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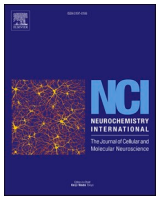

Differences in presynaptic hippocampal GABAergic terminals at the early stage of life in female and male mice: effect of an acute early inflammatory challenge

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ABSTRACT

GABA dictates the efficiency of synaptic connection, influencing its developmental complexity, but its role is tuned by developmental sex differences which affect the efficiency of its innervation. We investigated the efficiency of mechanisms of GABA storage and exocytosis in hippocampal terminals of male and female mice during the juvenile period (PND21), adolescence (PND36) or adulthood (PND90). The expression of mRNA encoding for the presynaptic GABA transporter type 1, (GAT1) and the vesicular GABA transporter (VGAT1) was analysed. A significant scaling-down in the GAT1 mRNA levels (SLC6A1) was detected at PND21 in both sexes until adulthood, while the SLC32A1-VGAT mRNA level was conserved. We also analysed the density of GAT1 and VGAT proteins. Western blot analysis unveiled the presence of a monomeric and an oligomeric form of GAT1. The density of the monomeric form was conserved at the different stages of development in both sexes. Differently, the oligomeric assembly was significantly overexpressed in hippocampal synaptosomal lysates from PND21 male and female mice, but recovered at PND36. VGAT density was largely conserved in PND21 and PND36 male hippocampal synaptosomal lysates when compared to adult particles, but significantly lower in PND21 female particles. Notably, these changes are consistent and support the altered vesicular storage of newly taken-up [^{3}H]GABA detected in PND21 male and female hippocampal synaptosomes as well as the different responsiveness of GABAergic male and female synaptosomes to increasing depolarizing stimuli (12, 20 and 30 mM KCl-enriched solutions) measured as efficiency of the [^{3}H]GABA exocytosis. Interstingly, an acute LPS treatment affects the efficiency of GABA exocytosis at PND36 in a sex-dependent manner. These results add new knowledge on the role of GABA as effector of central inhibitory plasticity at the early stage of development and its relevance in dimorphic adaptation in physio pathological conditions.

1. Introduction

Specific developmentally regulated molecular and biochemical processes occur in the rodent brain during the neonatal (postnatal day (PND)0–6), infant (PND7-14), juvenile (PND15-25), and adolescent (PND32-56) period (Semple et al., 2013). During this period, brain network undergoes a continuous re-modelling which depends on synaptic activity and contextual stimuli, and that primes the efficiency of central neuronal processes such as learning and memory, motor and executive skills as well as resilience to stress.

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Changes in these early events alter the efficiency of the neuronal communication, and, depending on their intensity and duration, could either strengthening synaptic connection, increasing the resilience to central insults, or weaking it, favouring pathological maladaptive responses. Understanding the mechanism(s) of control of synaptic communication at the early stages of life and how they respond to early noxious stimuli would help to predict the efficiency of synaptic plasticity in adults, also suggesting intervention to protect brain structures, and to preserve and even implement neuronal communication.

Most of the studies concerning age-dependent adaptations of neuronal circuits have focused on glutamate or, in general, onto the excitatory transmission, based on the fundamental role this amino acid plays in synaptic plasticity. Starting from the 2000, however, also the GABAergic innervation attracted interest (Chevaleyre and Castillo, 2003; Kullmann et al., 2012). Specifically, attention focusses on its ability to control the strength and the efficiency of the excitatory signal in most of the CNS regions, including the hippocampus, the frontal cortex, and the basal ganglia. It emerged that GABAergic tuning is highly plastic, to a level comparable to that of glutamate (Ji et al., 2024; Kullmann et al., 2012).

This conclusion added new vistas to the scenario and increased the complexity of the events involved in dictating central neuronal plasticity. New cellular and molecular events emerged that could be relevant to the control of neuronal circuitry and, GABA arised as a main player of synaptic communication (Sanes and Kotak, 2011).

The plastic role of GABA in controlling the efficiency of the synaptic connection depends on the developmental complexity of its innervation during lifespan. As a matter of fact, besides the shift from excitation to inhibition of neuronal $GABA_A$ receptor-mediated signalling at the late infantile stage (Akman et al., 2014; Ben-Ari et al., 2007; Vela et al., 2003), the efficiency of GABAergic signalling itself was hypothesized to change during early development (Akman et al., 2014; Davis et al., 1999), and to support significant adaptation in neonatal and in elder subjects (Bonfiglio et al., 2018; Cardoso et al., 2018; Taube et al., 2024). It was predicted that the efficiency of GABA exocytosis could be altered in juvenile-adolescent mice, because of an impaired expression of protein(s) that dictate GABA synthesis, i.e. the glutamate amino acid decarboxylase (GAD). Specifically, GAD was shown to be expressed in a sex-dependent manner during early development, around PND15, when its expression prevails in males when compared to females (Davis et al., 1999).GAD indirectly controls the efficiency of the vesicular storage of newly-synthetized GABA, then affecting the efficiency of GABA release and consequently the expression of GABA receptors (i.e. the $GABA_A$ and the $GABA_B$ receptors) located pre or postsynaptically (Behuet et al., 2019; Ryan et al., 2021; Tao et al., 2024).

These aspects are particularly relevant when considering that the efficiency of GABAergic transmission is tightly regulated by environmental stimuli and early pathological conditions as well, including, in the latter case, preterminal maternal immune-activation (Corradini et al., 2018), exposure to prenatal ((Maurer et al., 2025)and references therein) or juvenile stress (Albrecht et al., 2016) and even postnatal overfeeding (Amaro et al., 2023; Amaro et al., 2023). The impact of these conditions in most cases develops in a sex-dependent manner, determining therefore the individual variability in adulthood (Basu et al., 2020; Kotlinska et al., 2023; Santos-Silva et al., 2024).

To provide novel insights into the mechanisms subserving early neurodevelopmental sex-dependent changes in GABA transmission, the present study was dedicated to evaluating the efficiency of the storage and the exocytosis of GABA from hippocampal GABAergic nerve endings of both male and female mice during the juvenile period (PND21), adolescence (PND36) or adulthood (PND90). The expression of mRNA encoding for presynaptic proteins relevant to the efficiency of GABAergic transmission (the GABA transporter type 1, GAT1, (which has a preferential presynaptic distribution in the CNS (Ryan et al., 2021; Vitellaro-Zuccarello et al., 2003), and the vesicular GABA transporter (VGAT). The density of the respective proteins in hippocampal presynaptic nerve endings was also assessed in these animals and, functional studies were carried out to monitor the efficiency of GABA storage and exocytosis in the hippocampal synaptosomes (i.e. two events that strictky depend on GAT 1 and VGAT functioning).

Lastly, based on results in the literature showing that an early-life inflammation stimuli [acute lipopolysaccharide (LPS) injection] (Gomez et al., 2021) as well as a maternal immune activation (Harbi and Mouihate, 2025) alters the hippocampal excitatory synaptic transmission, we also asked whether acute LPS treatment could affect the efficiency of GABA exocytosis at PND36 and if changes occur in a sex-dependent manner.

2. Materials and methods

2.1. Animals and LPS treatment

Mice (male and female, strain C57BL/6J) were bred at the animal facility of the Department of Pharmacy, Section of Pharmacology and Toxicology, School of Medical and Pharmaceutical Sciences, University of Genoa (authorization n. 484 of 2004, June 8th) or at the Centro di Servizi Stabulario Interdipartimentale, University of Modena and Reggio Emilia. Animals were housed in polycarbonate cages (30 × 30 × 15 cm) with ad libitum access to food and water, under a 12/12 h light-dark cycle (lights on 8:00 a.m. to 8:00 p.m.), at room temperature of 21 ± 3 °C, with relative controlled humidity. Mice were handled once a week for habituation to the experimenters and checked daily for signs of discomfort [animal care and use guidelines "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council, 2003)]. The experimental procedures were in accordance with the European legislation (CEE, September 22, 2010, no. 2010/63/EU), the ARRIVE guidelines and they were approved by the Italian Ministry of Health (DDL 26/2014 with the approval of the local Ethical Committee; University of Genoa authorization n° 75F11.N.JP6 and University of Modena and Reggio Emilia, authorization n. 33220. 33.ext.39). In line with the 3Rs rules (replacement, refinement, and reduction), any effort was made to reduce the number of animals to obtain statistically reliable results.

Juvenile (PND21), adolescent (PND36), and adult (PND90) male and female animals were sacrificed, brains removed, and hippocampi were dissected and processed for further analyses (n = 56). A subset of adolescent animals (PND36) was injected i.p. with saline or 100 μg/kg (Escherichia coli, serotype 0127:B8; MerckKGaA, Germany) and sacrificed 6 h after treatment (n = 31).

This dosage has been shown to cause mild sickness for approximately 4.5 h in juvenile/adolescent rats and to induce long-term behavioural changes (Spencer et al., 2006a, 2006b, 2011) moreover in juvenile and adolescent mice it has been shown to induce a significant increase in hippocampal transcription of proinflammatory cytokines and markers of microglia activation that peaked between 3 and 6-h post injection and returned to basal level after 24 h ((Dinel et al., 2014); Benatti et al., in preparation). We therefore selected 6h as an informative time point to capture these transcriptional changes.

2.2. RNA extraction and RT-qPCR

After sacrifice a single hippocampus was dissected and immediately stored at −80 °C. Total RNA was then extracted using Gen Elute™ Mammalian Total RNA Miniprep Kit and DNASE 70-on Column DNase I Digestion Set (MerckKGaA, Germany). One microgram of total RNA was reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, MA) and RT-qPCR was performed in CFX Opus Real-Time PCR machine (Bio-Rad Laboratories, CA), using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, CA) and specific forward and reverse primers at a final concentration of 300 nM (Table 1). Cycling conditions followed protocols as previously described (Ciani et al., 2024; DOI: 10.2174/

Table 1
Nucleotide sequence of the forward and reverse primers used for Real-Time PCR.

Target name	Primer Sequence (5' -3')	Gene Bank Number
SLC6A1-GAT1	Fw: GCAATCGCCGTGAACTCTTC	NM_178703.5
	RV: AACCAGGAACAGCAAGCTCA	
SLC32A1-VGAT	Fw: GGGTCACGACAAACCCAAGA	NM_009508.3
	Rv: GAGGAACAACCCCAGGTAGC	
RPS29	Fw: TGAAGGCAAGATGGGTCAC	NM_009093.3
	Rv: GCACATGTTCAGCCCGTATT	
CypA	Fw: AGCATACAGGTCCTGGCATC	NM_008907.2
SLC6A1-GAT1	Fw: GCAATCGCCGTGAACTCTTC	NM_178703.5
	RV: AACCAGGAACAGCAAGCTCA	
SLC32A1-VGAT	Fw: GGGTCACGACAAACCCAAGA	NM_009508.3
	Rv: GAGGAACAACCCCAGGTAGC	
RPS29	Fw: TGAAGGCAAGATGGGTCAC	NM_009093.3
	Rv: GCACATGTTCAGCCCGTATT	
CypA	Fw: AGCATACAGGTCCTGGCATC	NM_008907.2

1570159X22666240705143649). Cycle threshold (Ct) values were determined using CFX Maestro™ Software (Bio-Rad). Specificity was confirmed via dissociation curve analysis and electrophoresis on a 2 % agarose gel. For normalization, two reference genes—cyclophilin A (CYPA) and ribosomal protein S29 (Rps29)—were evaluated for expression stability using NormFinder® (https://moma.dk/normfinder-software), which considers both intra- and intergroup variation. The geometric mean of their Ct values was used for normalization. Gene expression was quantified using the $2^{-}\Delta\Delta Ct$ method, as calibrators were used males PND21 for untreated animals (Figs. 1 and 2) or males PND36 saline for LPS exposed animals (Fig. 4A and B).

2.3. Isolation of synaptosomes

After sacrifice a single hippocampus was rapidly frozen in 0.32 M sucrose, buffered to pH 7.4 with Tris-(hydroxymethyl)-amino methane [Tris, final concentration 0.01 M]. Purified isolated nerve endings (synaptosomes) were then isolated from the hippocampus of male and female mice at 21, 36 and PND90 mice using a glass/Teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged at 1000×*g* for 5 min to remove nuclei and cellular debris, and the supernatant was stratified on a discontinuous Percoll® gradient (2 %, 6 %, 10 % and 20 % v/v in Tris-buffered sucrose (Dunkley et al., 1988, 2008, 1988; Pittaluga, 2019), and centrifuged at 33,500×*g* for 6 min. The layers between 10 % and 20 % Percoll® (synaptosomal fraction) were collected and washed by centrifugation at 19,000 g for 15 min.

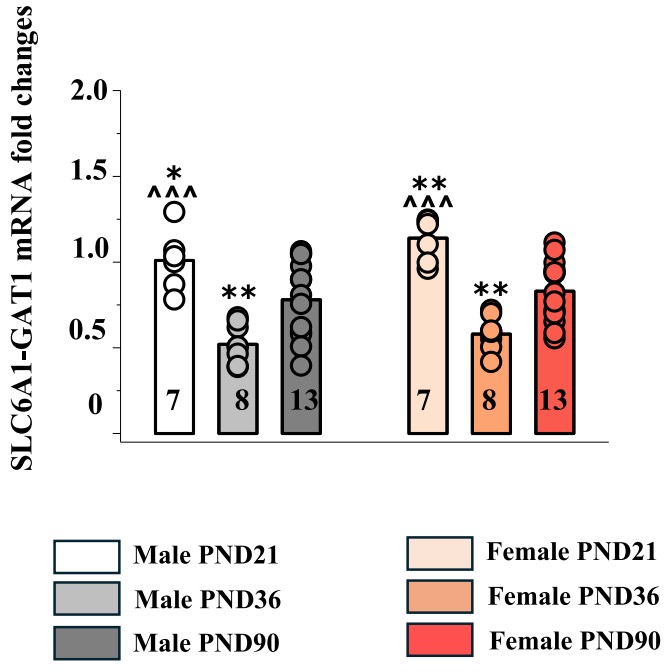

Fig. 1. SLC6A1-GAT1 expression and GABA uptake in female and male juvenile (PND21), adolescent (PND36) and adult (PND90) hippocampus. SLC6A1-GAT1 mRNA levels in the hippocampus of male and female mice at PND21, PND36 and PND90. Data are expressed as mean ± SEM (Standard Error of the Mean); the number of replicates is reported within each bar. Statistical analysis was performed by two-way ANOVA (sex*age) followed by Bonferroni: Main effect of age: *$p < 0.05$ vs respective PND90 mouse; **$p < 0.001$ vs respective PND90 mouse.^^^ $p < 0.001$ vs respective PND36 mouse.

2.4. ^{3}[H]GABA storage in hippocampal nerve endings

Hippocampal synaptosomes from male and female mice at PND21, 36 and 90 were resuspended in a physiological medium having the following composition (mM): NaCl, 140; KCl, 3; MgSO4, 1.2; CaCl2, 1.2; NaH2PO4, 1.2; NaHCO3, 5; HEPES, 10; glucose, 10; pH 7.4 and were incubated with [^{3}H]γ-aminobutyric acid (^{3}[H]GABA, final concentration 3 nM; NET191250MC, PerkinElmer) in the presence of 50 μM Aminooxy acetic Acid (AOA, C13408-1G, Sigma-Merck, Milano, Italy) to prevent GABA metabolism for 2 min at 37 °C in a rotating water bath. The synaptosomal suspensions were then quickly filtered and filters were counted for radioactivity. Unspecific labelling was quantified by incubating synaptosomes with ^{3}[H]GABA as above at 4 °C. Unspecific labelling was subtracted to the tritium content of synaptosomes incubated at 37 °C. The protein content of the synaptosomal suspension was quantified with Pierce™ BCA Protein Assay Kit (Thermo Scientific, Cat# 23225, Waltham, MA, USA). Results are expressed as nCi/mg protein.

2.5. Superfusion experiments

The release experiments were carried out with an experimental approach, the "superfusion of a thin layer of synaptosomes" (Olivero et al., 2019; Raiteri et al., 1974), which is recognized as a method of choice to study the mechanism of control of transmitter release. The main feature of this technique is the continuous up-down superfusion of a monolayer of synaptosomes, which minimizes autocrine/paracrine effects elicited by endogenous compounds (which are rapidly removed). Differently, the ligand(s) exogenously added to the superfusion medium can efficiently access the targeted receptors, modulating their activity. This would indirectly imply that the endogenous GABA released upon exposure of synaptosomes to a depolarizing stimulus cannot modify transmitter exocytosis.

Synaptosomes were resuspended in a physiological medium having the following composition (mM): NaCl, 140; KCl, 3; MgSO4, 1.2; CaCl2, 1.2; NaH2PO4, 1.2; NaHCO3, 5; HEPES, 10; glucose, 10; pH 7.4. They were then incubated for 15 min at 37 °C in a rotating water bath in the presence of ^{3}[H]GABA (final concentration 30 nM) in the presence of 50 μM aminooxi-acetic acdi (AOA) to prevent GABA metabolism. Identical aliquots of the synaptosomal suspensions were stratified on microporous filters at the bottom of parallel chambers in a Superfusion System (Ugo Basile, Comerio, Varese, Italy) and kept at 37 °C. Particles were then superfused with a standard physiological solution at 0.5 mL/min. After 39 min of superfusion to equilibrate the system, synaptosomes were transiently exposed (90 s) to a KCl-enriched medium (i.e. 12, 20 or 30 mM KCl-containing medium as indicated).

Four superfusate fractions [two 3-min fractions (basal release), one before (t = 36–39 min; b1) and one after (t = 45–48 min; b4) two 3-min fraction (t = 39–42 and 42–45 min; evoked release; b2 and b3)] were collected. The fractions and the superfused particles were then counted for radioactivity. The amount of radioactivity released into each fraction was expressed as a percentage of the total radioactivity. The KCl-evoked overflow was estimated by subtracting the neurotransmitter content in the basal fractions (b1 and b4) from that in the b2 and b3 fractions.

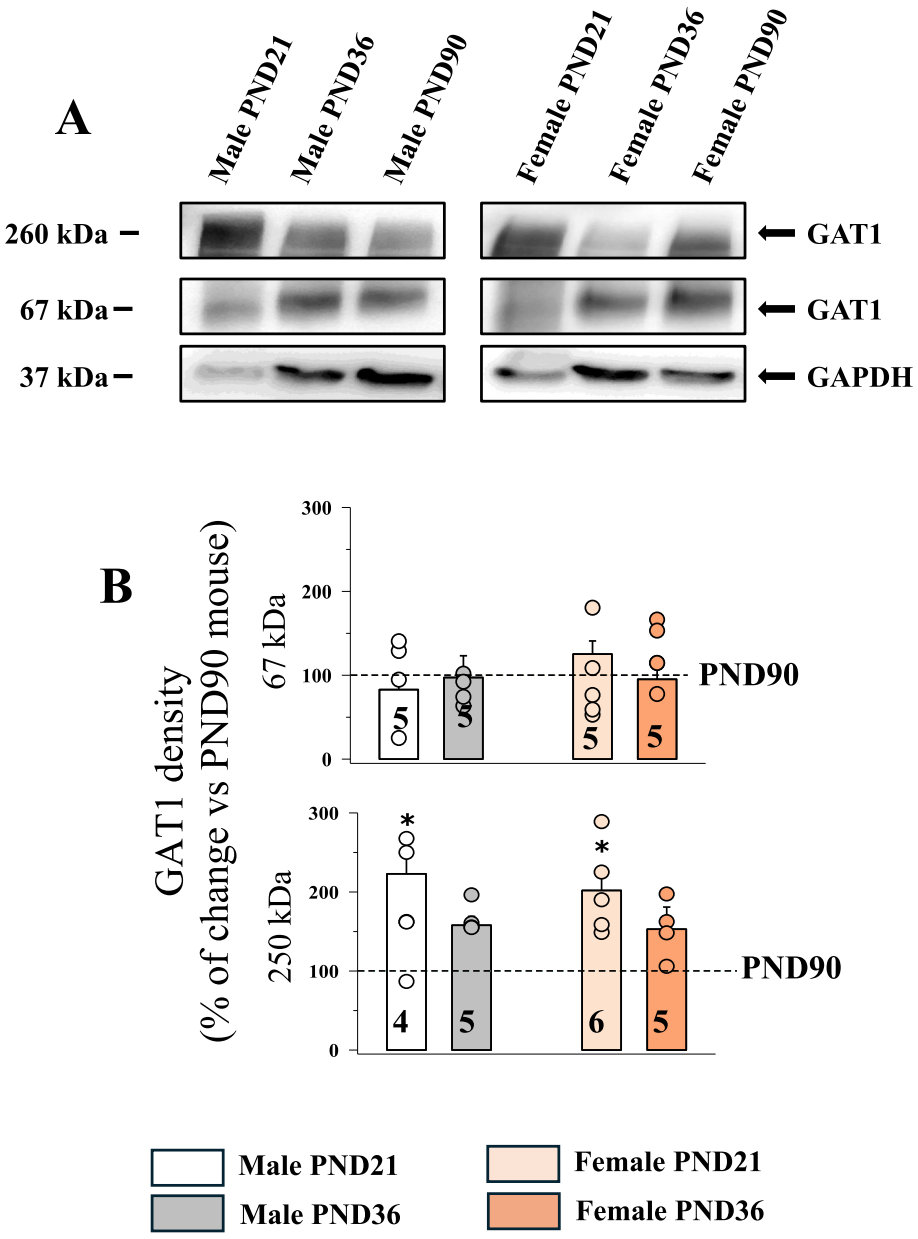

Fig. 2. GABA transporter type 1 (GAT1) density in hippocampal synaptosomes of male and female juvenile (PND21), adolescent (PND36) and adult (PND90) mice. **A**: representative Western Blot analysis of synaptosomal lysates from male and female mice at different PND days. **B**: the density of GAT1 immunostainings at 67 kDa and 260 kDa was calculated as GAT1/GAPDH ratio and changes in protein density were expressed as percentage of the GAT1/GAPDH ratio in adult animals (horizontal dashed line). Data were obtained in different experiments carried out on different days and are expressed as mean ± SEM; the number of replicates is reported within each bar. Statistical analysis was performed by one-way ANOVA followed by Tuckey test. *p < 0.05 vs respective PND90 mouse.

2.6. Western blot analysis

Hippocampal synaptosomes from male and female mice at PND21, 36 and 90 were lysed in modified RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1 % SDS, 1 % Triton X-100, protease inhibitors), sonicated, and centrifuged at 20,000 g for 10 min at 4 °C and the supernatant was kept for the immunoblot analysis. The protein content was quantified by using the colorimetric Pierce™ BCA Protein Assay Kit (Thermo Scientific, Cat# 23225, Waltham, MA, USA).

Samples were boiled for 5 min at 95 °C in SDS-PAGE sample buffer, separated by SDS-4-10 % PAGE (30 μg/lane) and blotted onto PVDF membrane. Membranes were blocked for 1 h at room temperature in Tris-buffered saline-Tween (t-TBS: 20 mM Tris, pH 7.4, 150 mM NaCl, and 0.05 % Tween 20), containing 5 % (w/v) non-fat dried milk, and then probed overnight at 4 °C with rabbit anti-GABA Transporter 1/GAT 1 (1: 15000, Abcam Cat# ab185205, RRID: AB_2889907), with VGAT (1:500, Invitrogen, Cat# PA5-27569, RRID: AB_2545045) or with anti-GAPDH (1:7000; Abcam, Cat # ab181602; RRID: AB_2630358). After washes in t-TBS, membranes were incubated for 1 h at room temperature with the proper horseradish peroxidase-linked secondary antibodies: anti-mouse (Sigma-Aldrich Cat# A9044, RRID: AB_258431); anti-rabbit (Sigma-Aldrich Cat# A9169, RRID:AB_258434). Immunoblots were visualized with an enhanced chemiluminescence Western blotting detection system Immobilon Forte Western HRP substrate (Sigma-Merck, Cat# WBLUF0500, Darmstadt, Germany). Images were acquired using the Alliance LD6 images capture system (Uvitec, Cambridge, UK) and analysed with UVI-1D software (Uvitec).

2.7. Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2022). Data represents the mean ± SEM of n independent values (n indicated in the figure legends), which correspond to the number of the animals used within each experiment. The sample size amounts at least at n = 5 in each group. Sigma Plot 15 data analysis and graphing software package was used for data handling/statistics and graph drawing. Analysis of variance was performed by ANOVA followed by multiple comparison tests (Tukey's or Dunnett's multiple-comparisons test, as appropriate); direct comparisons were performed by Student's t-test. Post hoc tests were done only if the F value was significant and there was no significant variance in homogeneity. Data were considered significant if $p < 0.05$.

Gene expression analysis: Main effects and interactions between the factors were analysed with a two-way ANOVA followed by post hoc contrasts of estimated marginal means, employing Bonferroni's correction implemented in SPSS (with a significance level set at $p < 0.05$). All statistical analyses were performed using SPSS software version 29 (IBM Corp., Armonk, NY, United States) and GraphPad Prism 10 (GraphPad Software Inc., La Jolla, CA, USA). Extreme outliers were excluded prior to statistical analysis using the boxplot tool in SPSS (more than 3x the interquartile range outside of the end of the interquartile box).

3. Results

SLC6A1-GAT1 mRNA hippocampal expression was analysed at different stages of development in male and female mice. Two-way ANOVA (age x sex) revealed a main effect of age [$F(2; 55) = 35.694$; $p < 0.0001$]. SLC6A1-GAT1 mRNA levels were significantly decreased in adolescent (PND36; n = 8 for both males and females) mice with respect to juvenile (PND21; n = 7 for both males and females) animals, irrespective of the biological sex (-48.07 ± 3.97 % in PND36 male with respect to PND21 ones; - 54.31 ± 3.45 % in PND36 female with respect to the PND21 ones ($p < 0.0001$)). SLC6A1-GAT1 expression slightly, although significantly, increased in adult (PND90; n = 13 for both males and females) animals with respect to PND36, while still being significantly lower than PND21 mice (Fig. 1). Notably, developmental changes were comparable in male and female mice. Specifically, the mRNA levels in adult male mice increased by 25.43 ± 3.97 % with respect to the PND36 males but were inferior by 22.65 ± 6.04 % with respect to PND21 males. Similarly, the mRNA level in PND90 mice increased by 25.43 ± 3.45 % with respect to the PND36 females but was reduced by 30.75 ± 4.58 % with respect to PND21 females.

The SLC6A1-GAT1 mRNA content regulates the expression of the GABA transporter type 1 The density of GAT1 protein was quantified in lysates of hippocampal synaptosomes from juvenile (PND21), adolescent (PND36), and adult (PND90) male and female mice. Western blot analysis confirmed the presence of an immunopositivity in hippocampal lysates (Fig. 2A), having an appropriate mass (~67 kDa) consistent with the presence of the monomeric form of GAT1. The analysis also unveiled the presence of GAT1-immunopositivity at around 260 kDa, that we propose could suggest the presence of oligomeric association of the transporter protein (i.e. dimers of dimers (Korkhov et al., 2004; Moss et al., 2009),). GAPDH was used as an internal loading control. The density of GAT1 protein was normalized over that of GAPDH and the GAT1/GAPDH ratio in synaptosomes isolated from the hippocampus of juvenile and adolescent mice was compared to the GAT1/GAPDH ratio of the corresponding male or female adult mice (Fig. 2B). Changes in juvenile and adolescent mice were expressed as percent change with respect to the adult animals. The analysis unveiled that the GAT1 density in the hippocampal synaptosomal lysates of juvenile and adolescent male and female mice at 67 kDa was largely conserved with respect to the adult ones (Fig. 2B); [male mice, $F(2, 12) = 0.19$; *n.s.*; female mice: $F(2, 12) = 0.57$; *n.s.*]. Differently, the GAT1 immunopositivity at 260 kDa was significantly more evident in hippocampal synaptosomal lysates from male and female juvenile animals when compared to the adult ones (Fig. 2A and B; PND21 vs PND90 male mice, $F(2; 13) = 3.80$, $p < 0.05$.; PND21 vs PND90 female mice: $F(2; 10) = 4.37$, $p < 0.05$). This increase, although not statistically significant, was observed in adolescent male and female mice with respect to their adult counterparts as well.

To verify the functional activity of the transporter, synaptosomes were isolated from the hippocampus of male and female mice at the different stages of development under study (PND21, PND36 and PND90) and the amount of [^{3}H]GABA taken up upon exposure of synaptosomes to a non-saturating concentration of the tritiated ligand (3 nM) was quantified (Fig. 3). The efficiency of [^{3}H]GABA uptake varied during development [$F(5,86) = 21.62$]. Specifically, [^{3}H]GABA taken up by hippocampal synaptosomes from PND21 male and female mice, was significatively higher than the respective tritium stored within the PND90 synaptosomal preparations and it was comparable in both sexes. The efficiency of [^{3}H]GABA uptake, decreased significantly [$F(5, 86) = 21,621$; $p < 0.001$] during development as indicated by the finding that the [^{3}H]GABA taken up by male PND36 hippocampal synaptosomes was significantly lower than that stored in PND21 male synaptosomes (male: 28.31 ± 2.08 %, n = 18, $p < 0.05$) and that the tritium taken up by PND36 female synaptosomes was significantly lower than that in PND21 female synaptosomes (female: 26.88 ± 3.11 %, %, n = 19, $p < 0.05$). [^{3}H]GABA storage slightly, but significantly, recovered in female PND90 hippocampal synaptosomes, when compared to the PND36 females, but not in PND90 male hippocampal particles (male: 24.99 ± 2.03 %, n = 14, $p < 0.05$; female: 14.55 ± 2.94 %, n = 17, $p < 0.05$)

The [^{3}H]GABA taken up in nerve terminals would preferentially accumulate into the readily releasable vesicular pool to be released upon exposure of synaptosomes to a mild depolarizing stimulus. Vesicular storage of GABA depends on the vesicular GABA transporter (VGAT), which is encoded by the SLC32A1-VGAT mRNA. Differently from the SLC6A1-GAT1 mRNA, the SLC32A1-VGAT expression was largely conserved in male and female during development (Fig. 4). The results allow us to conclude that this protein may not represent a limitative step in the compartmentalization of GABA in nerve terminals during the early stages of development.

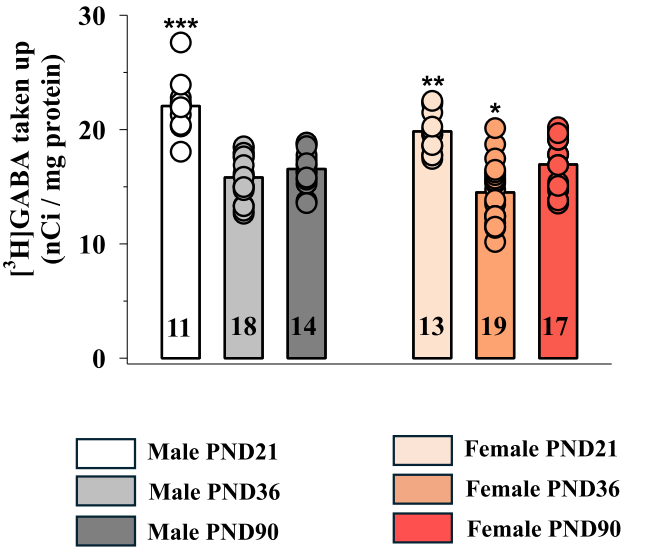

Fig. 3. Uptake of [^{3}H]GABA in hippocampal synaptosomes from juvenile (PND21), adolescent (PND36) and adult (PND90) mice. Data are expressed as mean ± SEM; the number of replicates is reported within each bar. Statistical analysis was performed by one-way ANOVA followed by Tuckey test; *$p < 0.05$ vs respective PND90 mouse; **$p < 0.01$ vs respective PND90 mouse; ***$p < 0.001$ vs respective PND90 mouse.

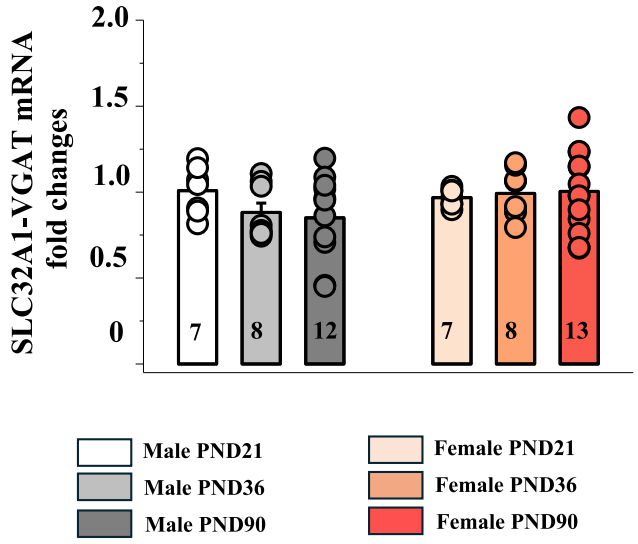

Fig. 4. SLC32A1-VGAT expression and [^{3}H]GABA release from female and male juvenile (PND21), adolescent (PND36) and adult (PND90) hippocampus. **A**: SLC32A1-VGAT mRNA in the hippocampus of male and female mice at PND21, PND36 and PND90. Real-time PCR was performed using specific primers and by applying $2^{-\Delta\Delta Ct}$ with RPS29 and CypA as housekeeping genes. Data were expressed as mean ± SEM; the number of replicates is reported within each bar. Statistical analysis was performed by two-way ANOVA followed by Bonferroni.

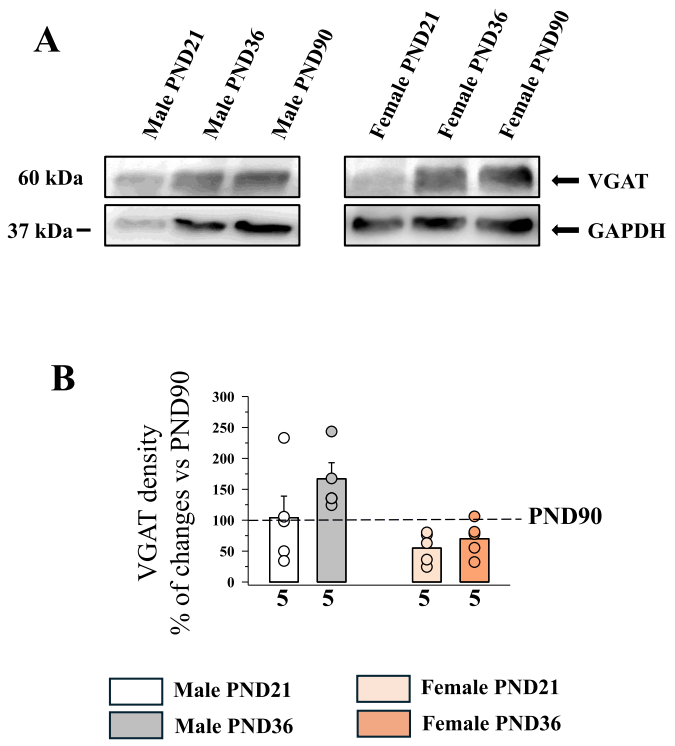

Fig. 5. Vesicular GABA transporter (VGAT) density in hippocampal synaptosomes from male and female juvenile (PND21), adolescent (PND36) and adult (PND90) mice. **A**: representative Western Blot analysis of synaptosomal lysates from male and female mice at different PND days. **B**: the density of VGAT immunostainings at 60 kDa was calculated as VGAT/GAPDH ratio and changes in protein density were expressed as already described (legend to Fig. 2). Data are expressed as mean ± SEM; the number of replicates is reported below each bar. Statistical analysis was performed by one-way ANOVA followed by Tuckey test Results represent the mean ± SEM of 6 determinations obtained in different experiments carried out on different days.' *p < 0.05 vs respective PND90 mouse.

Western blot analysis was then carried out to assess the VAGT density in the hippocampal synaptosomal lysates of mice of both sexes. Western blot analyses were carried out as already describe by using a specific antibody recognizing protein(s) having a mass (60 kDa) consistent with that of the VGAT protein. Again, GAPDH was used as an internal loading control, the density of VGAT protein was normalized over that of GAPDH and the VAGT/GAPDH ratio in synaptosomes isolated from the hippocampus of juvenile and adolescent mice was compared to the VGAT/GAPDH ratio of the corresponding male or female adult mice (Fig. 5B)-The results unveiled that the immunopositivity in the lysates from PND20 mice was largely conserved when compared to adult males, and that in PND36 hippocampal particles slightly, but not significantly increased [F2,9 = . 1.75; n.s.]. Differently, VGAT immunopositivity in the hippocampal synaptosomal lysates from PND21 females was significantly lower than that in adult female hippocampal synaptosomes [F2,9 = . 5.53; $p < 0.05$] but largely comparable to that in synaptosomal lysates from PND36 females (Fig. 5A and B).

Experiments were then carried out to quantify the impact of sex and age on the release of preloaded [^{3}H]GABA from hippocampal synaptosomes. Specifically, we analysed the release of the tritiated transmitter from superfused synaptosomes isolated from PND21, PND36 and PND90 male (Fig. 6A) and female (Fig. 6B) mice. Tritium overflow was elicited by exposing transiently synaptosomes in superfusion to a mild depolarizing stimulus (12 mM KCl-enriched solution for 90 s starting from t = 39 min of superfusion, red bar in Fig. 6A and B). We detected significant changes in the amount of the tritium release that emerged upon exposure of synaptosomes to the high-KCl solution. in the b2/b3 collected fractions (PND36: F(3, 18) = 33.09; $p < 0.001$; PND90: F(3, 20) = 18.33; $p < 0.001$) as well as in the b4 (i.e. the last fraction collected) (Fig. 6A, b4 fraction; PND21: F(3, 16) = 11.15; $p < 0.05$). The release of preloaded [^{3}H]GABA also was significantly modified upon exposure of particles to the depolarizing stimulus in hippocampal synaptosomes from female PND90 (Fig. 6B, b2 and b3 fractions, [F(3, 20) = 28.81; $p < 0.001$] but not in superfused particles from PND21 and PND36 female mice. In both male and female hippocampal synaptosomes, independently on the age, the amount of [^{3}H]GABA released in the fourth fraction collected is significantly lower than that in the first fraction (Fig. 6A and B) because of the continuous depletion of the internal tritium store in superfused synaptosomes. Actually, the continuous superfusion of the synaptosomes layered at the bottom of the superfusion chambers prevents any indirect effect including the reuptake of the released compound (i.e. [^{3}H]GABA (Pittaluga, 2019; Raiteri et al., 1974),).

The responsiveness of hippocampal synaptosomes PND21, PND36 and adult male and female mice to the 12 mM KCl stimulus was also analysed by comparing the total tritium overflow (i.e. the tritium release over the basal release in the b2 and b3 fractions, Fig. 6 C) from the different synaptosomal preparations. Accordingly to the results in Fig. 6A, the amount of tritium released upon exposure of male hippocampal synaptosomes to 12 mM KCl-containing medium increased arithmetically during development, being significantly low in hippocampal synaptosomes from juvenile males when compared to adult male mice [F(5, 28) = 17,97; $p < 0.001$] (Fig. 6C). Differently, the 12 mM KCl-evoked [^{3}H]GABA release from female hippocampal synaptosomes was low and conserved at PND21 and 36, but significantly increased at PND90, to a level largely comparable to that exocyted by male PND90 hippocampal synaptosomes (Fig. 6C). The 12 mM KCl-evoked [^{3}H]GABA exocytosis from PND36 female was significantly lower than that from PND36 males.

Based on the different responsiveness of male and female synaptosomes to the 12 mM KCl stimulus, we extended the functional study to 20 and 30 mM KCl-enriched medium, to investigate whether the responsiveness of the hippocampal synaptosomes from juvenile,

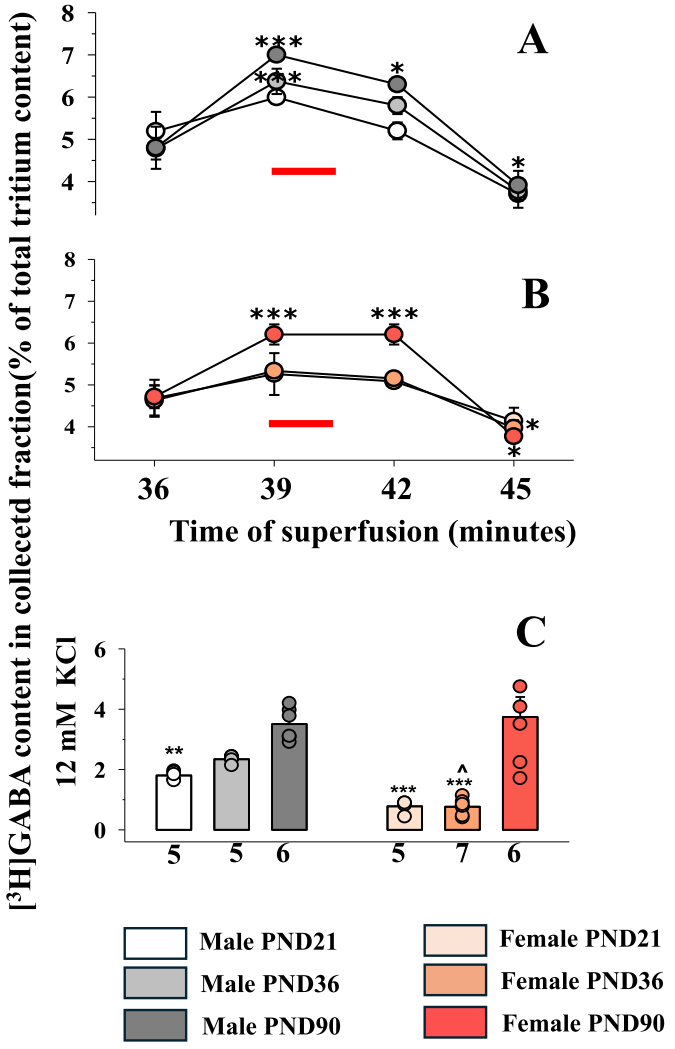

Fig. 6. [^{3}H]GABA release from hippocampal synaptosomes of male and female juvenile (PND21), adolescent (PND36) and adult (PND90) mice. **A** and **B**: [^{3}H] GABA release is reported as % of total radioactivity as a function of time from synaptosomes of male (**A**) and female (**B**) juvenile (PND21), adolescent (PND36) and adult (PND90) mice. Red bar: 12 mM KCl stimulation of synaptosomes in superfusion. Data were expressed as mean ± SEM of 5 (males) and 6 (females) experiments run in triplicate (three superfusion chambers for each experimental conditions).**C**: 12 mM KCl-evoked [^{3}H]GABA overflow (KCl-evoked release over basal, %) from hippocampal synaptosomes of male and female mice aged PND21, 36 and 90. The results are expressed as % of the total tritium synaptosomal content. Data are expressed as mean ± SEM. The number of replicates is reported below each bar. Statistical analysis was performed by one-way ANOVA followed by Tuckey test; **p < 0.01 vs respective PND90 mouse. ***p < 0.001 vs respective PND90 mouse; ^ p < 0.05 vs PND36 male.

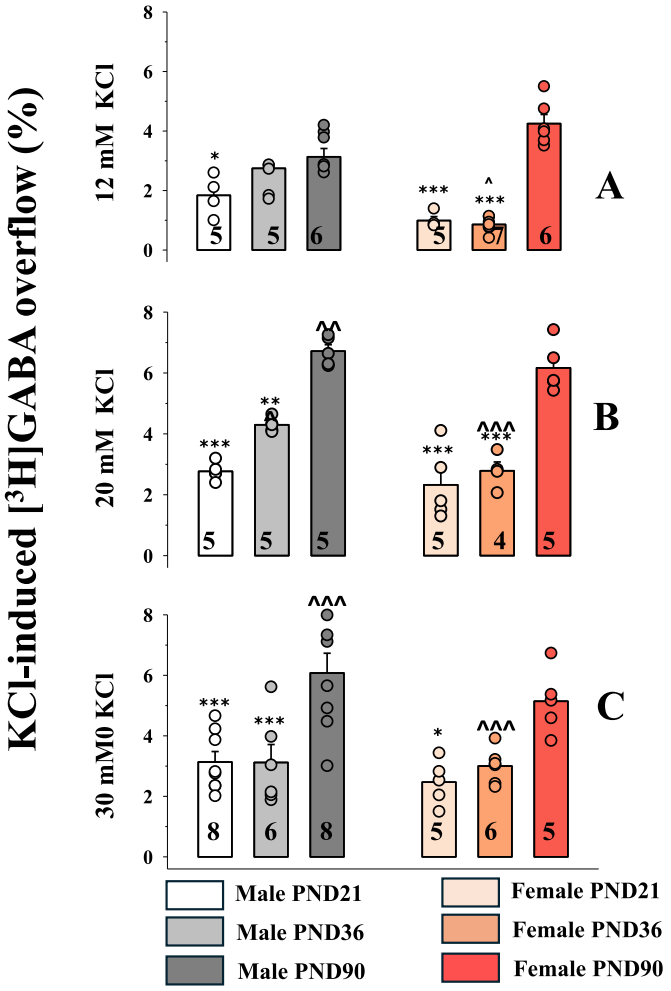

Fig. 7. [^{3}H]GABA overflow from hippocampal synaptosomes of male and juvenile (PND21), adolescent (PND36) and adult (PND90) mice. Hippocampal synaptosomes from PND21 (**A**), PND36 (**B**) and PND90 (**C**) male and female mice were exposed to different (12, 20 and 30 mM KCl) stimuli. Results are expressed as tritium release over the spontaneous outflow. Data are expressed as mean ± SEM; the number of replicates is reported within each bar. Statistical analysis was performed by one-way ANOVA followed by Tuckey test: *p < 0.05 vs respective PND90 mouse; **p < 0.01 vs respective PND90 mouse; ***p < 0.001 vs respective PND90 mouse; ^p < 0.05 vs respective PND36 mouse exposed to 12 mM KCl; ^^p < 0.01 vs respective PND36 mouse exposed to 12 mM KCl; ^^^p < 0.001 vs respective PND36 mouse exposed to 12 mM KCl.

adolescent or adult animals could depend on the strength of the applied depolarizing stimulus (Fig. 7A, B and C).

In PND21 male and female hippocampal synaptosomes, the amount of tritium exocytosis over the basal outflow elicited by 20 and 30 mM KCl was largely comparable to that obtained upon exposure of particles to 12 mM KCl, suggesting that, at this stage of development, the responsiveness of the GABAergic nerve endings slightly if ever does not depend on the intensity of the stimulus [male, F(2, 14) = 3,692, *n.s.*; female, F(2, 11) = 3970; *n.s.*;] (Fig. 7A, B and C).

Differently, the releasing activity elicited by 20, but not 30, mM KCl enriched solution from male PND36 hippocampal GABAergic nerve endings was largely conserved, but it was significantly higher than that elicited by the 12 mM KCl-enriched, [males, F(2, 13) = 4932; $p < 0.05$] (Fig. 7B and C vs A). Differently, the amount of tritium overflow from PND36 female hippocampal synaptosomes increased arithmetically, the 12 mM KCl-evoked tritium overflow being significantly lower than that elicited by the 20 mM or by 30 mM KCl-enriched solution and that caused by 20 mM KCl-evoked [^{3}H]GABA exocytosis being significantly lower than that elicited by 30 mM KCl [females, F(2, 14) = 39,863; < 0.001] (Fig. 7A,B and C).

The complexity of the scenario further increased when analysing the GABA exocytosis from PND90 male and female hippocampal synaptosomes. Exposure of PND90 male hippocampal synaptosomes to 20 mM or to 30 mM KCl-enriched solution elicited comparable tritium overflows, that were significantly higher than that elicited by 12 mM KCl [males, F(2, 16) = 10,904; $p < 0.01$] (Fig. 7B and C vs A). Inasmuch, the 12 mM KCl-evoked [^{3}H]GABA exocytosis from PND90 female hippocampal synaptosomes was significantly lower than that elicited by 20 mM. Furthermore, the 20 mM and the30 mM KCl-evoked tritium

overflows from PND90 female mice were largely comparable one each other [females, F(2, 13) = 6,55; *n.s.*] (Fig. 7B and C vs A).

Finally, experiments were dedicated to investigating the impact of acute LPS injection on the GABAergic parameters so far described in adolescent male and female animals (i.e. the mice showing the most relevant sex-dependent synaptic differences). Animals were acutely administered LPS and then sacrificed 6 h later, to collect tissues to analyse mRNA expression and to isolate synaptosomes to monitor [^{3}H] GABA uptake/release.

Six hours after LPS exposure, two way ANOVA (sex x LPS treatment) revealed a main effect of sex for both SLC6A1-GAT1 and SLC32A1-VGAT hippocampal expression [F(1; 30) = 6.650; p = 0.016 and F(1; 30) = 7.188; p = 0.012, respectively]. For SLC6A1-GAT1 mRNA a main effect of LPS treatment was present as well [F(1; 30) = 5.025; p = 0.033] (Fig. 8A and B). Post hoc analysis revealed that mRNA levels of SLC32A1-VGAT were slightly increased in female animals exposed to LPS with respect to their male counterparts (p = 0.012), a similar not statistical trend was observed for SLC6A1-GAT1 (p = 0.055). As far as the efficiency of [^{3}H]GABA uptake is concerned, this parameter was largely conserved in both male and female mice [F(3, 24) = 0.567; *n.s.*]

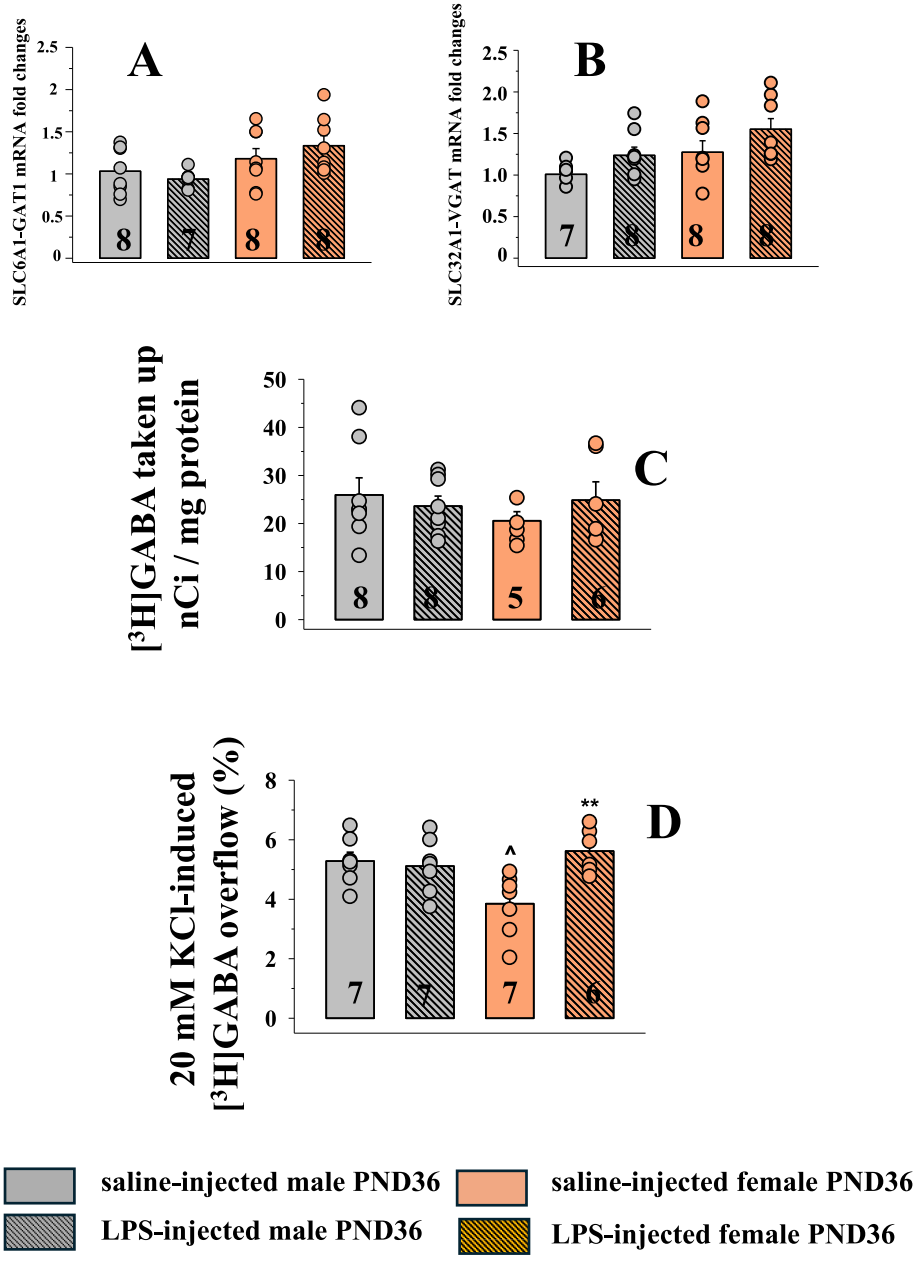

Fig. 8. Impact of LPS treatment on SLC6A1-GAT1 and SLC32A1-VGAT mRNA expression, on GABA uptake and on GABA exocytosis in the hippocampus of adolescent female and male saline-injected or LPS-injected mice. **A,** SLC6A1-GAT1 and **B,** SLC32A1-VGAT mRNA levels in the hippocampus of saline-injected or LPS-injected PND36 male and female mice (n = 8 for each group). Data were expressed as mean ± SEM; the number of replicates is reported within each bar. Statistical analysis was performed by two-way ANOVA followed by Bonferroni: *p < 0.05 vs male saline -injected PND36 mouse. **C**: Uptake of [^{3}H]GABA in hippocampal synaptosomes in male and female PND36 saline-injected or LPS-injected mice. Results are expressed as nCi/mg synaptosomal protein. Data represent the mean ± SEM; the number of replicates is reported within each bar.; **D**: 20 mM KCl-evoked ^{3}H]GABA overflow (KCl-evoked release over basal, %) from hippocampal synaptosomes of male and female PND36 saline-injected or LPS-injected mice. The results are expressed as % of the total tritium synaptosomal content. Data are expressed as mean ± SEM. The number of replicates is reported below each bar. Statistical analysis was performed by one-way ANOVA followed by Tuckey test; ^p < 0.05 vs male PND36 saline-injected mouse; **p < 0.01 vs female PND36 saline-injected mouse.

(Fig. 8C). Furthermore, [^{3}H]GABA exocytosis elicited by the transient exposure to a medium containing 20 mM KCl (the stimulus that unveiled the functional differences between male and female hippocampal synaptosomes) was significantly more pronounced in hippocampal PND36 male synaptosomes when compared to PND36 female particles (Fig. 8D). Quite surprisingly, however, the acute LPS injection modified the efficiency of [^{3}H]GABA exocytosis in PND36 female hippocampal nerve endings. Specifically, the 20 mM KCl-evoked [^{3}H]GABA exocytosis from LPS-injected hippocampal synaptosomes was significantly increased when compared to that from untreated particles, to a level comparable to that detected in male hippocampal synaptosomes [$F(3, 23) = 5.167$; $p < 0.01$] (Fig. 8D).

4. Discussion

GABA is the main player of the "plasticity of inhibition", since it mediates a large part of the synaptic, cellular and molecular events of control of synaptic transmission. The "plasticity of inhibition" relies on presynaptic inhibitory tuning which alleviates the destabilization of synaptic connection elicited by recurrent, disorganized, neuronal excitation (Kullmann et al., 2012). It preferentially involves GABA spillover from GABAergic structures (Kaczor et al., 2015) onto neuronal processes (most of which are glutamatergic in nature). The efficiency of this synaptic tuning depends on the amount of GABA released upon depolarization of GABAergic nerve endings, and, consequently, on the capability of these terminals to synthetize, store and take-up the inhibitory amino acid.

The impact of GABA-mediated control onto synaptic communication has been shown to vary during early development and to occur in a sex-dependent manner (Behuet et al., 2019; Davis et al., 1999; Vela et al., 2003). In particular, its developmental heterogenicity in certain sexually dimorphic brain regions (i.e. substantia nigra, hippocampus and hypothalamus) has been ascribed to a switch from facilitation to inhibition of GABA signalling around PND 10–15 (Akman et al., 2014 and references therein). The switch is proposed to depend on changes in the "receptor-mediated" transduction of the GABA signalling, as well as on sex and development-dependent adaptation of cellular events prodromic to GABA release, namely the synthesis, uptake and vesicular storage of the inhibitory amino acid within nerve endings (Akman et al., 2014; Basu et al., 2020; Corradini et al., 2018; Harbi and Mouihate, 2025; Ji et al., 2024; Kotlinska et al., 2023; Taube et al., 2024; Losi et al., 2014). In this view, the principal aim of the present study was to implement our knowledge on the sex-dependency of some specific presynaptic effectors (namely GAT1 and VGAT) of GABAergic signal and on their relationship to GABA storage and release at the early stages of development (Losi et al., 2014).

First, we focussed on the GAT1 transporter. We analysed the mRNA level of the membrane GABA transporter GAT1, SLC6A1, that dramatically scales down from PND21 to PND36, then remaining largely conserved until adulthood. The changes in mRNA expression were largely conserved in both males and females, suggesting that this adaptation is a common threat across development. To strengthen the relevance of the observations, we also analysed the density of the GAT1 protein in hippocampal synaptosomal lysates of both males and females at PND21, 36 and 90, considering that altered mRNA levels would not necessarily lead to changes in the density of the corresponding protein. Before discussing the results from these experiments, however, it is important to remind that the GABA transporter type 1 and 3 (GAT1 and GAT3) belong to the neurotransmitter/sodium symporter family which are expressed in several brain area, including the hippocampus (Vitellaro-Zuccarello et al., 2003). GAT1 has a preferential presynaptic distribution, and it exists in both constitutive monomeric and oligomeric forms (Korkhov et al., 2004; Moss et al., 2009; Ryan et al., 2021), which have different roles and distribution in neurons. The oligomeric form of GAT1 preferentially consists of dimers of dimers ((Moss et al., 2009; Scimemi, 2014)) and undergoes constitutive trafficking from the endoplasmic reticulum to cell membranes to control the efficiency of GABA uptake.

The analysis of the GAT1 immunopositivity in hippocampal synaptosomal lysates (which consists of protein from cytosolic structures, including those located in the endoplasmic reticulum, and from plasma membranes) unveiled a clear immunopositivity at 67 kDa, i.e. the expected weight of the monomeric form of the transporter. Immunostaining at 67 kDa was largely conserved in both male and female synaptosomal hippocampal lysates, independently of the age of animals. Surprisingly, however, the Western blot analysis also highlighted a clear immunopositivity at about 260 kDa, which would suggest the presence of an oligomeric form of the transporters having possibly a tetrameric assembly. The intensity of the immunostaining at 260 kDa varied according to the age of the animal, being clear evident in lysates of hippocampal synaptosomes of juvenile male and female mice, but less pronounced in lysates from adolescent and adult animals. These observations are in our opinion best interpreted by assuming that hippocampal GABAergic terminals of juvenile mice are enriched with functional GAT1 that would assure an efficient uptake of GABA, more efficient than that in adolescent and adult terminals.

The GAT1 transporter is a specific marker of those cellular structures that can take up GABA, including nerve terminals and astrocytic arborizations. The fate of the newly taken-up GABA depends on the structures involved. In astrocytic processes newly taken-up GABA is largely metabolized to produce glutamine which in turn is released to neuronal processes to synthesize either glutamate or GABA (Lee et al., 2019; Borden, 1996). Differently, GAT1 in presynaptic neuronal structures is essential to end the GABA-mediated tuning within the synaptic cleft. As a matter of fact, the efficiency of GABA uptake trough the GAT1 transporter was reported to inversely correlate with the GABA-mediated activation of GABA receptors (either $GABA_A$ and $GABA_B$ receptors) at presynaptic and postsynaptic sides of the active symmetric synapses. In short, a lower efficiency of GABA uptake would prolong the permanence of GABA into the synaptic cleft and the activation of the related receptor repertoire, strengthening its role in the "plasticity of inhibition" (Kullmann et al., 2012). Conversely, the reduced $[GABA]_{out}$ elicited by an efficient GABA uptake would causes a shift in the balance between GABA and glutamate in the synaptic cleft, favouring abnormal augmentation of excitatory signalling (Tao et al., 2024).

Interestingly, our results indicate that changes in the SLC6A1-GAT1 mRNA expression and GAT1 density were paralleled by changes in the amount of [^{3}H]GABA stored into hippocampal GABAergic nerve endings (synaptosomes) isolated from adolescent (PND36) and adult (PND90) mice when compared to juvenile (PND21) animals. These findings are best interpreted by proposing that the transcriptional differences detected in PND21 and in PND36 in male and female mouse hippocampus account for functional phenotypic adaptation (which emerges as reduced GAT1 protein density), which may affect the efficiency of GABA uptake into nerve terminals. This would imply that starting from PND36 the "plasticity of inhibition" gains efficiency with respect to juvenile animals.

Differently from the SLC6A1-GAT1 mRNA, however, the hippocampal expression of SLC32A1-VGAT as well as that of the VGAT protein density in male hippocampal synaptosomal lysates was largely conserved from the juvenile period to adulthood, suggesting that the vesicular storage and, perhaps, the releasing efficiency of GABAergic terminals would not undergo dramatic developmental changes in these animals. Accordingly, the amount of [^{3}H]GABA released upon exposure of male hippocampal synaptosomes to 12 mM KCl-enriched solution (which causes a calcium-dependent release of the inhibitory amino acid (Olivero et al., 2021)) underwent an arithmetic progression during development. Furthermore, the exposure of male synaptosomes to stronger stimuli (i.e. 20 and 30 mm KCl) did not caused dramatic changes in GABA exocytosis at least in hippocampal synaptosomes from PND21 and PND36 animals.

The scenario changed when considering females. VGAT density was

significantly lower in PND21 female hippocampal synaptosomal lysates when compared to PND90 particles, but slightly recovered at PND36. Concomitantly, the efficiency of GABA exocytosis in females unveiled differences in the releasing activity at the different stages of development, also with respect to males. Specifically, the tritium exocytosis elicited by exposing female synaptosomes to a mild, physiological depolarizing stimulus (12 mM KCl-enriched solution) was significantly less pronounced in PND21 and PND36 female synaptosomes with respect to PND90 female particles. Furthermore, the releasing activity from PND21 female synaptosomal preparations was significantly lower than that from juvenile male hippocampal synaptosomes, although differences overcome in adolescent mice. The scenario seems best interpreted by proposing that the low VGAT density detected in PND21 female hippocampal synaptosomes reduces the GABA vesicular storage in juvenile females, hampering its responsiveness to depolarizing stimuli, as indeed observed. Notably the efficiency in GABA exocytosis recovers in synaptosomes isolated from PND36 female animals, also depending on the stimulus applied (12–20 mM KCl), the lower responsiveness being nulled in synaptosomes exposed to stronger stimuli (i.e. 30 mM KCl). In a whole these findings are best interpreted by assuming that, up until adolescence, the GABAergic inhibitory tuning that control hippocampal synaptic activity is less efficient in female mice when compared to male mice but recovers during development becoming comparable to that in male at adulthood.

The possible impact of the mechanism(s) of vesicular GABA storage on the sex-dependent responsiveness of hippocampal GABAergic terminals to different releasing stimuli deserves in our opinion some more comments. GABA has been reported to accumulate in nerve terminals in at least two different intracellular Ca^{2+}-dependent stores, one of which corresponds to a readily releasable vesicular pool sensitive to low depolarizing stimuli and/or hypertonicity (Ashton and Ushkaryov, 2005; Lonart and Südhof, 2000), while the other supports a delayed exocytotic component, more sensitive to higher stimuli (Szerb, 1984; Bonanno et al., n.d.). The efficiency of GABA vesicular store depends on the VGAT protein, which based on our present finding, is less expressed in juvenile female hippocampal terminals. GABA vesicular store, however, also depends on GAT1, which account for the reuptake of GABA into nerve terminals (Bonanno et al., n.d.; Szerb, 1984; Ashton and Ushkaryov, 2005) and that, based on our observation, it would take up more efficiently the amino acid in juvenile male and female hippocampal terminals when compared to adults. Differently, the delayed exocytotic component of vesicles could preferentially store the newly synthetized GABA (Szerb, 1984) which is produced by GAD that decarboxylases glutamate. Interestingly, the mRNA of GAD was reported to have a higher expression in male when compared to female in the adolescent/juvenile period (Davis et al., 1999). This would imply that, differently from females, male GABAergic nerve terminals would possess an enzymatic repertoire sufficient to assure the replenishment of the delayed exocytotic vesicular pool, assuring an efficient GABAergic tuning in male already at the early stage of development. The lower responsiveness of female PND21 and PND36 hippocampal nerve endings to the depolarizing stimuli in term of GABA exocytosis might therefore represent the consequence of at least two concomitant events (the low VGAT1 density here described an the low GAD expression proposed in the literature (Davis et al., 1999)), that would penalize the storage of GABA in the vesicular pool, reducing the efficiency of GABA exocytosis.

Taking into consideration that GABA-mediated synaptic inhibition has been related to sexual maturation (Zheng, 2009) and that testosterone production (which account for masculinization and control GABA tuning) start from PND20, we cannot exclude that the different release efficiency of GABA release starting from PND21 (i.e. after the period when GABA acts as an excitatory transmitter) might directly depend on hormone maturation and its role in sexual dimorphism (Davis et al., 1999).

The second main finding of this work is that the delayed maturation of the GABAergic inhibitory tuning (measured as reduced release efficiency) detected in female hippocampal synaptosomes is overcame after an acute inflammatory challenge at the adolescence. The changes in the GABA release efficiency were paralleled by a modest but significant increase in SLC32A1-VGAT and SLC6A1-GAT1 hippocampal expression in female animals receiving the immune challenge at PND36. Future studies examining how LPS exposure in adolescent mice impact VGAT and GAT1 protein levels would help to address this point, although Wu and coworkers demonstrated that the effects of LPS on VGAT protein levels differ between the early or later stage of neuroinflammation ((Wu et al., 2025)). Furthermore, the possibility should be considered that the observed effects on GABA release may depend on a temporal lag between transcriptional and functional changes, as post-transcriptional mechanisms and protein trafficking may be affected by the immune challenge as well. To note, Gomez and co-worker in 2021 (Gomez et al., 2021) demonstrated that a very early (PND14) challenge with LPS increases seizure susceptibility in male and female mice, consistent with the conclusion that inflammation contributed to dictate the efficiency of GABA transmission and brain vulnerability. It is important to stress that in their work, Gomez and co-workers administered LPS at a stage of development when GABA is expected to be excitatory rather than inhibitory. The increased susceptibility to seizure might therefore related to an increased release of GABA elicited by the inflammatory challenge (as indeed observed in our case) that however at that stage of development mediates excitation instead of inhibition. Differently, in our case, the increased GABA exocytosis from female hippocampal nerve endings would assure a more efficient “plasticity of inhibition”.

5. Conclusion

To conclude, the GABAergic innervation is typified by a scope of functions, the complexity of which originates from the heterogenicity of the GABA system (Ben-Ari et al., 2007). The variety of GABAergic interneurons but also the intrinsic complexity of the GABA-mediated signalling account for this complexity. Beside the GABA switches from excitation to inhibition at the very early stage of development, we here provide evidence describing a sex and age-dependent maturation of GABA release efficiency during adolescent stage, that we propose possibly associate to developmental and dimorphic brain maturation and that could aim at equilibrating synaptic connections. We also provide evidence that an acute inflammatory challenge in female accelerates the completion of the maturation of GABA exocytosis, promoting conditions that would support mature “plasticity of inhibition”, to a level comparable to that detected in males. In this view, the inflammatory challenge would represent a positive stress signalling to promote and accelerate the maturation of the GABA system to exert a mature “plasticity of inhibition” essential to the control of synaptic plasticity.

CRediT authorship contribution statement

Cristina Benatti: Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Alessandra Roggeri:** Methodology, Investigation, Data curation. **Ylenia Toscano:** Methodology, Investigation, Data curation. **Veronica Torre:** Methodology, Investigation, Data curation. **Nicoletta Brunello:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Fabio Tascedda:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Johanna Maria Catharina Blom:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Anna Pittaluga:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Ethics

All animal procedures were approved by the Italian Ministry of Health (DDL 26/2014 with the approval of the local Ethical Committee;

University of Genoa authorization n° 75F11.N.JP6 and University of Modena and Reggio Emilia, authorization n. 33220. 33.ext.39).

Declaration of AI use

We have not used AI-assisted technologies in creating this article.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2025.106062.

Data availability

Data will be made available on request.

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