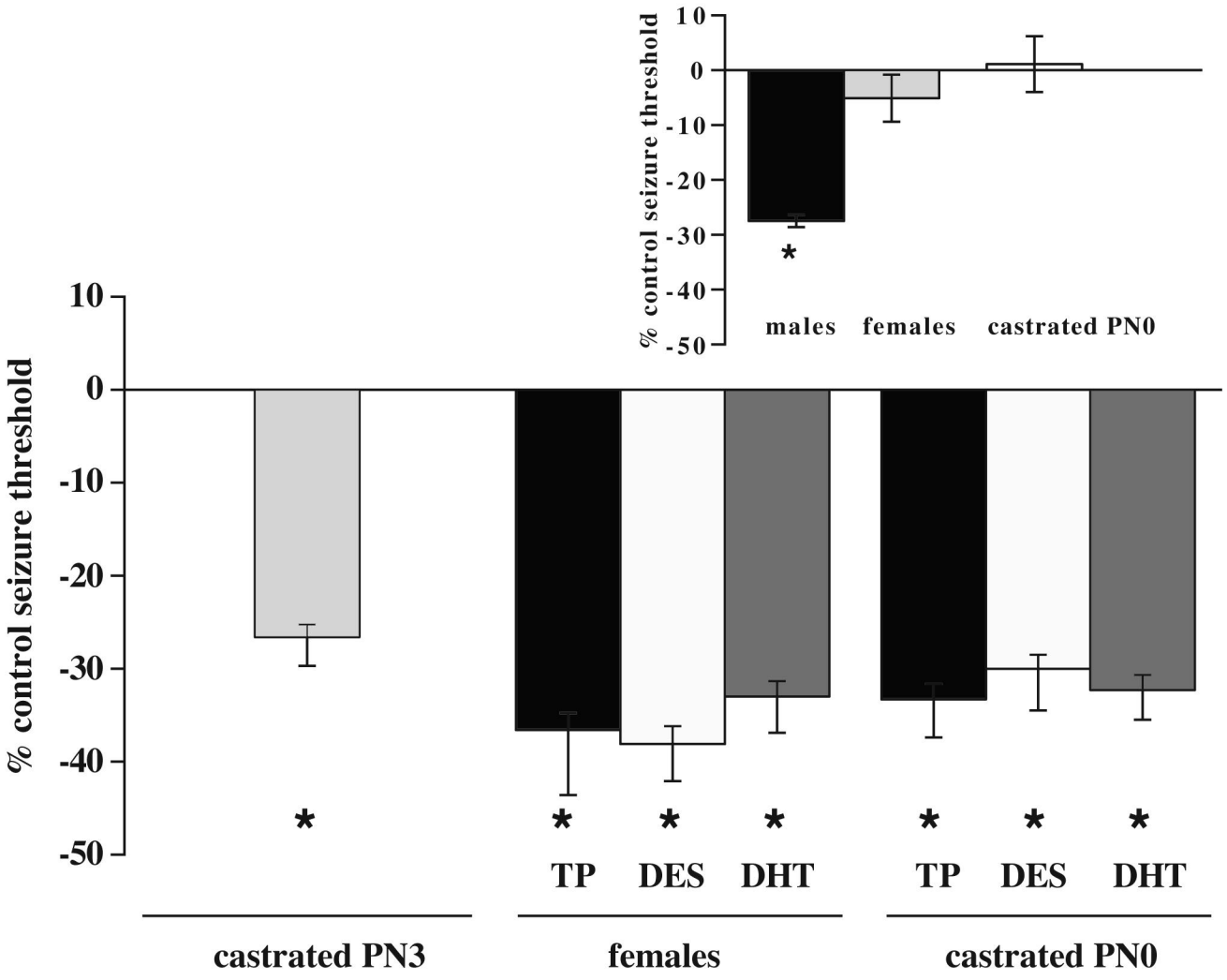

Figure 2.

Effects of administration of exogenous testosterone or its metabolites. The flurothyl seizure threshold was determined for the first clonic seizure in muscimol-infused (normalized to saline-infused) rats at PN15. Muscimol infusions in the SNR had proconvulsant effects in all groups compared to saline infused controls suggesting that exposure to testosterone or its metabolites during the early postnatal period (irrespective to the sex) is responsible for the male proconvulsant phenotype of seizure-controlling SNR effects. The groups included male rats with early life natural testosterone exposure (castrated at PN3; taken from Figure 1), females and males castrated at PN0, which were hormonally treated with TP, DES, or DHT between PN0-PN2.

Inset: For clarity, we included the already published data on SNR muscimol effects on flurothyl-induced seizures in intact males, females, and males castrated at PN0 (Velíšková and Moshé, 2001). These data show that SNR muscimol had a proconvulsant effect in intact males but not in intact females or castrated males at PN0.

An asterisk indicates significant differences ($P < 0.05$) from respective sex- and treatment specific saline controls.

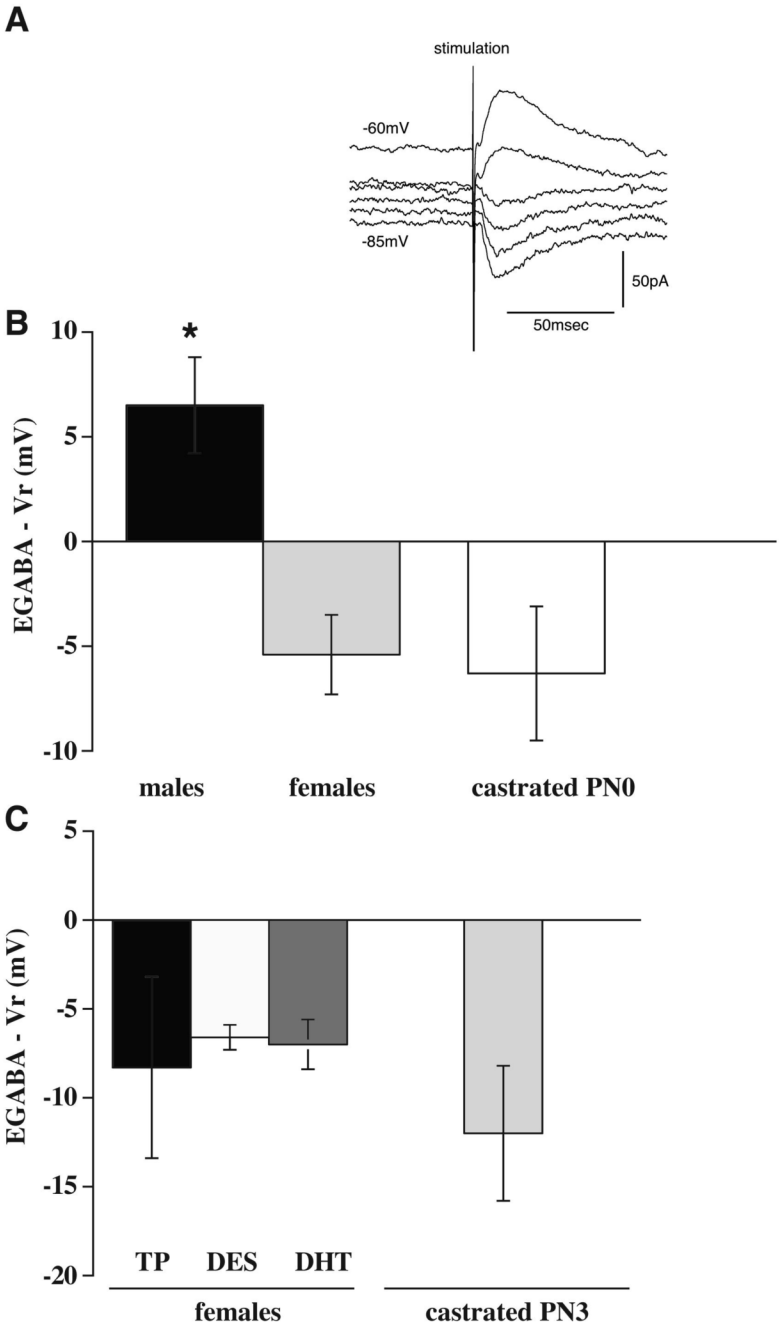

Figure 3.

***In vitro* perforated patch clamp recordings.** Changes in driving forces (EGABA -Vr; Vr = resting potential) of $GABA_A$ receptor mediated currents in SNR GABAergic neurons in slices from PN13-16 intact males, females, castrated males at PN0, females with TP, DES, or DHT administered at PN0-2 and males castrated at PN3. **A.** $GABA_A$ receptor mediated PSCs in a GABAergic neuron from the SNR of a male castrated at PN0. Raw traces of current are shown in voltage clamp mode, holding at a series of voltages (-85 to -60mV at intervals of 5mV). In this neuron, EGABA = -68mV. **B.** In intact males, the driving force is depolarizing (positive); in females or neonatally castrated males it is hyperpolarizing (negative; mean + SEM). **C.** In female rats treated with exogenous testosterone or its metabolites during PN0-2, the driving

force is hyperpolarizing. In PN3 castrated male rats, which were exposed to natural testosterone (and its metabolites) for three days, the driving force is also hyperpolarizing.

Table 1

Summary of the experimental design and hormonal treatments for both the *in vivo* and *in vitro* experiments.

Sex	Castration	Hormonal status			Testing in vivo	In vitro patch clamp
		Testosterone (natural or exogenous)	DES(exogenous)	DHT(exogenous)		
Male		Natural PN0-15			PN15*	PN13-16
Female					PN15*	PN13-16
Male	PN0				PN15*	PN13-16
Male	PN3	Natural PN0-3			PN15	PN13-16
Female		Exogenous daily PN0-2			PN15	PN13-16
Female			Daily PN0-2		PN15	PN13-16
Female				Daily PN0-2	PN15	PN13-16
Male	PN0	Exogenous daily PN0-2			PN15	NT
Male	PN0		Daily PN0-2		PN15	NT
Male	PN0			Daily PN0-2	PN15	NT

NT = not tested.

Asterisks represent the groups, which were previously reported (Velíšková and Moshé, 2001).

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Sex-dependent maturation of $GABA_A$ receptor-mediated synaptic events in rat substantia nigra reticulata

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Abstract

The substantia nigra pars reticulata (SNR) plays important roles in movement and, in an age- and sex-dependent manner, in seizure control. GABAergic synaptic transmission is critical in both normal development and seizures. In many neuronal types it is excitatory early in development and later switches to the mature hyperpolarizing type. We assessed the time course of the switch of $GABA_A$ receptor-mediated postsynaptic currents (PSCs) in anterior SNR neurons of male and female developing rats using the gramicidin perforated patch clamp technique. The switch occurred in males around postnatal day (PN) 17 and in females around PN10. This sex dimorphism may play a role in several other recognized sex differences in the development of SNR and in its regulatory role in seizures.

Keywords: Epilepsy; PSP; Postsynaptic potentials; Chloride; KCC2; Potassium–chloride cotransporter; Calcium

The substantia nigra pars reticulata (SNR) is a major output center of the basal ganglia and plays an important role in the control of axial posture and movement [1,8] as well as in saccadic eye movements in animals and humans (reviewed in [18]). Additionally, it exerts a regulatory role on seizures in animal models [7,20,27,29] and probably in humans [24].

A considerable degree of sexual dimorphism has been documented in the normal development of the SNR (reviewed in [41]), especially concerning the expression and signaling of $GABA_A$ receptors [5,11,12,14,25,31,42] and sex hormone receptors [15,32], chloride transport [14], and circling behavior [40]). Dimorphism is also present in the control of seizures [43].

One of the factors known to play an important role in development and likely to contribute to the dimorphism of the SNR is the GABAergic system and especially the electrical direction of the synaptic events it mediates [13]. Synaptic potentials mediated by $GABA_A$ receptors comprise the bulk of fast inhibitory transmission in the mature nervous system, but are usually depolarizing early in development due to high intracellular chloride concentration. In fact, in a developmental window when glutamatergic transmission is not yet established, GABA functions as the major excitatory system, eliciting giant depolarizing potentials with overriding action potentials [4,6,30,33]. Additionally, depolarization potently activates voltage-sensitive calcium channels, resulting in activation of a multitude of signaling cascades, including gene expression, critical in several aspects of neuronal differentiation and neuronal network construction (review [3]).

Maturation of chloride homeostasis and driving force towards hyperpolarization has been shown to depend on the chloride-extruding transporter potassium–chloride cotransporter 2 (KCC2) [19,36]. In most studied neurons, the $GABA_A$ receptor functional switch takes place prior to the third postnatal week, as assessed either directly by physiological methods in vitro ([14,19,21,36,44]; see also review [3]) or indirectly by KCC2 expression assessment [14,28,37,45]. Most, although not all, neuron types in the mature nervous system, including SNR

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GABAergic neurons [17], exhibit high KCC2 expression levels and inhibitory responses to GABA.

In the SNR, we have shown that the developmental increase in KCC2 occurs earlier in female than in male GABAergic neurons. Furthermore, applications of the $GABA_A$ receptor agonist muscimol on slices of postnatal day (PN) 14–16 rats induce depolarization in males (immature type response) and hyperpolarization in females (mature type response) [14]. We here test whether the sexual dimorphism applies to $GABA_A$ receptor mediated synaptic responses as well and we characterize the time course of male and female maturation.

We used 27 male PN 7–26 and 21 female PN 4–19 Sprague–Dawley rats born to mothers purchased from Taconic farms (NY) while pregnant. The day the litter was born was defined as PN0. Rats were kept in a 12 h dark/12 h light cycle with food and water ad libitum. All procedures and experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

After sacrifice under ketamine anesthesia, coronal slices of 300 μm thickness including the SNR were cut with a vibratome (OTS 4000, Electron Microscopy Sciences, Hatfield, PA). Dissection solution (mM): 210 sucrose; 3.5 KCl; 1 $CaCl_2$; 4 $MgCl_2$; 26 $NaHCO_3$; 1.25 NaH_2PO_4 and 10 D-glucose. Supplemented with ascorbic acid (100 μM), pyruvate (500 μM) and kynurenate (1 mM). We chose only slices containing the anterior (rostral) SNR because its neuronal population is essentially homogeneous, comprising immunohistochemically characterized GABAergic neurons, whereas in the posterior SNR a significant proportion of neurons are dopaminergic and display distinct electrophysiological properties [35].

For electrophysiology, slices were allowed to recover for at least 1 h in a chamber with artificial cerebrospinal fluid (ACSF), which contained (mM): 126 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4 and 10 D-glucose. Perforated whole cell patch clamp recordings from SNR neurons were performed with the cation-permeable ionophore gramicidin (0.5–1 μg/ml; DMSO 0.1%, v/v), to avoid alteration of intracellular chloride concentration by the electrode [10,22]. Electrode solution: 140 K gluconate, 6 NaCl, 4 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES, pH 7.3; 295 mosm. Junction potential (−14 mV) was electronically compensated. ACSF was supplemented with ascorbic acid (100 μM) and pyruvate (500 μM). All bath solutions were bubbled with 95% O_2/5% CO_2 gas mixture. Their pH was 7.3–7.4 and osmolarity 300–310 mosm. Experiments were performed at room temperature.

Acceptance criteria for recordings included resting potential (Vr) ≤ -55 mV and stable (fluctuation $< \pm 10\%$) input resistance. Since almost all neurons were not at rest but were spontaneously firing action potentials, Vr was taken as the mean value between the action potential threshold and the trough of the afterhyperpolarization.

To obtain postsynaptic currents (PSCs) generated by $GABA_A$ receptor activation, electrical pulses of 100 μs duration were delivered through a stainless steel bipolar stimulating electrode (Frederick Haer Co., Bowdoingham, ME) inserted in the slice at a distance of 200–400 μm from the recorded neuron, usually ventromedially to the neuron. 6-Cyano-7-nitroquinoxalene-2,3-dione (CNQX; 10 μM) and 2-amino-5-phosphonopentanoic acid (AP5; 50 μM) or kynurenic acid (1 mM) were present to block PSCs generated by synaptic glutamate release.

Voltage clamp measurements were made when series resistance had reached values less than 100 MΩ. Series resistance was partially compensated electronically by about 40%. To assess the reversal potential of PSCs, voltage steps were applied from a holding potential of −70 mV and stimulations were delivered at each voltage. In neurons where frequent action potential firing impeded PSC assessment, more positive holding potentials were used. A straight line was fitted to the *I–V* points and its intersection with zero current was taken as reversal potential.

The software pClamp (Axon Instruments/Molecular Devices, Sunnyvale, CA) was used for data acquisition and analysis. Kaleidagraph (Synergy Software, Reading, PA) was used for *I–V* linear regression and sigmoid curve least squares fitting. Data points were fitted with sigmoid curves using the equation

$$V = \frac{m1 + m2}{\{1 + \exp(-(\text{age} - m3)/m4)\}}$$

where $m1$, $m2$, $m3$ and $m4$ are numerical parameters whose optimal values are calculated by the fitting process.

A total of 54 neurons (30 from male and 24 from female animals) from 27 male and 21 female animals were included in the results.

The resting potentials and spike thresholds did not differ significantly depending on sex or age within the ages the experiments were performed. Vr were -62.8 ± 5.8 mV in males and -61.2 ± 4.7 mV in females (mean ± S.D.). All except 3 neurons exhibited spontaneous rhythmic firing. Firing rates in both sexes increased with age but did not show sex dependence for animals of the same age. Specifically, firing rates were <10 Hz at PN < 15 in 15 of 16 male and all of 19 female neurons; >10 Hz at PN ≥ 15 in 10 of 14 male and 4 of 5 female neurons.

In the presence of glutamate receptor antagonists, synaptic stimulation under voltage clamp evoked PSCs (Fig. 1A) whose reversal potential (Egaba) was determined by constructing *I–V* curves. The PSCs were invariably eliminated after the addition of the $GABA_A$ receptor antagonist bicuculline (50 μM), demonstrating that they were generated by $GABA_A$ receptors (not shown).

Scatter diagrams of the driving force Egaba–Vr as a function of age were constructed for neurons from both male and female animals (Fig. 1B and C). The average switch age (point where the sigmoid fits dissect the $\Delta V = 0$ axis) was PN 16.6 days for males and PN 9.8 days for females.

We here established the developmental time course of the switch of $GABA_A$ receptor-induced synaptic responses to hyperpolarization and showed that it takes place earlier in females than males. This is, to our knowledge, the first characterization of the ontogeny of sex-dependent developmental switch in chloride-dependent electrophysiology. Our experiments targeted a slightly earlier age range in females than males because our main goal was to obtain enough data on either side of the switch time for each sex, a time, which (as had been apparent from preliminary data) was earlier for females.

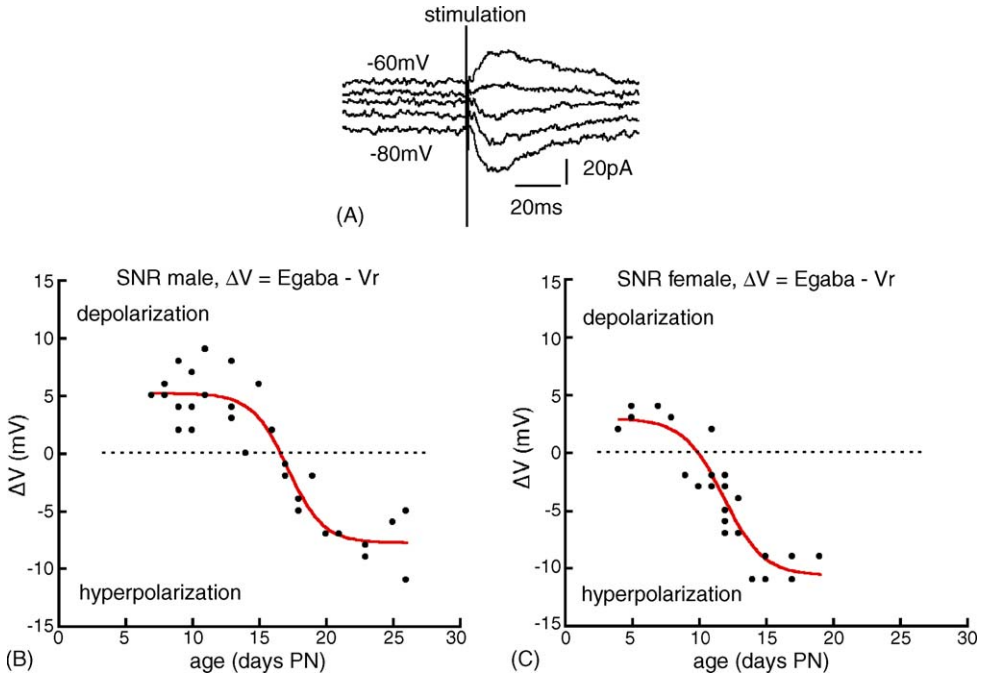

Fig. 1. (A) Raw traces of current in voltage clamp recording of an anterior SNR neuron. $GABA_A$ receptor-mediated PSCs at a series of voltages (−80 to −60 mV at intervals of 5 mV). In this neuron (from a female PN12 animal), Egaba = −67 mV. (B and C). Scatter diagrams of driving force (ΔV = Egaba − Vr) of (B) male and (C) female neurons as a function of age. Each point represents a neuron for which Egaba was obtained as in panel A. Positive values indicate depolarization from the resting potential. Data points were well fitted by sigmoid curves. The average switch age (point where the sigmoid fits dissect the $\Delta V = 0$ axis) was PN 16.6 for males and PN 9.8 for females.

The mechanisms causing this sex dimorphism are not well understood, but may depend on the different hormonal environment in male and female neonates, especially in regards to estrogen. We have shown that exogenous estrogen administration decreases KCC2 expression in SNR neurons exhibiting immature $GABA_A$ receptor physiology (excitatory responses) [15]. At birth, a systemic testosterone surge occurs in males, but not females [9]. This testosterone surge may lead, through local testosterone aromatization [26,34], to higher local levels of estrogens in the male SNR. Moreover, male neonates have more estrogen receptor alpha immunoreactivity in SNR neurons compared to females, which would render them possibly more sensitive to estrogens [32].

Another factor that may accelerate the $GABA_A$ receptor switch in females could be a stronger GABA-mediated excitatory synaptic activity. GABA-mediated depolarizations of immature neurons can accelerate the switch of its own effect from depolarizing to hyperpolarizing [16,23,38], probably by a mechanism involving depolarization-induced calcium influx and a subsequent increase in KCC2 mRNA expression [16]. Although this applies to both sexes during the neonatal period, PN15 female rat SNR neurons show more abundant mRNA for the alpha-1 subunit of $GABA_A$ receptors and GABA content compared to males [31], suggesting possible higher amplitude synaptic activity in females. If such a difference was present before the female switch period, it might, in part, account for the earlier switch in females.

The longer developmental window during which male SNR neurons are subjected to the excitatory and differentiating effects of GABA may affect the sex-specific development of the SNR and its role in physiological and pathological processes [13]. We have already shown that $GABA_A$-mediated excitation can lead to calcium entry and transcriptional regulation of genes [14]. Sexual dimorphism in the role of the SNR in seizure control has been documented in our laboratory. Bilateral infusions of muscimol in the SNR of male PN15 animals reduce the latency to onset of clonic seizures induced by flurothyl (proconvulsant effect). Similar infusions in females have no significant effect [43]. The underlying mechanisms are complex, but the GABA–chloride system maturation state is one of the likely candidates, in view of the important role it is known to play in development [3]. Lending further support to the role of this maturation in seizure control, genetically controlled reduction of KCC2 expression in mice leads to frequent generalized seizures and death shortly after birth [46]. Finally, besides a role in seizures, sexual dimorphism in SNR development may influence movement stereotypies that are present in cases of autism and may change as a function of age [39] and sex [2].

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