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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,

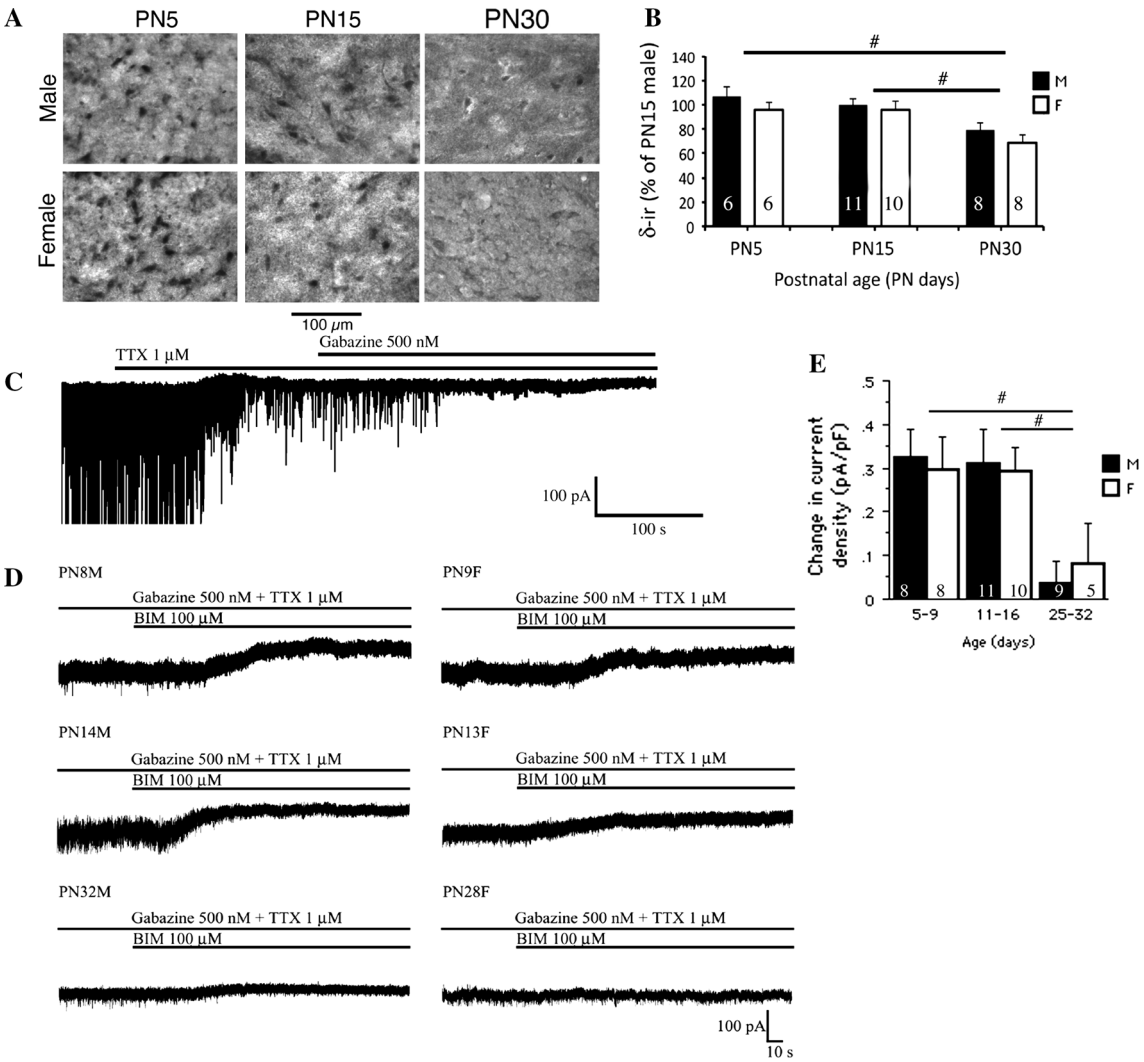

Fig. 1 Developmental changes in δ-ir and BIM-sensitive tonic currents in rat SNR_A neurons. **a** Representative photographs from SNR_A sections from male and female rats show strong δ-ir expression at PN5 and PN15 and significant reduction at PN30, without any sex differences. Significant reduction 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 [$F_{age}(2, 41) = 9.89$, $P < 0.05$; $F_{sex}(1, 41) = 9.78$, $P < 0.05$; $F_{age*sex}(2,$

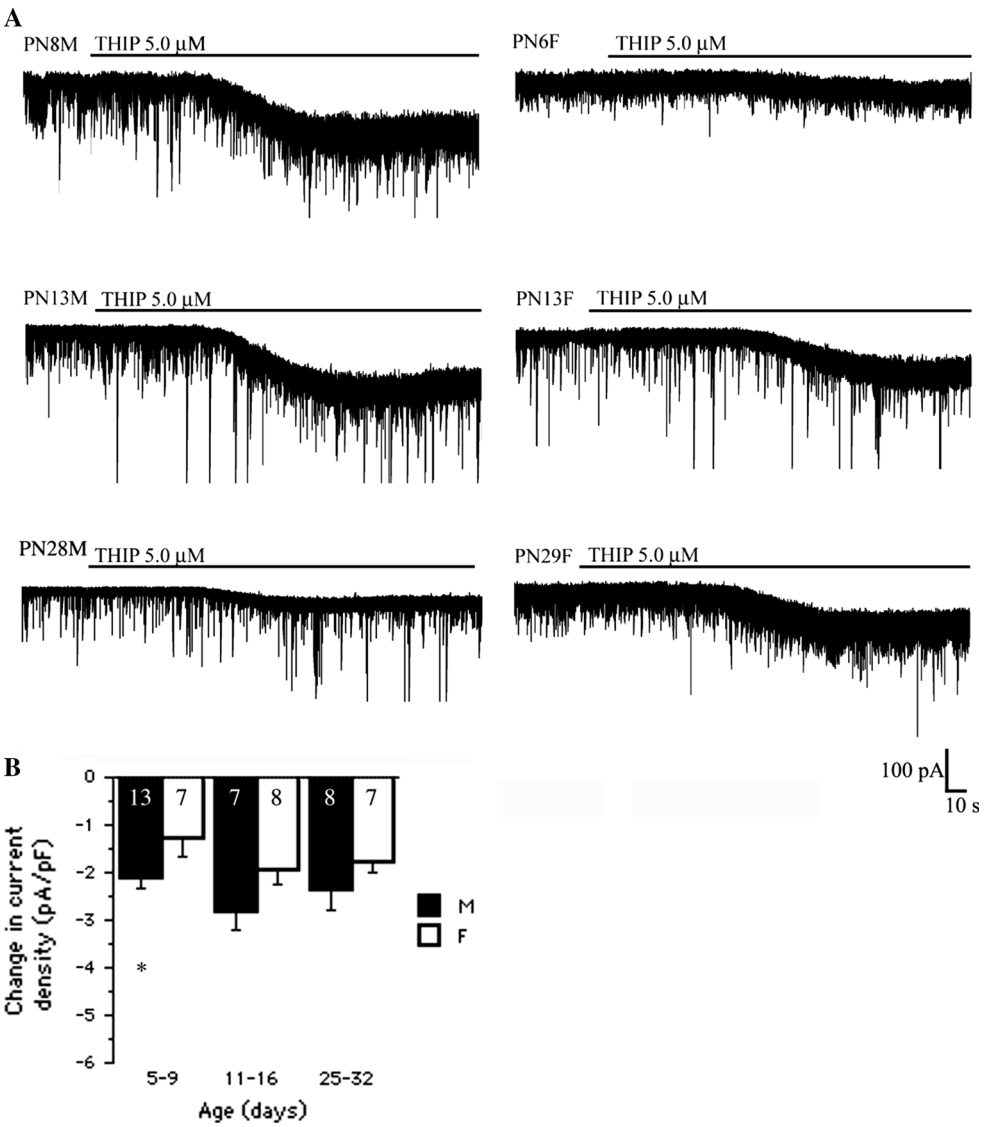

Fig. 2 Sex-specific changes in THIP-induced tonic current in rat SNR_A neurons. **a** Representative recordings demonstrating THIP (5 μM) induced changes in the tonic current in SNR_A GABAergic neurons in the presence of D-AP5 50 μM and CNQX 10 μM. **b** When the cell size was taken into consideration, 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

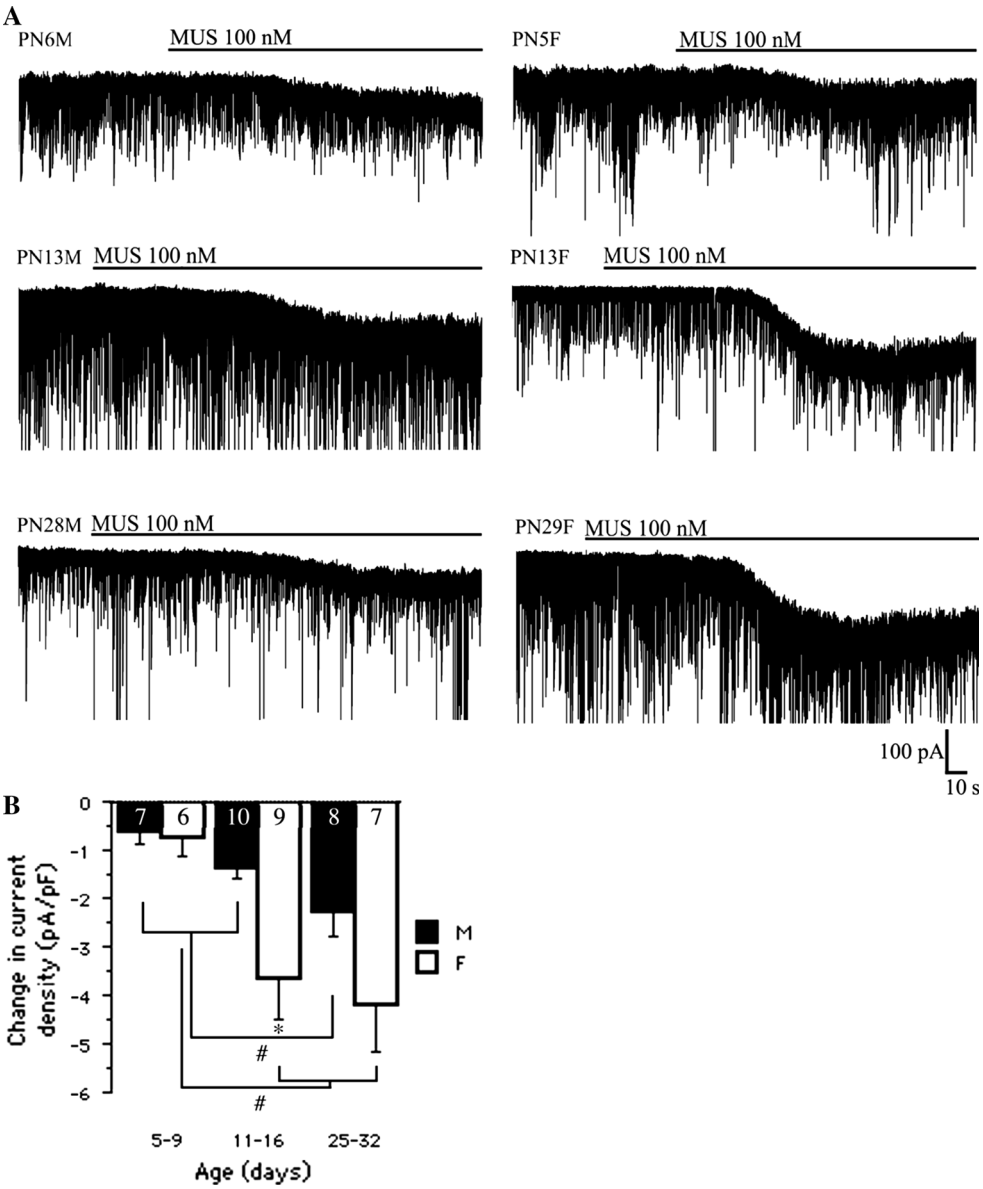

Fig. 3 Sex and age-specific changes in muscimol-induced tonic currents in rat SNR_A. **a** Representative traces demonstrating that muscimol 100 nM induced an inward current in all groups. Recordings were performed in the presence of D-AP5 50 μM and CNQX 10 μM. Note the larger deflection in PN13 and 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

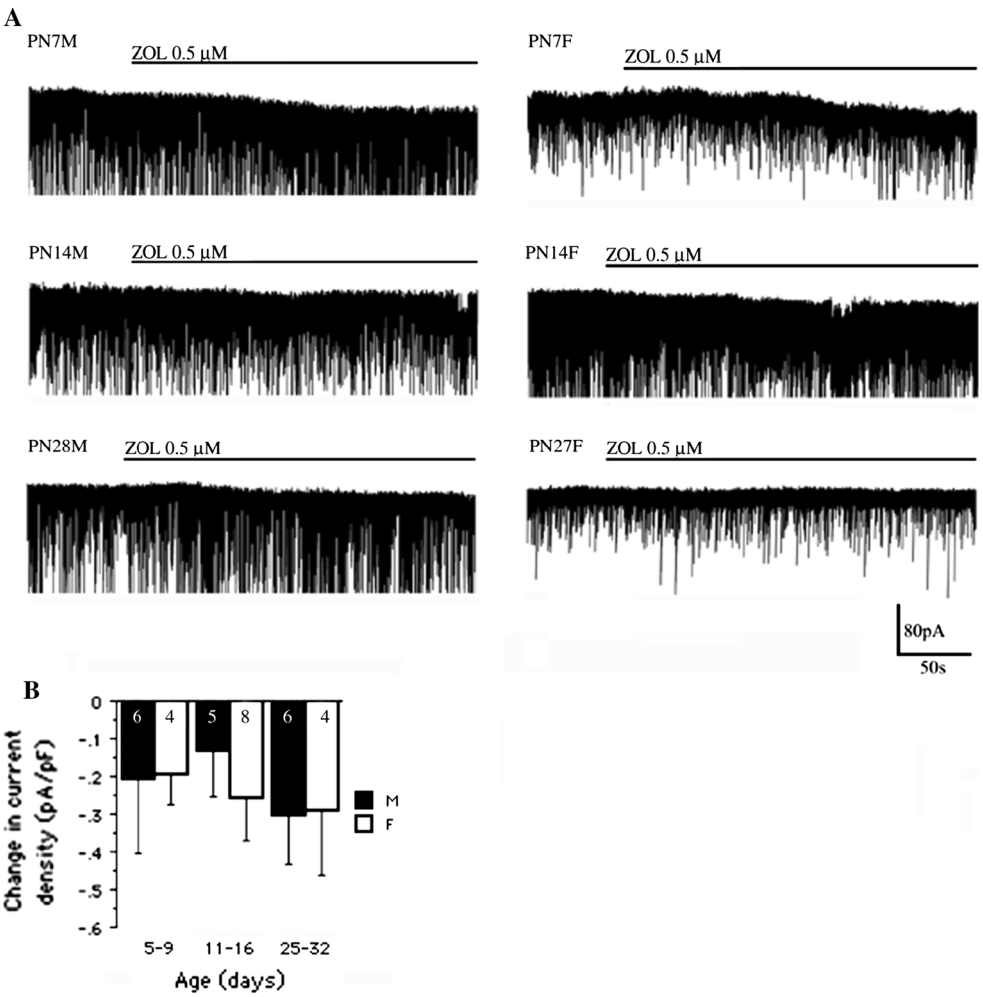

Fig. 4 Zolpidem-induced tonic responses in rat SNR_A neurons. **a** Representative traces show that zolpidem 0.5 μM gave rise to a small inward current in SNR_A neurons across all age groups in the presence of D-AP5 50 μM and CNQX 10 μM. **b** The zolpidem-induced changes in current density measurements 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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