Korean J Physiol Pharmacol 2017;21(6):695-702
https://doi.org/10.4196/kjpp.2017.21.6.695

KJPP

Original Article

Developmental changes in $GABA_A$ tonic inhibition are compromised by multiple mechanisms in preadolescent dentate gyrus granule cells

Sudip Pandit[#,†], Gyu Seung Lee[#,¶], and Jin Bong Park*

Department of Physiology, School of Medicine and Brain Research Institute, Chungnam National University, Daejeon 35015, Korea

ARTICLE INFO

Received August 28, 2017
Revised September 20, 2017
Accepted September 21, 2017

***Correspondence**
Jin Bong Park
E-mail: jinbong @cnu.ac.kr

Key Words
Ages
Dentate gyrus granule cells
Extrasynaptic $GABA_A$ receptors
GABA transporter

[#]These authors contributed equally to this manuscript.

[†]Current address: Department of Pharmacology, University of Virginia, 1340 Jefferson Park Ave, Charlottesville, VA 22908, USA
[¶]Current address: Dong-Gu Public Health Center, Daejeon Metropolitan City 30 Bogeunso Avenue, Samseung-dong, Dong-gu, Daejeon 34691, Korea

ABSTRACT The sustained tonic currents (I_{tonic}) generated by γ-aminobutyric acid A receptors ($GABA_A$Rs) are implicated in diverse age-dependent brain functions. While various mechanisms regulating I_{tonic} in the hippocampus are known, their combined role in I_{tonic} regulation is not well understood in different age groups. In this study, we demonstrated that a developmental increase in GABA transporter (GAT) expression, combined with gradual decrease in $GABA_AR$ α_5 subunit, resulted in various I_{tonic} in the dentate gyrus granule cells (DGGCs) of preadolescent rats. Both GAT-1 and GAT-3 expression gradually increased at infantile (P_{6-8} and P_{13-15}) and juvenile (P_{20-22} and P_{27-29}) stages, with stabilization observed thereafter in adolescents (P_{34-36}) and young adults (P_{41-43}). I_{tonic} facilitation of a selective GAT-1 blocker (NO-711) was significantly less at P_{6-8} than after P_{13-15}. The facilitation of I_{tonic} by SNAP-5114, a GAT-3 inhibitor, was negligible in the absence of exogenous GABA at all tested ages. In contrast, I_{tonic} in the presence of a nonselective GAT blocker (nipecotic acid, NPA) gradually decreased with age during the preadolescent period, which was mimicked by I_{tonic} changes in the presence of exogenous GABA. I_{tonic} sensitivity to L-655,708, a $GABA_AR$ α_5 subunit inverse agonist, gradually decreased during the preadolescent period in the presence of NPA or exogenous GABA. Finally, Western blot analysis showed that the expression of the $GABA_AR$ α_5 subunit in the dentate gyrus gradually decreased with age. Collectively, our results suggested that the I_{tonic} regulation of altered GATs is under the final tune of $GABA_AR$ α_5 subunit activation in DGGCs at different ages.

INTRODUCTION

The activation of synaptic and extrasynaptic γ-aminobutyric acid A receptors ($GABA_A$Rs) generates phasic and tonic forms of inhibition (tonic $GABA_A$ current, I_{tonic}), respectively [1,2], and has a profound influence on the hippocampal neural circuitry. I_{tonic} is particularly interesting in the context of different ages because extrasynaptic $GABA_AR$ signaling is implicated in brain physiology and rage of pathophysiologies [3-7]. Changes in extracellular GABA concentrations alter the relative contribution of specific $GABA_A$Rs to I_{tonic} as different receptor populations are recruited [8]. $GABA_A$Rs containing the α_5 subunit (α_5-$GABA_A$Rs) contribute to I_{tonic} when the ambient GABA concentration increases, while at low ambient GABA concentrations the activation of δ subunit-containing receptors predominates [9]. In dentate gyrus granule cells (DGGCs), I_{tonic} increases during initial postnatal

Author contributions: S.P. and G.S.L. conduct experiments and wrote the manuscript. J.B.P. designed experiments and wrote the manuscript.

maturation [10,11], and further increases as adolescents mature into adulthood [12]. The age-dependent increase of I_{tonic} in DGGCs may mirror the increased expression of δ-$GABA_A$Rs in adults [13], which raises the question of whether and how a developmental change in α_5-$GABA_A$Rs alter I_{tonic} at different ages.

GABA transporters (GATs) are members of a family of Na^+-dependent neurotransmitter reuptake proteins. To date, four different GATs (GAT-1, GAT-2, GAT-3, and Betain/GABA transporter type 1) have been described in rat brain. Of these, GAT-1 is a primary neuronal GAT, while GAT-3 is commonly associated with glial cells [14]. Accordingly these two GAT subtypes are responsible for controlling extracellular GABA released from vesicular and non-vesicular sources, respectively [15]. In the hippocampus, GAT-1 predominantly determines the GABA concentration surrounding neurons, while GAT-3 activity is apparent with increased extracellular GABA concentration, especially when GAT-1 is blocked [16]. However, GAT-1 expression is low at early postnatal age, with GAT-3 expression dominating in that period [17]. Overall, it remains unknown whether and how the interaction between GAT-1 and GAT-3 modulates I_{tonic} during postnatal brain maturation. In this study, we investigated the combined role of GAT-1 and GAT-3 in I_{tonic} regulation in DGGCs at different ages; the results suggested that I_{tonic} mirrored the changes in expression of extrasynaptic $GABA_A$Rs activated by elevated extracellular GABA, according to the interrelationship between neuronal and glial GATs.

METHODS

Experimental animals

Male Sprague-Dawly rats purchased from Samtako Bio (Kyung Gi-Do, Korea) were housed under a 12/12 h light/dark schedule with free access to food and water until used. Animals were grouped by postnatal day (P), as follows: infantile (P_{7-9} and P_{14-16}), juvenile (P_{21-23} and P_{28-30}), adolescence (P_{36-37}), and young adulthood (P_{42-44} and P_{49-51}). Brains were rapidly extracted for electrophysiological recordings or Western blotting from animals anesthetized with ketamine and xylazine (80 mg/kg and 12 mg/kg, i.p., respectively). Animals in early infantile stage (P_{7-9}) were euthanized by decapitation without anesthesia. All animal experimentation was conducted in compliance with the policies of Chungnam National University regarding the use and care of animals.

Electrophysiological recordings and data analysis

Patch-clamp recordings were obtained in acutely prepared coronal hippocampal slices from male rats, as described previously [6,18]. Briefly, slices were perfused with artificial cerebrospinal fluid (aCSF; in mM: NaCl 126, KCl 2.5, $MgSO_4$ 1, $NaHCO_3$ 26, NaH_2PO_4 1.25, glucose 20, ascorbic acid 0.4, $CaCl_2$ 1, pyruvic acid 2; pH 7.3~7.4; saturated with 95%O_2-5%CO_2) at a ~3 ml/min flow. Recordings were obtained at 32°C using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). The series resistance was motored throughout the experiments. Neurons localized in the outer half of the granule cell layer were selected to minimize the effects of neurogenesis [19]. Patch pipettes were filled with a high Cl^- containing solution (in mM): KCl 140, HEPES 10, Mg^{2+}ATP 5, $MgCl_2$ 0.9, and EGTA 10. Current output was filtered at 2 kHz and digitized at 10 kHz (Digidata 1322A, pClamp 9 software, Axon Instruments). I_{tonic} was defined as the difference between the holding current ($I_{holding}$) before and after application of the $GABA_A$ receptor blocker bicuculline (20 μM). Drugs were added to the perfusing aCSF solution at known concentrations. All drugs except NO-711 (Tocris, UK) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Western blotting

All proteins from the dissected dentate gyrus (DGs) were lysed with 1× passive lysis buffer (Cell Signaling Technology, Danvers, MA, USA) and quantified using a Coomassie Protein assay kit (Bio-Rad, Hercules, CA, USA). Approximately 50 μg of protein was electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS–PAGE) and transferred onto nitrocellulose membranes. The blots were blocked with 1× Tris buffered saline (TBS)-Tween 20 containing 3% bovine serum albumin (BSA) +2% heparan sulfate (HS) for 1 h at room temperature (5% TTBS; Gibco, USA). The blots were then incubated at 4°C with primary antibodies against $GABA_A$R α_5 subunit, GAT-1, and GAT-3 (1:1,000; Millipore, USA) in 5% TTBS, respectively. The next day, the blots were incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2,000; Santa Cruz Biotechnology, USA). An enhanced chemiluminescence detection kit (ECL; Pierce, USA) was used to visualize antibody binding, and the intensity of the bands was measured using Image J software 1.42q (NIH, USA).

Statistical analysis

Numerical data are presented as means±standard error of the mean (SEM). Student's t-tests and analysis of variance, followed by post-hoc tests, were used as appropriate.

RESULTS

Electrophysiological recordings were obtained from a total of 114 DGGCs. In addition to blocking synaptic transmission, the $GABA_A$R antagonist, bicuculline (BIC, 20 μM), induced a variable outward shift in the holding current ($I_{holding}$) in DGGCs.

Increased expression of GAT-1 and GAT-3 in preadolescence

Altered expression of neuronal and glial GATs could contribute to I_{tonic} changes by altering the extracellular GABA concentration at different ages. To understand the age-dependent changes in GAT expression, we directly compared the expression of GAT-1 and GAT-3 in the DGs at different ages. Western blot analysis showed that the expression of GAT-1 and GAT-3 in the DGs gradually increased at P_7, P_{14}, and P_{21}, and thereafter stabilized at P_{28}, P_{35}, and P_{42} (Fig. 1).

Effect of GAT-1 and GAT-3 blockers on I_{tonic} according to age

Changes in the expression and /or relative contribution of GAT-1 and/or GAT-3 may cause I_{tonic} changes at different ages. To discriminate the functional role of neuronal and glial GATs in I_{tonic}, we measured and compared the I_{tonic} of DGGCs in the presence of selective GAT-1 and GAT-3 blockers at different ages.

Bath application of NO-711 (5 μM), a selective GAT-1 blocker [20], failed to induce consistent changes in $I_{holding}$ at P_{6-8}, while it induced a similar inward shift in $I_{holding}$ (I_{NO-711}) at P_{13-15} (21.8±4.4 pA, n=7), P_{20-22} (20.8±6.1 pA, n=6), P_{27-29} (21.4±3.6 pA, n=5), and P_{34-36} (27.3±3.4 pA, n=7) (Fig. 2A). In a subset of experiments, we measured I_{NO-711} in the presence of 1 μM GABA to confirm that GAT-1 activity facilitates I_{tonic} at the early infantile age. GABA (1 μM)-induced $I_{holding}$ shift were decreased with age (P_{6-8}, 16.3±4.2 pA, P_{20-22}, 7.6±1.7 and P_{34-36}, 3.3±1.2 pA, n=5~6). Additional application of NO-711 (GABA+NO-711) caused a significant inward shift in $I_{holding}$ even at P_{6-8}, which was still much smaller than at P_{20-22} and P_{34-36} ($p<0.01$ in both cases; Fig. 2B).

To investigate the functional significance of changes in GAT-3 expression, a selective GAT-3 blocker, SNAP-5114, was used [14]. Because SNAP-5114 (100 and 300 μM) alone failed to elicit changes in $I_{holding}$, we sequentially applied NO-711 and NO-711+SNAP-5114. In the presence of NO-711, 300 μM SNAP-5114 induced a significant inward shift in $I_{holding}$ ($I_{SNAP-5114}$) at P_{6-8}, P_{20-22}, and P_{34-36}. $I_{SNAP-5114}$ was significantly smaller at P_{6-8} than at P_{20-22}, and P_{34-36} ($p<0.05$ in both cases; Fig. 2C). Even in the presence of NO-711, 100 μM SNAP-5114 did not induce significant changes in $I_{holding}$ at all tested age groups (Fig. 2C). Thus, in a subset of experiments, we assessed $I_{SNAP-5114}$ in the presence of 1 μM GABA at different ages. GABA induced significant $I_{holding}$ changes at P_{6-9} (22.6±3.5 pA, n=6), while it induced minimal changes in $I_{holding}$ at P_{20-22} (1.3±1.0 pA, n=5) and P_{34-36} (1.9±0.9 pA, n=5). An additional 100 μM SNAP-5114 efficiently induced an inward shift in $I_{holding}$ at P_{6-9}, while it failed to cause significant changes in $I_{holding}$ at P_{20-22} and P_{34-36} in the presence of 1 μM GABA (Fig. 2D). These results support the idea that GAT3 activity is apparent with an increased extracellular GABA concentration in the hippocampus [16].

Nipecotic acid-induced I_{tonic} in preadolescence

To investigate the combined role of GAT-1 and GAT-3 in developmental I_{tonic} changes, we measured and compared the I_{tonic} of DGGCs in the presence of the nonselective GAT blocker, nipecotic acid (NPA), at different ages.

In contrast to selective GAT-1 or GAT-3 blockade, NPA (100 μM) induced a significant inward shift in the $I_{holding}$ of DGGCs (I_{NPA}), blocked by BIC, at all tested age groups. Interestingly, I_{NPA} gradually decreased during the infantile and juvenile periods (P_{6-8}, P_{13-15}, P_{20-22}, and P_{27-29}), and stabilized in adolescence and young adulthood (P_{34-36} and P_{41-43}) (Fig. 3A and B).

We hypothesize that the age-dependent I_{NPA} change may mirror the functional maturation of $GABA_A$Rs rather than the up-regulation of GAT expression in preadolescence, and that the large I_{NPA} may be due to a high ambient GABA level. Therefore,

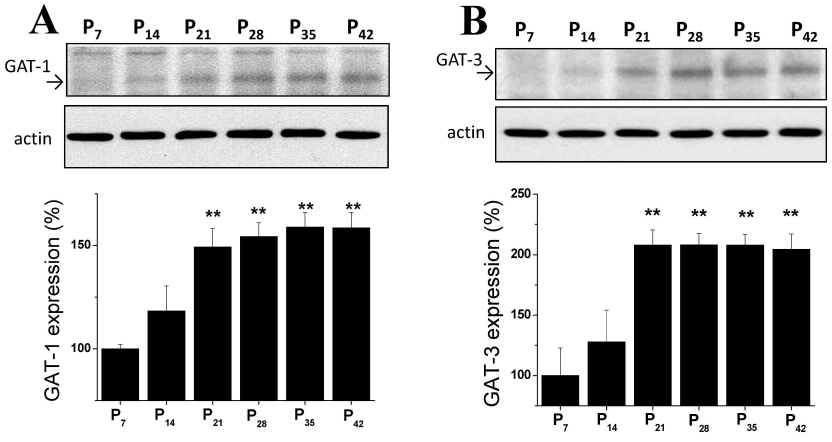

Fig. 1. Expression of GABA transporter-1 (GAT-1) and GABA transporter-3 (GAT-3) in the dentate gyrus at different ages. Western blot analysis showing neuronal GAT-1 (A) and glial GAT-3 expression (B) in the dentate gyrus at different postnatal days (P). The expression was normalized to the level detected at P_7 and compared with the expression at each age group (n=5), shown by the bar graphs. **p<0.01 compared with P_7 expression.

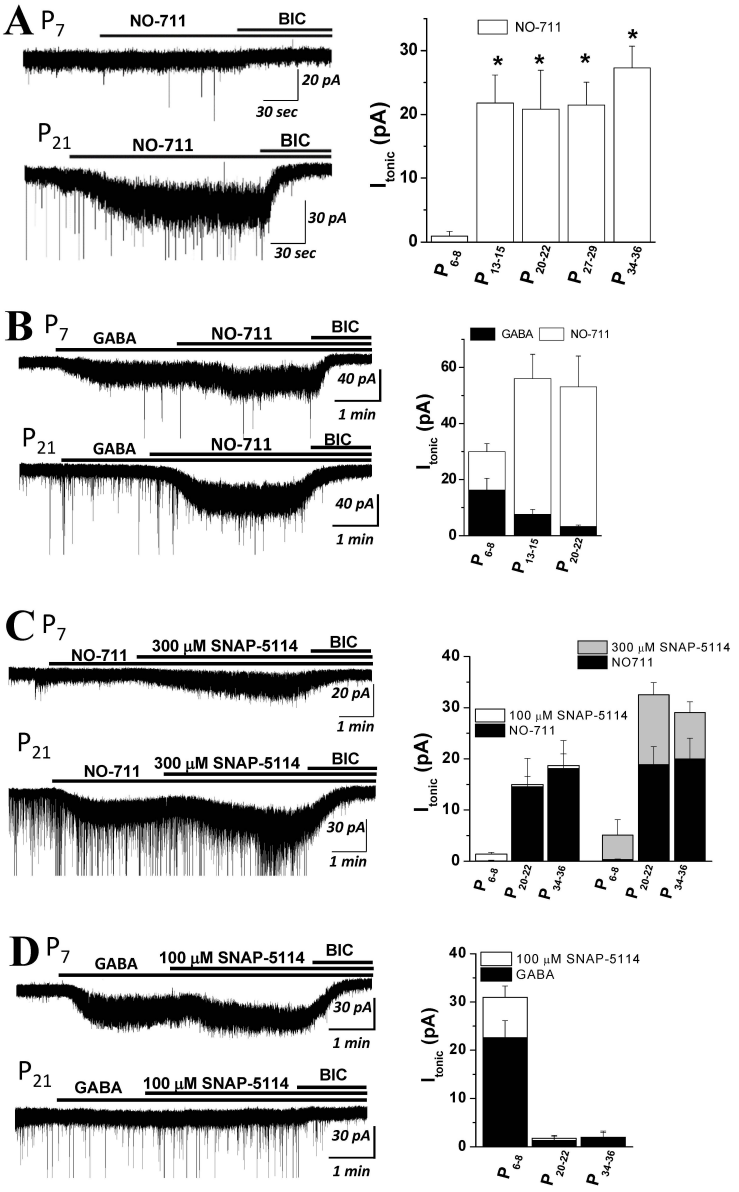

Fig. 2. Effects of NO-711 and SNAP-5114 on I_{tonic} at different ages. (A) Representative current traces of dentate gyrus granule cell (DGGC) showing the effect of NO-711 (5 mM), a GAT-1 blocker. Note that the effects of NO-711, which were blocked by bicuculline (BIC), were smaller at P_{6-8} than in older age. Mean changes in the holding current ($I_{holding}$) after NO-711 application are summarized in the bar graph. (B) Representative current traces showing the effect of NO-711 in the presence of GABA (1 μM). Mean $I_{holding}$ changes after the application of GABA and GABA+ NO-711 are summarized in the bar graph. (C) Representative current traces showing the effect of SNAP-5114 (300 μM), a GAT-3 blocker, in the presence of NO-711 (5 μM). Note that the effects of SNAP-5114 were smaller at P_{6-8} than in older age. Mean changes in $I_{holding}$ after by the sequential application of NO-711 and NO-711+SNAP-5114 (100 or 300 μM) are summarized. (D) Representative current traces showing the effect of SNAP-5114 (100 μM) in the presence of GABA (1 μM). Mean changes in $I_{holding}$ after the application of GABA and GABA+SNAP-5114 (100 μM) are shown in the bar graph. Summarized data are shown as means±standard error of the mean (SEM) (n=5~6). *p<0.05, **p<0.01 compared with P_{6-8}.

we directly compared INPA inhibition of L-655,708, an inverse agonist at the benzodiazepine binding site of α_5-$GABA_A$Rs [21]. The bath application of L-655,708 (5 μM) efficiently blocked I_{NPA} at all tested ages (p<0.01 in all cases; Fig. 3A). Interestingly, in agreement with gradual I_{NPA} attenuation with age, L-655,708-sensitive I_{NPA} also gradually decreased during preadolescence (Fig. 3B). As a result, the portion of L-655,708-sensitive total I_{NPA} did not differ by age.

Age-dependent decease in the $GABA_AR$ α_5 subunit in preadolescence

To understand the functional changes in $GABA_A$Rs according to age, we directly compared I_{tonic} activated by exogenous GABA (5 μM) and their sensitivity to L-655,708 at different ages.

I_{tonic} gradually decreases as infants mature into adolescence. The large I_{NPA} that characterized infantile periods (P_{6-8} and P_{13-15})

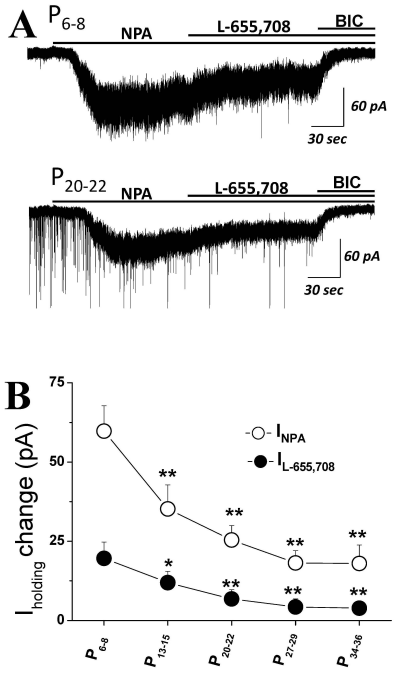

Fig. 3. Effects of nipecotic acid (NPA) on I_{tonic} according to age. (A) Representative current traces before and during the sequential application of NPA (100 μM), a nonselective GAT blocker, and NPA+L-655,708, an inverse agonist of the $GABA_A$ receptor α_5 subunit. Note that the effects of NPA, which were blocked by BIC, were larger at P_{6-8} than in older age. (B) Mean changes in the inward $I_{holding}$ shift after NPA (I_{NPA}), and the outward $I_{holding}$ shift following additional application of L-655,708 ($I_{L655-708}$), are summarized. Summarized data are shown as means±SEM (*n*=6~7). *p<0.05, **p<0.01 compared with P_{6-8}.

gradually decreased at the juvenile (P_{20-22}, and P_{27-29}) and adolescent (P_{34-36}) stages, and thereafter stabilized in young adults (P_{41-43}) (Fig. 4A and B). The bath application of L-655,708 (5 μM) partially blocked I_{tonic} in the presence of GABA at all tested ages (p<0.01 in all cases; Fig. 4A). In agreement with I_{tonic} attenuation, L-655,708-sensitive I_{tonic} gradually decreased in preadolescence (Fig. 4A and B). We observed a tendency for the portion of L-655,708-sensitive total I_{tonic} to decrease with age, although this did not reach statistical significance.

In further experiments, we directly compared the expression of the $GABA_AR$ α_5 subunit in DGs in the different age groups (Fig. 4C and D). Although we detected very low level of $GABA_ARs$ α_5 subunit immune reactivity at P_7 and P_{14} in DGs, Western blot analysis showed that the expression of the $GABA_ARs$ α_5 subunit gradually decreased in the juvenile (P_{21} and P_{28}) and adolescent (P_{35}) periods, and thereafter stabilized in the young adults (P_{42}). The degree of $GABA_ARs$ α_5 subunit expression was not further changed at P_{49} and P_{56} (data not shown).

DISCUSSION

The main findings of the present study were as follows: 1) age-dependent increase in the I_{NO-711} and $I_{SNAP-5114}$ of DGGCs at infantile stages were partially consistent with the gradual increase in GAT-1 and GAT-3 expression in infantile and juvenile DGs; 2) the age-dependent decrease in I_{NPA} was compromised as $GABA_AR$ α_5 subunit expression gradually decreased during preadolescence.

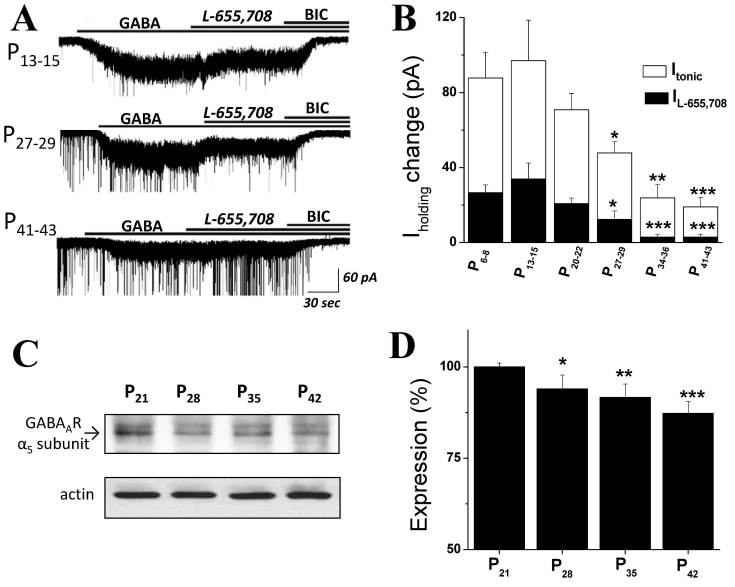

Fig. 4. Age-dependent decrease of $GABA_AR$ α_5 subunit expression in preadolescent rats. (A) Representative current traces before and after the sequential application of GABA (5 μM) and GABA+L-655,708, an inverse agonist of the $GABA_A$ receptor α_5 subunit. (B) Mean changes in the inward $I_{holding}$ shift by GABA and outward $I_{holding}$ shift following additional application of L-655,708 ($I_{L655-708}$) are summarized. Summarized data are shown as means±SEM (n=6~7). *p<0.05, **p<0.01 **, ***p<0.001 compared with P_{6-8}. (C) Representative Western blot analysis showing $GABA_AR$ α_5 subunit expression at different ages. (D) Summarized $GABA_AR$ α_5 subunit expression at different ages. The protein expression was normalized to the level detected at P_{21}. Summarized data are shown as means±SEM (n=4). *p<0.05, **p<0.01, ***p<0.001 compared with P_{21}.

Together, these findings suggest that, in addition to regulation of the ambient GABA concentration according to GAT activity, the change in age-dependent I_{tonic} mirrored the altered expression and/or composition of extrasynaptic $GABA_A$Rs during preadolescence.

Greater role of GAT-1 versus GAT-3 in regulating the I_{tonic} of DGGCs

GATs embedded in axon terminal membranes and/or astrocyte plasma membranes regulate ambient GABA levels. In many neural systems, GAT-1 antagonists alone result in a smaller I_{tonic} versus that observed when both GAT-1 and GAT-3 are blocked [22,23]. This was explained by the involvement of both GAT-1 and GAT-3 transporters in regulating extracellular GABA concentrations around neurons. Similarly, I_{NPA} was much larger than I_{NO-711} in infantile and juvenile DGGCs in the present study. Interpretation of this difference is complicated because, as opposed to NO-711, NPA is a GAT substrate. In addition, NPA could result in heteroexchange for GABA by GATs [24]. However, given that the concentrations of NPA and NO-711 used in this study were, respectively, about five and ten times that of the IC_{50} used for GAT-1 blockade [14], a simple interpretation could be that NPA blocked GAT-3 more efficiently than NO-711 during the infantile stage. However, our results showed that the GAT-1 blocker alone, and the GAT-1 blocker with additional application of SNAP-5114 (100 μM), resulted in a similar average I_{tonic} of 20 pA in DGGCs after the late infantile periods; these results contradict the idea that GAT-3 actively contributed to the I_{tonic} of DGGCs. In the present study, SNAP-5114 facilitated I_{tonic} at the concentration of 300 μM, which was near to the IC_{50} for GAT-1 blockade [14], further confounding the role of GAT-3 in the I_{tonic} of DGGCs. Combined with the fact that both GAT-1 and GAT-3 expression increased until the late juvenile periods, these results appear to support a major role of GAT-1 in the GABA uptake regulating I_{tonic} in the adult hippocampus [16]. In general, our results suggest that GAT-1 and GAT-3 play a primary and adjunctive role, respectively, in regulating I_{tonic} of DGGCs in preadolescece.

As with other neurotransmitter transporters, GATs can also act in reverse mode and thus release GABA from cells. Indeed, GABA can be secreted from cells by the reversed transport direction of GATs, particularly during early postnatal stages [25,26]. Thus, it is possible that GABA release via reversed GAT activity is integral in maintaining GABA levels that activate I_{tonic} [27] in the infantile and early juvenile stages. However, our finding that GAT-1 and GAT-3 blockers always enhanced the I_{tonic} of DGGCs suggest that the two transporters operate synergistically to promote GABA uptake, which was seen at all tested ages in our experiments.

Role of α_5-$GABA_A$Rs in age-dependent I_{NPA} attenuation during preadolescence

Both the α_5 and δ subunit are key mediating components of the I_{tonic} of DGGCs [28]. Alterations in GABA concentrations affect the relative contribution of specific $GABA_A$Rs to I_{tonic} as different receptor populations are recruited [8]. In the present study, BIC uncovered basal I_{tonic} shown by the outward $I_{holding}$ shift went over the initial level, especially when the inward shift in $I_{holding}$ by exogenous GABA and GAT blockers was less than ~30 pA. α_5–$GABA_A$Rs contribute to I_{tonic} when ambient GABA concentrations increase, while at low ambient GABA concentrations the activation of δ–$GABA_A$Rs predominates [9]. In the present study, I_{NPA} was larger than I_{NO-711} during preadolescence, which could be explained by an ambient GABA concentration sufficient to recruit additional α_5–$GABA_A$Rs in the presence of NPA, but not in NO-711. Our results showed that the portion of L-655,708-sensitive I_{NPA} ranged from ~27% to ~32%; this is consistent with previous findings showing that I_{tonic} is mediated by α_5–$GABA_A$Rs and is responsible for ~29% of the total I_{tonic} in DGGCs [28]. Thus, α_5–$GABA_A$Rs mediated I_{NPA} at all tested ages. Combined with the results whereby I_{NPA} and $GABA_A$R α_5 subunit expression gradually decreased with age, our results suggest that the age-dependent I_{NPA} decrease mirrors the functional decrease of α_5–$GABA_A$Rs rather than changes in GATs activity, during preadolescence.

However, there may be an as-yet undiscovered, non α_5–containing $GABA_A$Rs responsible for the large I_{NPA} observed during preadolescences. Indeed, in the present study, $GABA_A$R α_5 subunit immunoreactivity was not detectable in DGs at infantile stages. It is also notable that the small amplitude of I_{NO-711} prevented us from comparing the sensitivity of I_{NPA} and that of I_{NO-711} to L-655,708 in DGGCs. Regarding $GABA_A$Rs activated in the presence of NPA, it is also noteworthy that NPA can directly activate $GABA_A$R-like channels [29]. However, to the best of our knowledge, there is no information on the composition of $GABA_A$Rs directly activated by NPA. However, it is still of interest that $GABA_A$R α_5 subunit expression gradually decreased with the postnatal development in various brain regions.

Functional consequences

Overall, our results showed that GATs blockades elevated ambient GABA level sufficiently to harmonize with α_5-$GABA_A$Rs, resulting in an age-dependent I_{tonic} decrease in preadolescent brains. Combined with the fact that $GABA_A$R α_5 subunit expression in the hippocampus is closely related to learning and memory in young adults [30,31], selective pharmacological modulators, such as α_5-$GABA_A$R selective inverse agonists, may be effective in increasing cognitive performance in memory disorders [32]. Future studies are warranted to elucidate the pathophysiology of α_5-$GABA_A$Rs generating I_{tonic} combined with GAT blockades in the developing brains of preadolescents.

ACKNOWLEDGEMENTS

This work was supported by Chungnam National University and National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (NRF-2015R1D1A1A02059430).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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