Available online at www.sciencedirect.com
ScienceDirect
Neuropharmacology 52 (2007) 1342–1353
NEURO PHARMACOLOGY
www.elsevier.com/locate/neuropharm

17 β-estradiol modulates GABAergic synaptic transmission and tonic currents during development in vitro

Maria Pytel [a], Tomasz Wójtowicz [a,*], Katarzyna Mercik [a], Isabella Sarto-Jackson [b], Werner Sieghart [b], Chrysanthy Ikonomidou [c], Jerzy W. Mozrzymas [a]

[a] *Laboratory of Neuroscience, Department of Biophysics, Wroclaw Medical University, Chalubinskiego 3, 50-368 Wroclaw, Poland*
[b] *Division of Biochemistry and Molecular Biology, Center for Brain Research, Medical University of Vienna, Spitalgasse 4, Vienna A-1090, Austria*
[c] *Department of Pediatric Neurology, Medical Faculty Carl Gustav Carus, Technical University Dresden, Fetscherstrasse 74, 01307 Dresden, Germany*

Received 14 November 2006; received in revised form 17 January 2007; accepted 18 January 2007

Abstract

Estrogens exert a variety of modulatory effects on the structure and function of the nervous system. In particular, 17 β-estradiol was found to affect GABAergic inhibition in adult animals but its action on GABAergic currents during development has not been elucidated. In the present study, we investigated the effect of 17 β-estradiol on hippocampal neurons developing in vitro. In this model, mIPSC kinetics showed acceleration with age along with increased α1 subunit expression, similarly as in vivo. Long-term treatment with 17 β-estradiol increased mIPSC amplitudes in neurons cultured for 6–8 and 9–11 DIV and prolonged the mIPSC decaying phase only in the 9–11 DIV group. The time needed for the onset of 17 β-estradiol effect on mIPSC amplitude was approximately 48 h. In the period of 9–11 DIV, treatment with 17 β-estradiol strongly reduced the tonic conductance activated by low GABA concentrations. The effects of 17 β-estradiol on mIPSCs and tonic conductance were not correlated with any change in expression of considered $GABA_AR$ subunits (α1–3, α5–6, γ2) while α4 and δ subunits were at the detection limit. In conclusion, we provide evidence that 17 β-estradiol differentially affects the phasic and tonic components of GABAergic currents in neurons developing in vitro.

Keywords: $GABA_A$ receptor; Tonic conductance; mIPSC; Patch-clamp

1. Introduction

Estrogens are ovarian steroids that participate in induction of preovulatory surge of gonadotrophins and in the onset of sexual behavior. Extensive studies provided a wealth of evidence indicating involvement of estrogens in a variety of modulatory effects on the structure and function of the nervous system. In our recent study we have described a neuroprotective effect of β-estradiol (BE) in young brains in which apoptotic neurodegeneration was induced (Asimiadou et al., 2005). In particular, we have found that β-estradiol ameliorated neurotoxicity of drugs that activate or up-regulate $GABA_A$ receptors (γ-amino-butyric-acid A-type receptors). To test whether 17 β-estradiol was related to a direct modulation of $GABA_A$ receptors, current responses to exogenous GABA were recorded, but no significant effect of 17 β-estradiol was observed (Asimiadou et al., 2005). Moreover, neuroprotective effect of 17 β-estradiol was found to be correlated with increased levels of phosphorylated ERK1/2 (extracellular signal regulated kinase) and AKT, indicating that, at least in part, 17 β-estradiol effect was related to activation of these pro-survival proteins. However, it cannot be excluded that a long-term treatment with 17 β-estradiol could affect GABAergic currents via indirect mechanisms. Indeed, long-term treatment with estrogens was found to profoundly regulate the structure of neuronal cells and to modulate the GABAergic and glutamatergic neurotransmission. Murphy et al. (1998a,b) have reported that estradiol down-regulated

* Corresponding author. Tel.: +48 71 784 1550; fax: +48 71 784 1399.
E-mail address: twojtow@biofiz.am.wroc.pl (T. Wójtowicz).

0028-3908/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuropharm.2007.01.014

BDNF (brain-derived neurotrophic factor) in cultured hippocampal cells and this effect was associated with a decrease in inhibition, as evidenced by decrease in GAD (glutamate decarboxylase) and GABA level, and increase in excitatory tone leading to a 2-fold increase in dendritic spine density. In adult animals, estradiol was found to transiently reduce GAD immunoreactivity, to decrease the amplitude and to increase the duration of spontaneous IPSCs (inhibitory postsynaptic currents) (Rudick and Woolley, 2001, 2003; Rudick et al., 2003). However, the estrogen effect on mIPSCs (miniature inhibitory postsynaptic currents) (in the presence of tetrodotoxin, TTX) was qualitatively different: there was no significant change in amplitude and decay time course but their frequency was diminished (Rudick and Woolley, 2001; Rudick et al., 2003). Modulation of GABAergic currents by long-term treatment with 17 β-estradiol might potentially involve changes in $GABA_A$ receptor subunit expression patterns but this issue has not been fully explored. Pierson et al. (2005) used semiquantitative RT PCR to assess the RNA level of $GABA_A$ receptor subunits in NT2-N neurons and found that treatment with 17 β-estradiol for 2 days increased α2 and decreased α5 subunit but after 7 days this effect was not observed any more. Shen et al. (2005) have reported that treatment with 17 β-estradiol and progesterone enhanced expression of receptors containing δ subunit giving rise to increased tonic currents in CA1 hippocampal neurons. Weiland and Orchinik (1995) have found no effect of estradiol on $GABA_AR$ subunit expression but treatment of animals with 17 β-estradiol together with progesterone did affect the subunit expression of these receptors.

Vast majority of data describing the modulation of GABAergic system by 17 β-estradiol was obtained in adult animals. Much less is known about 17 β-estradiol modulatory effects on the GABAergic drive during development. Based on analysis of calcium signals in response to muscimol, Perrot-Sinal et al. (2001) have concluded that treatment of neurons with 17 β-estradiol enhanced muscimol-evoked calcium transients and prolonged the period during which GABA is excitatory. Wang and Tsai (1999) have reported that treatment with 17 β-estradiol augmented GABA content in developing tilapia brain. Taking into account that 17 β-estradiol exerts neuroprotective effect in young animals (Asimiadou et al., 2005) and the fact that GABAergic system undergoes profound changes during development (Cherubini et al., 1991) it seems interesting to consider the impact of this compound on developing neurons. For this purpose we have considered a model of cultured rat hippocampal neurons developing in vitro. We found that 17 β-estradiol clearly modulates both phasic (mIPSC) and tonic currents in this model and that this effect strongly depends on the developmental stage.

2. Materials and methods

2.1. Cell culture for electrophysiological recordings

Primary cell culture was prepared as described in details by Andjus et al. (1997). Briefly, P1–P3 days old Wistar rats were decapitated. This procedure is in accordance with the regulation of the Polish Animal Welfare Act. Hippocampi were dissected, sliced, treated with trypsin, mechanically dissociated and centrifuged twice at $40 > \times g$, plated in the Petri dishes and cultured. Recordings were made from pyramidal cells (judging from shape) between 6 and 16 days in culture.

17 β-estradiol was from Sigma (1,3,5(10)-Estratriene 3,17β-diol, E-2758, Sigma-Aldrich, Germany) and stock was prepared by dissolving in DMSO (dimethyl sulphoxide) at final concentration of 10 mM. Treatment of cultured neurons with 17 β-estradiol was performed using two protocols. In the long-term treatment protocol, cultured neurons were supplemented with 17 β-estradiol at final concentration of 1 μM 48 h after the culture was prepared (2nd DIV). Culture medium was exchanged (ca. 50% of volume) two or three times a week. Each time 17 β-estradiol was added to the medium from a freshly prepared stock. In parallel, in separate petri dishes, cultured neurons were supplemented with DMSO at the same final concentration as in 17 β-estradiol-treated group (0.01% vol/vol). In order to observe the time needed for the onset of 17 β-estradiol effect, we considered groups of neurons treated with 17 β-estradiol (1 μM) for 24 and 48 h in each of the considered age groups (see Section 3).

The estrogen receptor antagonist tamoxifen ([*Z*]-1-[*p*-Dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene) was used at 0.5 μM for 48 h in the presence of 1 μM of 17-β-estradiol and DMSO as a solvent (Sigma, Germany). This concentration of tamoxifen is sufficient to block estrogen receptors (Murphy and Segal, 1996) and within considered duration of treatment was non-toxic to neuronal culture (O'Neill et al., 2004).

2.2. Electrophysiological recordings

Currents were recorded in the whole-cell and outside-out mode of the patch-clamp technique using the Multiclamp 700B (Molecular Instruments, Sunnyvale, CA, USA) at a holding potential (V_h) of −70 mV. The intrapipette solution contained (in mM) CsCl 137, $CaCl_2$ 1, $MgCl_2$ 2, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′-tetraacetic acid (BAPTA) 11, ATP 2, HEPES 10 (pH 7.2 with CsOH). The composition of the standard external solution was (in mM) NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 20, HEPES 10 (pH 7.2 with NaOH).

Miniature IPSCs were recorded in the whole-cell configuration in the presence of TTX (1 μM) and kynurenic acid (1 mM). For the whole-cell recordings, patch pipettes had 2.5–3.4 MΩ when filled with internal solution. The whole cell recordings were considered for analysis when the access resistance was below 10 MΩ. Typically access resistance was in the range 4–8 MΩ. Occasionally, the series resistance compensation procedure was applied to bring access resistance below 10 MΩ but in most cases it was not necessary. The cells in which series resistance showed instability during recordings were not considered in the analysis. Any recordings in the whole-cell mode were started at least 3 min after the patch rupture. This time was sufficient to stabilize the recording conditions. In the case of the whole-cell recordings, solutions were supplied by gravity using a glass tube (1 mm i.d.) positioned with a manipulator in the vicinity of the recording site.

Using similar experimental configurations (whole-cell mode and perfusion with pipette placed close to the recording site) a special protocol was considered to assess GABAergic tonic conductance. In order to diminish the amount of neurotransmitters released due to the activity of neuronal network, TTX (1 μM) and kynurenic acid (1 mM) and CGP 55845 ((2*S*)-3-[[(1*S*)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)-phosphinic acid) (1 μM) to block $GABA_B$ receptors) were applied in the washing solution supplied directly on the neuron from which the recording was made. It is expected that rapidly flowing washing solution (with nominally zero [GABA]) directly onto a well exposed neuronal cell (the presence of glia was diminished by Ara-C (cytosine-D-arabinofuranoside, C1768, Sigma-Aldrich, Germany) applied 2 days after preparation at concentration of 1 μM) around which synaptic GABA release was reduced to the minimum (due to TTX) would provide the most efficient control of GABA concentration around the neuron. To assess the tonic conductance, solutions containing 0.1, 0.3 and 1 μM GABA were consecutively applied for 3–5 min. Before and after GABA applications, wash solution (with

TTX and kynurenic acid) was applied to check the stability of recordings and then 100 μM bicuculline was applied and the shift of the baseline was recorded.

For acquisition and analysis, the pClamp 9.2 software (Molecular Instruments, Sunnyvale, CA, USA) was used. For the analysis of the synaptic currents, recorded in the whole-cell configuration, the current signals were low-pass filtered at 3 kHz with a Butterworth filter and sampled at 20 kHz using the analog-to-digital converter Digidata 1322 (Molecular Instruments, Sunnyvale, CA, USA) and stored on the computer hard disk. Current responses to rapid GABA applications were filtered at 10 kHz and sampled at 100 kHz.

Current responses to exogenous GABA were recorded in the outside-out configuration using the ultrafast perfusion system based on a piezoelectric-driven theta-glass application pipette (Jonas, 1995). The piezoelectric translator was from Physik Instrumente (preloaded HVPZT translator 80 μm, Waldbronn, Germany) and theta-glass tubing from Hilgenberg (Malsfeld, Germany). The open tip recordings of the liquid junction potentials revealed that a complete exchange of solution occurred within 40–60 μs.

2.2.1. Analysis

The decaying phase of the currents was fitted with a function in the form:

$$y(t) = \sum_{i=1}^{n} A_i \exp(-t/\tau_i) \quad (1)$$

where, A_i are the fractions of respective components, τ_i are the time constants. For normalized currents, $\Sigma A_i = 1$. Deactivation time course was well fitted with a sum of two exponentials ($n = 2$). The desensitization onset was fitted with either one or two exponentials plus a constant factor describing the steady-state currents. The deactivation kinetics was additionally assessed by averaged time constant calculated according to the formula: $\tau_{mean} = \Sigma A_i \tau_i$.

Data are expressed as mean ± SEM.

All experiments were performed at room temperature 22–24 °C.

2.3. SDS-PAGE, Western-blotting, and chemiluminescence detection

Cell cultures were prepared from eight to nine animals using the same protocols as described above (Andjus et al., 1997). Rat hippocampal neurons were collected in ice-cold phosphate buffer saline ($CaCl_2$ * H_2O 0.9 mM, KCl 2.6 mM, KH_2PO_4 1.5 mM, $MgCl_2$ 0.5 mM, NaCl 138 mM, Na_2HPO_4 8.1 mM) and centrifuged 15 min 240 × *g*. Pellets were resuspended in extraction buffer containing NaCl 150 mM, Tris 50 mM pH = 7.4, EDTA 1 mM, Triton X-100 1% and containing one Mini complete protease inhibitor cocktail tablet per 7 mL buffer (Roche Diagnostics, Mannheim, Germany) and shaken overnight at 4 °C. Total protein content was established with BCA method (Smith et al., 1985). Total lysates were denatured (15 min 65 °C) in sample buffer (Invitrogen life technologies, Carlsbad, CA, USA) under reducing conditions yielding a final protein concentration of 1 μg/μl. SDS-PAGE was performed according to Neville and Glossmann (1974) with 10% acrylamide. Samples (5–15 μg total protein) were run on NuPAGE Bis-Tris gels with a MOPS running buffer (Invitrogen, Life technologies, Carlsbad, CA, USA) and semidry-blotted onto prewetted polyvinylidene fluoride membranes (Immobilon Transfer Membranes, Millipore, USA). After blocking with 1.5% non-fat dry milk powder in PBS and 0.1% Tween 20 for 1 h at room temperature membranes were incubated overnight 4 °C with rabbit anti-α1 (1–9) (Zezula et al., 1991), anti-α2 (322–357) (Mossier et al., 1994), anti-α3 (1–11) (Ogris et al., 2006), anti-α4 (1–14) (Bencsits et al., 1999), anti-α5 (337–388) (Mossier et al., 1994), anti-δ (334–393) (Pöltl et al., 2003), anti-γ2 (1–33) (Ebert et al., 1999) primary antibodies (1 μg/1 ml). After extensive washing (1.5% (w/v) dry milk powder and 0.1% Tween 20 in PBS), the membranes were incubated with $F_{(ab')2}$ fragments of goat anti-rabbit IgG, coupled to alkaline phosphatase (Dianova, Jackson ImmunoResearch Labs., USA) for 50 min at room temperature. Membranes were washed extensively and equilibrated in assay buffer (diethanolamine 0.1 M, $MgCl_2$ 1 mM pH 10.0) for 10 min. Then membranes were incubated with 1 ml of 0.25 mM CDP-star reagent (Tropix, Bedford, USA) diluted in assay buffer. After 1 min the fluid was removed and the membranes were sealed in a foil. The chemiluminescent signal was quantified by densitometry after exposing the immunoblots to the Fluor-S MultImager (Bio-Rad Laboratories, Hercules, CA, USA) and evaluated using the Quantity One Quantitation Software (Bio-Rad Laboratories, Hercules, CA, USA). Under the experimental conditions used, the immunoreactivities were within the linear range, and this permitted a direct comparison of the amount of antigen per gel lane between samples (Sarto-Jackson et al., 2006). Data were generated from several different gels and expressed as mean ± standard error. Student's unpaired *t*-test was used for comparing groups, and significance was set at $p < 0.05$.

For re-analyzation of the PVDF membranes, they were washed twice for 30 min at 55 °C in stripping buffer (62.5 mM Tris, 3% SDS, pH 6.8) to which 7.7 mg/mL dithiothreitol was added. The membranes were extensively washed in PBS and 0.1% Tween 20 at room temperature and the whole procedure starting with the blocking reaction was repeated as described above.

3. Results

3.1. Model of development in vitro

As explained in Section 2, the neuronal cultures were prepared from P1–3 pups i.e. at very early stage of postnatal development. Ortinski et al. (2004) and Barberis et al. (2005) provided evidence that key developmental changes related to IPSCs characteristics can be fairly reproduced in vitro. In particular, mIPSCs decay was clearly accelerated between 6 and 12 days in vitro (DIV) and this change was accompanied by a switch in subunit expression that typically occurs during development in vivo (Ortinski et al., 2004). Taking this into account, we examined the kinetics of mIPSCs during development in vitro for the considered model of hippocampal neurons. As shown in Fig. 1, synaptic currents showed a pronounced acceleration of the decaying phase with time in vitro. This difference was particularly clear when comparing the neurons cultured for up to 8 DIV and those kept in culture for 12–16 (τ_{mean} for 6–8 DIV: 47.9 ± 3.5 ms, $n = 14$, for 9–11 DIV: 39.3 ± 1.4 ms, $n = 29$, and for 12–16 DIV: 36.7 ± 1.7 ms, $n = 22$, $p < 0.05$). There is a general agreement that such acceleration of mIPSCs is a clear indicator of maturation and is commonly observed in physiological conditions (Hollrigel and Soltesz, 1997). Thus, neurons cultured for 6–8 DIV (below 6 DIV there was very low spontaneous synaptic activity) were defined as "Young" and those cultured for 12–16 days as "Old". In the period between 9–11 DIV, the kinetics of IPSCs showed a substantial variability, and this period of culture was defined as "Intermediate".

On average, the frequency of mIPSCs showed a monotonic and significant increase with age (Fig. 1C). The averaged amplitude of mIPSCs did not show any clear trend during considered period of culture (not shown).

The developmental acceleration of mIPSC decay kinetics is known to be associated with a switch in subunit composition of the postsynaptic receptors (e.g. Hollrigel and Soltesz, 1997; Cherubini and Conti, 2001). In particular, it is widely accepted that development is associated with increased expression of α1 subunit (Overstreet et al., 2005). To test whether the observed acceleration of mIPSCs during development in vitro

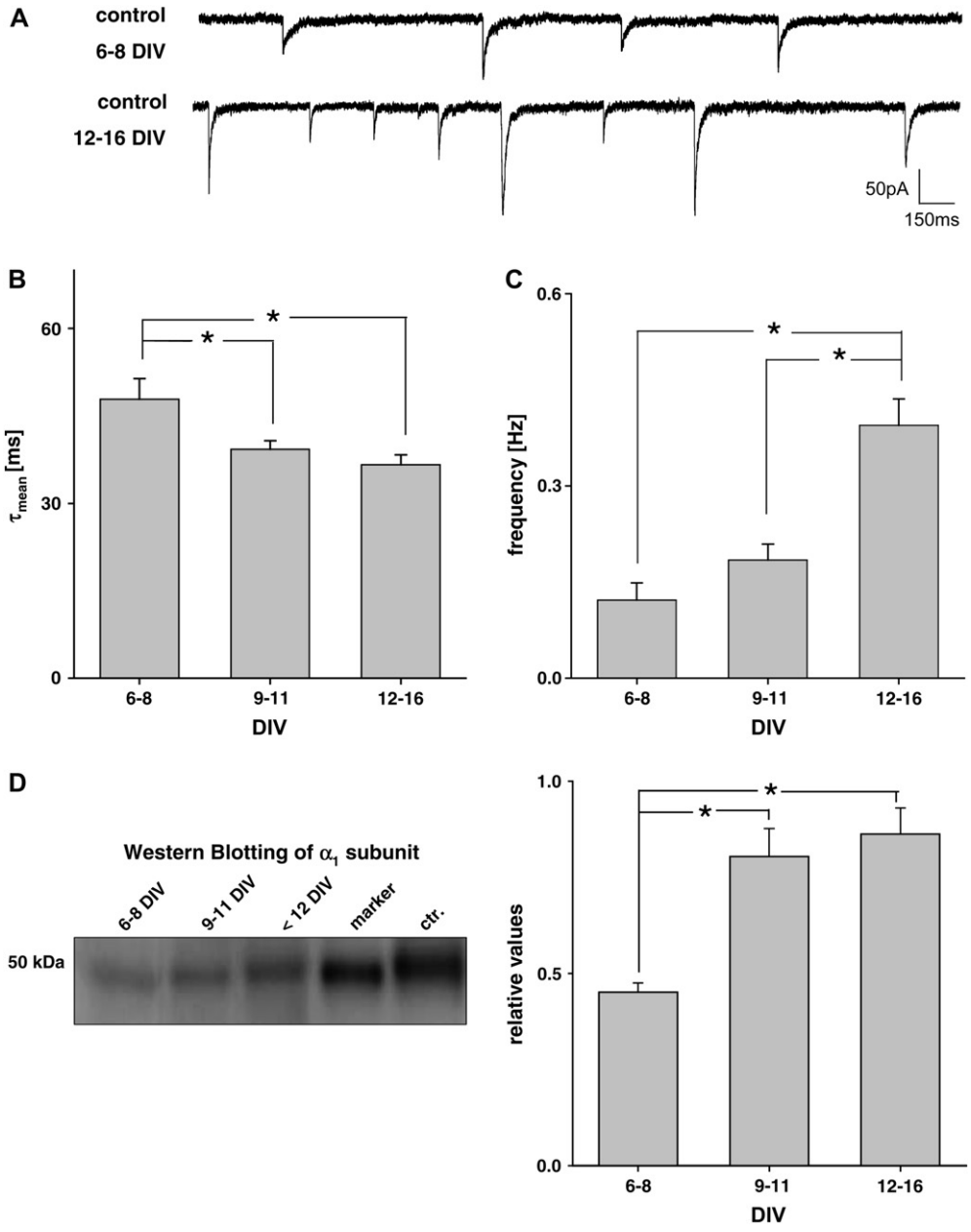

Fig. 1. The model of hippocampal neurons developing in vitro reproduces basic developmental features of GABAergic mIPSCs. A, Typical recordings of mIPSCs obtained from a neuron from the Young (6–8 DIV) age group (upper trace) and from Old (12–14 DIV) age group (lower trace). B, Average decay time constant (τ_{mean}) calculated for mIPSCs recorded from neurons in the Young, Intermediate (9–11 DIV) and Old age groups. The reduction of τ_{mean} with age is clearly seen. C, Averaged frequencies of mIPSCs recorded from neurons at different stages of development in vitro. The frequency of mIPSCs increases during development. D, expression of α1 subunit assessed using the technique of Western Blotting in respective age groups. For control conditions a purified $GABA_A$ receptor was used. In the left panel exemplary Western blot of α1 subunit is shown at different developmental ages while the right panel shows statistics of these results. Asterisks above the bars indicate statistically significant difference.

(Fig. 1) is associated with alterations in the α1 $GABA_A$ receptor subunit expression, the technique of Western blotting was applied. For this purpose, neuronal extracts were collected from the same culture at different time windows corresponding to the considered age groups. As shown in Fig. 1D, the expression of α1 subunit clearly increased during the considered period of culture in vitro, demonstrating that the developmental pattern of expression of this key $GABA_AR$ subunit is reproduced in our model.

3.2. Acute application of 17 β-estradiol does not affect mIPSCs

In our previous study (Asimiadou et al., 2005), we did not find any significant direct 17 β-estradiol effect on current responses elicited by rapid GABA applications. It seems interesting, however, to test whether 17 β-estradiol exerts any direct modulatory action on synaptic currents in the three considered age groups. To this end, synaptic currents were

recorded under control conditions and, while recording from the same cell, 17 β-estradiol was added at final concentration of 1 μM. mIPSCs recorded in control conditions and in the presence of 17 β-estradiol had indistinguishable averaged amplitudes and decay kinetics in Young, Intermediate and Old groups (not shown). Thus, these experiments further demonstrate that in our model 17 β-estradiol does not exert any detectable direct effect on GABAergic currents.

3.3. Long-term treatment with 17 β-estradiol affects both amplitude and kinetics of mIPSCs

In order to test the impact of the long term treatment with 17 β-estradiol, neuronal cultures were continuously treated with this compound at a final concentration of 1 μM starting from the 2nd DIV (see Section 2 for details). For each considered age group, mIPSCs were recorded from control neurons, neurons treated with 17 β-estradiol and neurons treated with DMSO (0.01%). DMSO at this concentration did not induce any detectable alteration in mIPSCs characteristics (not shown). As presented in Fig. 2A, the amplitudes of mIPSCs recorded from the same neuron showed substantial variability. To characterize the properties of these currents, mIPSCs in each individual record were averaged (Fig. 2B) and standard cumulative distributions were additionally constructed for each age group (Fig. 2C–E).

In all considered age groups, there was a trend for 17 β-estradiol to increase the mIPSCs amplitude (Fig. 2C–F). Significant effects were observed in the Young and Intermediate age groups (Fig. 2C,D,F). A nearly parallel shift of mIPSC amplitude cumulative distribution induced by treatment with 17 β-estradiol in the Young and Intermediate groups (Fig. 2C, D) indicates that 17 β-estradiol produces a relatively proportional up regulation of the entire population of mIPSC amplitudes rather than gives rise to appearance of mIPSCs with extra large amplitudes.

In parallel to amplitude analysis, the effect of 17 β-estradiol on the mean mIPSC decay time constant (τ_{mean}) was investigated. For this purpose, we considered the averaged (for each trace) and normalized mIPSCs and their decaying phases were fitted with the sum of two exponential functions (Eq. (1)) and the averaged time constant (τ_{mean}) was calculated as explained in Section 2. In the Intermediate group, the mean value of τ_{mean} of mIPSCs recorded from neurons treated with 17 β-estradiol was significantly longer than in respective controls (Fig. 3A, B).

Interestingly, while in all the age groups treated with 17 β-estradiol there was a tendency to increase the amplitude (Fig. 2F), in the case of decay kinetics a clear effect was seen only in the Intermediate group (Fig. 3).

While, as mentioned, the frequency of mIPSCs increased with age (Fig. 1C), treatment with 17 β-estradiol had no significant effect on this parameter (not shown).

3.4. Effect of 24 and 48 h treatment with 17 β-estradiol on mIPSCs

The experiments described above revealed that long-term treatment of neurons with 17 β-estradiol resulted in a modulation of mIPSCs amplitude (Fig. 2) and decay kinetics (Fig. 3). As explained in Section 2, in these experiments, 17 β-estradiol was continuously present in culture starting from the 2nd DIV. Thus, the time needed for the onset of 17 β-estradiol effect cannot be deduced from these data. In order to asses the time needed for 17 β-estradiol to exert its modulatory action on mIPSCs, we have recorded from neurons from all the age groups, treated for 24 and 48 h before recordings. As shown in Fig. 4A, 24 h treatment with 17 β-estradiol in the Young and Intermediate group resulted in a negligible effect on mIPSCs amplitude but after 48 h of treatment, the averaged amplitudes were significantly increased in both of these age groups. The extent of mIPSC amplitude enhancement after 48 h treatment with 17 β-estradiol was similar to that observed in experiments in which neurons were subjected to long term-treatment (see Figs. 2 and 4). These observations indicate that the time window needed for 17 β-estradiol to induce its modulatory effects on neurons from Young and Intermediate age group is close to 2 days. In the Old age group, after 24 h and 48 h of 17 β-estradiol treatment, a tendency to increase the mIPSC amplitude was observed (by roughly 10% for 24 and 48 h treatment) but this effect did not reach statistical significance (Fig. 4). In parallel, the analysis of the decay mIPSC kinetics was performed on control and on Intermediate and Old age groups but no effect of 17 β-estradiol was found (not shown). Altogether, these data show that 17 β-estradiol requires approximately 48 h to develop its effect on mIPSC amplitudes in the Young and Intermediate age groups. In addition, for the effect of 17 β-estradiol on decaying kinetics in the Intermediate group (such as in Fig. 3), a long-term treatment is required.

In order to check whether observed effect of 17 β-estradiol was due to activation of estrogen receptors, a series of experiments was performed on neuronal cultures treated with tamoxifen, a blocker of estrogen receptors.

In our experiments, when applied in a long-term fashion (more than 4 days), tamoxifen exhibited toxic effect on neuronal cells even at submicromollar concentrations. However, tamoxifen treatment (0.5 μM) up to 48 h did not induce any visible effect on neurons and the averaged frequency of mIPSCs was not affected by this drug (data not shown). Taking this into account we have restricted tests with tamoxifen (0.5 μM) to 48 h treatment of ''Intermediate'' neurons (where BE had its strongest effect). At this concentration, tamoxifen is known to effectively block estrogen receptors (Murphy and Segal, 1996). 48 h treatment with tamoxifen (co-applied with 17 β-estradiol at 1 μM) abolished the enhancement of mean mIPSC amplitude observed in the presence of 17 β-estradiol alone in the intermediate age group (in control conditions: 47.6 ± 5.7 pA, $n = 3$, and for tamoxifen and 17 β-estradiol treated neurons: 49.7 ± 3.4 pA, $n = 5$, $p > 0.05$). Deactivation parameters did not show significant difference as well (e.g.: control τ_{mean}: 32.0 ± 4.9 ms, $n = 3$, treated τ_{mean}: 31.1 ± 2.3 ms, $n = 5$).

3.5. Tonic conductance is altered by long-term treatment with 17 β-estradiol

The tonic conductance was assessed as explained in details in Section 2. Application of 100 μM of bicuculline, dissolved in

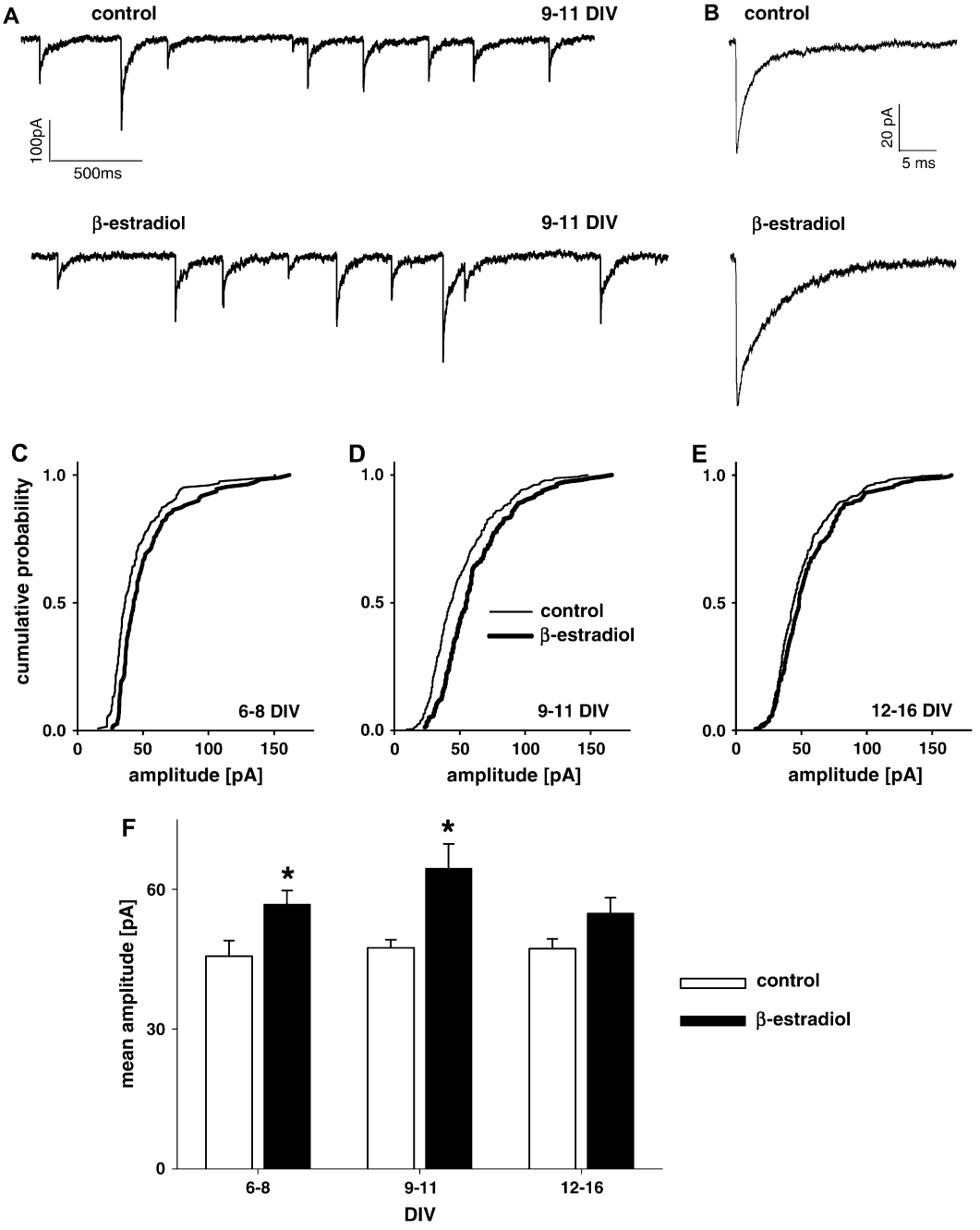

Fig. 2. Long-term treatment with 17 β-estradiol affects the amplitudes of mIPSCs. A, Typical traces with mIPSCs recorded from neurons in the Intermediate age group in control conditions (upper trace) and treated with 17 β-estradiol (lower trace). B, Example of averaged control mIPSC (upper trace) and treated with 17 β-estradiol (lower trace). C, D, E, Examples of typical cumulative histograms for mIPSC amplitudes in controls and 17 β-estradiol-treated neurons in Young, Intermediate and Old age groups, respectively. F, Statistics of averaged mIPSCs amplitudes in Young, Intermediate and Old age groups. Asterisks above the bars indicate statistically significant difference. The averaged values were obtained from at least $n = 24$ cells.

nominally GABA-free solution, produced a clear shift in the base line that was similar in all considered age groups (Fig. 5). Since the concentration of ambient GABA in brain is believed to be in the range of up to 1 μM (Farrant and Nusser, 2005), to further characterize the putative tonic conductance of considered neurons, GABA was applied at concentration of 0.1, 0.3 and 1 μM. GABA applications at these concentrations produced clear dose-dependent inward current (Fig. 5A, B). As shown in Fig. 5A–D, in Young and Intermediate age groups there was a clear trend of tonic current reduction in neurons treated with 17 β-estradiol but statistical significance was reached for each considered agonist concentration only in the Intermediate age group (Fig. 5D). In the Old age group there was no clear trend of modulation by 17 β-estradiol (Fig. 5E).

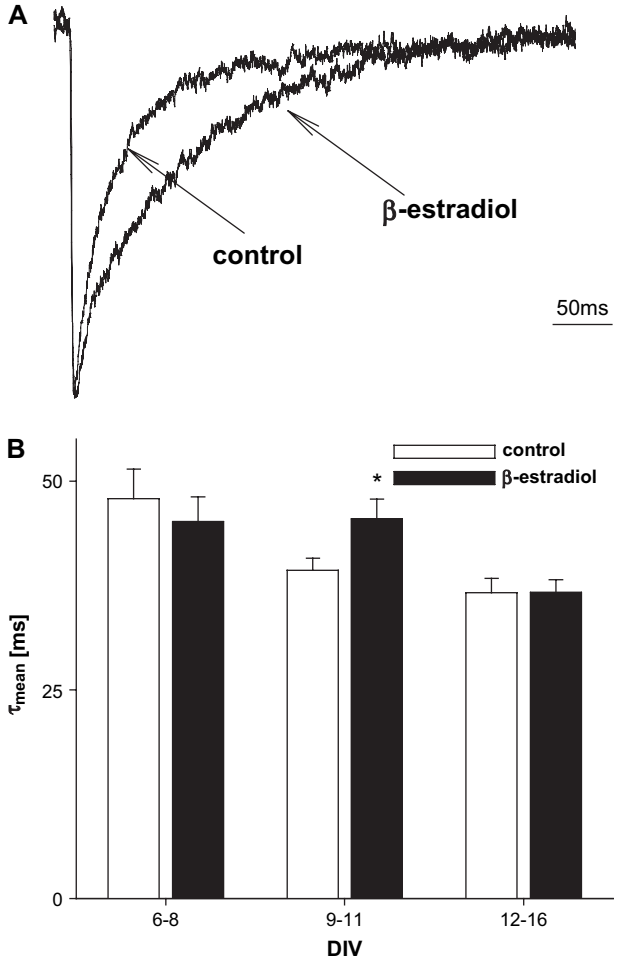

Fig. 3. Long-term treatment with 17 β-estradiol slows down the decay mIPSC kinetics in the Intermediate age group. A, Normalized and superimposed averaged mIPSCs recorded from control and 17 β-estradiol-treated culture in the Intermediate age group. B, Statistics of the long-term treatment with 17 β-estradiol on τ_{mean} in young, Intermediate and Old age groups. Asterisks above the bars indicate statistically significant difference.

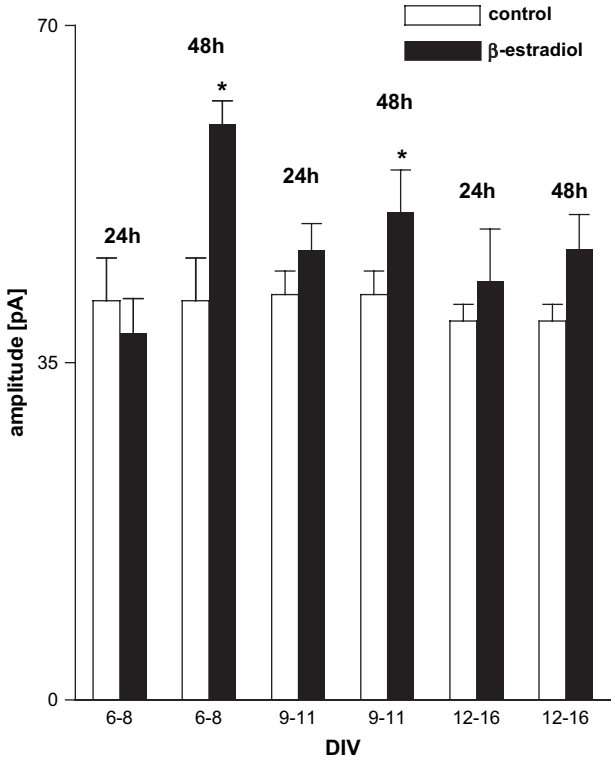

Fig. 4. 17 β-estradiol requires 48 h to exert its modulatory effect on the mIPSC amplitudes in the Young and Intermediate age groups. The graph shows statistics of mIPSCs amplitudes recorded in control conditions, after 24 and 48 h of 17 β-estradiol treatment in Young, Intermediate and Old age groups. Statistically significant increase in mIPSC amplitude is seen in Young and Intermediate age groups after 48 h but not after 24 h of treatment with 17 β-estradiol. mIPSC's amplitudes in control conditions were averaged in particular age group. Asterisks above the bars indicate statistically significant difference. The averaged values were obtained from at least $n = 4$ cells.

3.6. Effect of long term treatment with 17 β-estradiol on current responses to rapid GABA applications

The effect of treatment with 17 β-estradiol on amplitude and kinetics of synaptic currents (Figs. 2 and 3) as well as on whole-cell conductance activated by low [GABA] (Fig. 5) might indicate that 17 β-estradiol modifies the number and kinetic properties of $GABA_A$ receptors. In order to get additional insight into this issue, current responses to rapid GABA applications were recorded for control neurons and those long-term treated with 17 β-estradiol. Short (2 ms) applications of saturating [GABA] (10 mM) were used to characterize the deactivation kinetics and longer (up to 50 ms) applications of saturating [GABA] were employed to describe the desensitization process. However, a thorough analysis of these current responses did not reveal any significant effect of 17 β-estradiol treatment on these currents. For instance, in the Intermediate group, in which 17 β-estradiol exerted most apparent effects on mIPSCs (Figs. 2, 3 and 5), the mean amplitude of currents elicited by saturating [GABA] in the control group was 932 ± 118 pA ($n = 12$) and was only slightly larger than that measured from the 17 β-estradiol treated neurons from the same age group (829 ± 143 pA, $n = 12$) but this difference was far from reaching statistical significance ($p > 0.05$). Similarly, neither deactivation parameters (τ_{mean}, fast and slow time constants and respective percentages) nor desensitization characteristics (time constant, steady-state to peak) showed any significant difference between controls and the group treated with 17 β-estradiol. It needs to be taken into consideration, however, that excised patches may contain an unknown mixture of synaptic and extrasynaptic receptors while mIPSCs and "tonic" responses to low [GABA] are most likely mediated by receptors with different kinetic properties and different subcellular localization (Farrant and Nusser, 2005; see also Section 4).

3.7. 17 β-estradiol does not alter the subunit expression levels in the cultured hippocampal neurons

Standard Western-blotting protocol was used to check whether treatment with 17 β-estradiol affected the $GABA_A$ receptor subunit expression in neuronal culture during

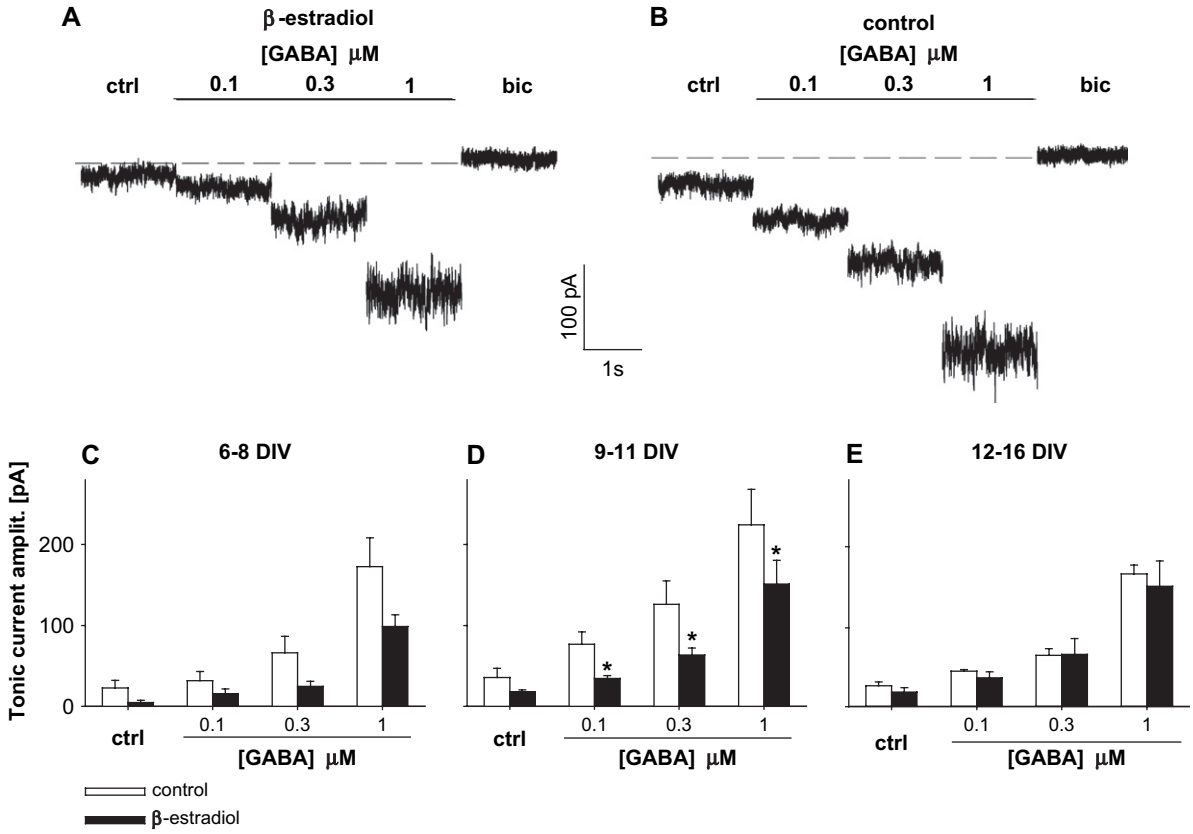

Fig. 5. Treatment with 17 β-estradiol reduces the tonic conductance elicited by applications of low [GABA]. A, B Typical whole-cell currents recorded when applying the following solutions: nominally GABA-free solution, 0.1, 0.3, 1.0 μM of GABA and wash containing 100 μM of bicuculline in 17 β-estradiol-treated neurons (A) and control neurons (B) from the intermediate age group. C, D, E, Statistics of currents recorded using the protocol presented in A and B for Young, Intermediate and Old age groups, respectively. In each group, the values of currents were calculated with respect to the baseline recorded in the presence of 100 μM of bicuculline. A particularly strong effect of 17 β-estradiol was observed in the Intermediate age group. Asterisks above the bars indicate statistically significant difference. The averaged values were obtained from at least $n = 3$ cells.

development in vitro. In order to verify whether 17 β-estradiol affected the expression of a considered $GABA_AR$ subunit, a direct comparison on a single gel of neuronal extracts originating from control, 17 β-estradiol-treated and DMSO-treated cultures was performed. Using this approach, we tested the effect of 17 β-estradiol on expression of α1, α2, α3, γ2 subunits in all considered age groups and no significant effect of this drug was found (data not shown, α1, $n = 7$; α2, $n = 3$; α3, $n = 7$; γ2, $n = 5$, where n corresponds to the number of repeats performed for each age group). Since in our experiments, 17 β-estradiol was found to affect the tonic conductance (Fig. 5), we have additionally checked for the effect of 17 β-estradiol on subunits that are known to form the receptors involved in tonic GABAergic currents (α4, α5, δ). We did not detect any 17 β-estradiol-induced change in expression of α5 subunit in any of the considered age groups ($n = 9$). When using the usual amount of total protein (see Section 2), no signal for α4 and δ subunits could be observed and when doubling the quantity of total protein, a faint signal at detection level was observed for both subunits in controls and in a group treated with 17 β-estradiol (not shown). In the groups of neurons treated with DMSO, no change in expression pattern of any considered $GABA_AR$ subunit was observed with respect to controls (not shown).

4. Discussion

The major finding of the present study is that long-term treatment with 17 β-estradiol alters GABAergic synaptic transmission during development in vitro. Interestingly, while phasic mIPSCs showed a trend towards up-regulation by long-term treatment with 17 β-estradiol (Figs. 2–4), the tonic conductance was reduced by this drug (Fig. 5). These effects, however, were not invariant during the considered developmental period (Figs. 2–5). Interestingly, the most intense 17 β-estradiol-induced alterations of GABAergic currents took place at the Intermediate age (9–11 DIV). It seems thus that neurons are mostly susceptible to modulation by 17 β-estradiol in the period in which a transition from ''young'' to ''adult'' phenotype of mIPSCs is taking place. While in control conditions the averaged time constant of mIPSC decaying phase (τ_{mean}) shows a clear acceleration with development (Fig. 1), in cultures treated with 17 β-estradiol this trend appears delayed (Fig. 3). This observation could suggest that long-term treatment with 17 β-estradiol delays the processes involved

in the development of the GABAergic system. A similar interpretation was suggested by Perrot-Sinal et al. (2001) who measured calcium signals elicited by muscimol applications to developing neurons and found that treatment with 17 β-estradiol prolonged the period in which GABA was excitatory. However, although it cannot be excluded that 17 β-estradiol interferes with some mechanisms underlying development of GABAergic system, our data obtained using Western Blotting technique indicate that the expression of several $GABA_AR$ subunits is not affected by this drug in the considered model of development in vitro. In particular, while acceleration of mIPSCs decay is correlated with a clear increase in α1 subunit expression (Fig. 1), the prolongation of mIPSCs at the Intermediate age by 17 β-estradiol (Fig. 3) is not coincident with any significant alteration of α1 or any other subunit expression. Moreover, the lack of any clear effect of long-term treatment with 17 β-estradiol on current responses recorded from excised patches provides further arguments that this drug does not induce any massive effect on expression of $GABA_AR$ subunits in our model. The lack of evidence for any clear 17 β-estradiol effect on expression of $GABA_A$ receptor subunits is surprising as some previous studies implicated such 17 β-estradiol action in other models. In particular, Pierson et al. (2005) has found that treatment with 17 β-estradiol increased RNA level of α2 and β3 as well as decreased RNA for α5 subunit. However, it needs to be emphasized that Pierson et al. (2005) have used a different experimental model – neuronal cell line (NT2-N) and that RNA level does not always mirror the expression of proteins. The most apparent difference with respect to the data presented here is the observation by Shen et al. (2005) that treatment with 17 β-estradiol and progesterone increased expression of δ subunit and tonic current in CA1 hippocampus from adult rats. In our system, δ subunit was at the detection limit both in control and in 17 β-estradiol-treated cultures but tonic current was clearly decreased by treatment with this drug (Fig. 5) suggesting that this effect could be unrelated to expression of δ subunit. There are, however, several reasons why these differences between our data and those of Shen et al. (2005) could occur. In the present study, 17 β-estradiol alone was used, while Shen et al. (2005) additionally supplied progesteron. In addition, Shen et al. used adult animals that were treated in vivo. Similarly to our findings, Weiland and Orchinik (1995) did not find any alteration of subunit expression when treating neurons with 17 β-estradiol alone.

The observation that treatment with 17 β-estradiol up regulated mIPSCs (Fig. 2) is reminiscent of finding of Wang and Tsai (1999) who considered young tilapia brain and found that treatment with this drug increased GABA content. However, increased mIPSC amplitudes could result from several mechanisms (e.g. modulation of postsynaptic receptors) that could be unrelated to the mean GABA content in the brain. Moreover, our finding that treatment with 17 β-estradiol induced a drop in the tonic conductance indicate that at least a part of 17 β-estradiol-induced modulation concerns intrinsic membrane properties of treated neurons.

Most studies concerning the effect of 17 β-estradiol were carried out on neurons coming from adult animals and therefore their comparison to the data presented here is not straightforward. In most reports, 17 β-estradiol was implicated as a factor promoting disinhibition. In animals treated with 17 β-estradiol, Rudick and Woolley (2001) observed a transient decrease in the number of GAD puncta, decrease in amplitude and in decay rate of evoked IPSCs but mIPSCs were not affected except for reduction of their frequency. However, in a more recent study from this group (Rudick et al., 2003), it was found that treatment with 17 β-estradiol produced a skew in distributions of amplitudes and decay times due to appearance of mIPSC subpopulations with extra large amplitudes and slower decays. It is thus possible that increase in mIPSC amplitudes and prolongation of decay observed in the present study could be partly reproduced in adult animals in the in vivo model. It is of note, however, that in our experiments, contrary to Rudick and Woolley (2001) and Rudick et al. (2003) we did not observe any effect of 17 β-estradiol treatment on mIPSCs frequency.

The most novel finding of the present study is the reduction of bicuculline-sensitive tonic conductance by long term treatment with 17 β-estradiol. This effect may have an important impact on neuronal signaling, as tonic GABAergic conductance plays a crucial role in neuronal inhibition (Farrant and Nusser, 2005). The charge transfer due to tonically active GABAergic conductance can considerably exceed the one mediated by phasic (synaptic) events (Nusser and Mody, 2002; Mody and Pearce, 2004). It is worth mentioning in this context that occurrence of epilepsy was found to be correlated with the level of circulating 17 β-estradiol (Backstrom, 1976) and that this drug can be considered as a pro-convulsive agent (Lason, 2000). It cannot be excluded that the reduction of tonic GABAergic conductance by 17 β-estradiol observed in the present study could reflect a pro-convulsive mechanism or represent a factor favoring occurrence of epilepsy. However, when considering the GABAergic system in the context of development, it needs to be kept in mind that in the early postnatal life, GABA is excitatory because, at this developmental stage, the equilibrium potential for chloride is less negative than the resting potentials of neurons (Cherubini et al., 1991; Khazipov et al., 2004). Nevertheless, it seems likely that, if enhancement of $GABA_ARs$ activity was a factor inducing neurodegeneration, then a reduction of tonic GABA conductance by 17 β-estradiol could be a good candidate to rescue the neurons from apoptosis. However, the verification of this hypothesis is not straightforward because it would require testing the pro-survival action of 17 β-estradiol together with factors specifically affecting the tonic conductance. This, however, seems difficult because the population of $GABA_ARs$ underlying tonic conductance appears to be heterogeneous (Stell et al., 2003; Semyanov et al., 2004).

Long-term treatment with 17 β-estradiol reduced currents elicited by low doses of GABA (Fig. 5) and had no significant effect on current responses to rapid applications of saturating [GABA] (see Section 3). The reason for this apparent discrepancy could be that $GABA_ARs$ that are responsible for tonic

conductance have, as mentioned, higher affinity for GABA than the synaptic ones. Thus, while low [GABA] would activate mainly receptors mediating tonic component, saturating [GABA] would activate $GABA_A$Rs underlying both tonic and phasic currents. It is thus possible that, at saturating [GABA], the effects of 17 β-estradiol on tonic and phasic conductances would partially compensate each other. Verification of this possibility, however, would require elucidation of mechanism of mIPSCs modulation by long-term treatment with 17 β-estradiol. In particular, our present data do not allow us to discriminate between pre- and postsynaptic mechanisms of 17 β-estradiol modulation of mIPSCs.

As shown in Fig. 5, a clearly visible tonic current was present both in control and in 17 β-estradiol-treated cells when applying normal, nominally GABA-free washing solution. This might be regarded surprising because the solution was intensely applied with a large pipette (i.d. 1 mm) placed right in front of the cell and precautions were taken to avoid any contamination with GABA. Moreover, in our experimental model of cultured neurons, the cells are well exposed to applied solutions and therefore it is not difficult to control the environment of the neuron from which the recording is made. It might be suspected that the neuronal cells in our model could contain a proportion of $GABA_A$ receptors characterized by a marked spontaneous activity. It has been recently reported that receptors containing ϵ subunit show a pronounced spontaneous open probability (Wagner et al., 2005) but this subunit has not been found in the hippocampus (Sinkkonen et al., 2000). However, it is possible that the bicuculline-sensitive tonic current in a nominally GABA-free solution simply reflects the activity of $GABA_A$Rs whose spontaneous open probability is low but due to large number of receptors they build up a detectable spontaneous current. It needs to be emphasized that cultured neurons in our model contain a very high number of $GABA_A$Rs. The fact that application of [GABA] as low as 3–10 μM elicit whole-cell responses of several nanoampers (not shown) and that responses to saturating [GABA] from tiny excised patches are typically of several hundreds of picoampers (at −70 mV), indicate that a single neuron in our model may contain several tens of thousands of $GABA_A$Rs. Thus, with such a high number of receptors, a spontaneous open probability of less than 0.001 would be able to produce a spontaneous bicuculline-sensitive current observed in the present experiment. The mechanism and the role of spontaneous activity of $GABA_A$Rs and related tonic conductance in regulating the neuronal excitability will require further studies including single channel recordings at low [GABA] or in GABA-free solutions.

Concentration of free 17 β-estradiol used in the present pharmacological investigations exceed that present in vivo (10^{-12} M up to 10^{-9} M, e.g. Rune et al., 2006; Montano et al., 1995). However, in vivo, due to adsorption to a variety of binding sites, vast majority of estradiol is bound, and only few percent of estradiol is directly accessible (free) (Montano et al., 1995). It is thus a common practice to use 17 β-estradiol in excess in the in vitro studies (see e.g. Murphy and Segal, 1996).

The major question that remains to be elucidated is the molecular mechanism of 17 β-estradiol-induced effects described in the present study. The observation that tamoxifen abolishes the enhancement of mIPSCs amplitude in the presence of 17 β-estradiol in the Intermediate age group provides evidence that the modulatory effect of 17 β-estradiol is initiated by the activation of estrogen receptors. However, the downstream cascade of this processes remains unclear. Moreover, our tests with tamoxifen had to be limited to short-term treatments (48 h) due to toxic effect of this drug on the long-term cultures. As mentioned in the Introduction, 17 β-estradiol activates or interferes with several signaling pathways and affects both function and morphology of the nervous system. Our data suggest that treatment with this drug differentially affects mechanisms determining mIPSC amplitude and its time course (decay kinetics). While, 48 h treatment with 17 β-estradiol of cultures in Young and Intermediate age groups was sufficient to reproduce the effect of the long-term treatment of this drug on mIPSC amplitude (Figs. 2 and 4), no effect of 17 β-estradiol on mIPSC decay was observed after 48 h treatment in the Intermediate age group. It seems thus that while for the onset of 17 β-estradiol effect on mIPSC amplitude approximately 48 h of treatment are needed, the effect of 17 β-estradiol on mIPSC decay kinetics requires longer interaction with this drug starting from early developmental stage. Western blotting performed here did not confirm the hypothesis that 17 β-estradiol might affect the expression level of considered here $GABA_A$R subunits. There are, however, several other possibilities of pre- or postsynaptic mechanisms that might modulate the synaptic currents. For instance presynaptic site of action might be suggested by the observation that β-estradiol down-regulates GAD (Murphy et al., 1998a). Functioning of a neuron is determined not only by averaged expression levels of different $GABA_A$R subtypes but also by their subcellular distribution. For instance, enhancement in clusterization of synaptic $GABA_A$Rs or altered positioning of synapses could affect the GABAergic signaling without any change in the overall expression of respective receptors. In addition, a presynaptic action of 17 β-estradiol cannot be excluded although the lack of 17 β-estradiol effect on the mIPSC frequency is not supporting this possibility. On the other hand, it is known that even slight modifications in the neurotransmitter releasing machinery or in the synapse geometry could result in profound alterations in the synaptic current properties (Clements, 1996; Mozrzymas, 2004). Pre- and postsynaptic mechanisms could be also modulated by 17 β-estradiol-dependent posttranslational mechanisms such as phosphorylation or dephosphorylation by respective kinases and phosphatases. We cannot exclude, for instance, that the lack of difference in current responses to rapid GABA applications in control and 17 β-estradiol-treated neurons could be due to washout of intracellular milieu upon patch excision.

In conclusion, we provide evidence that 17 β-estradiol differentially affects the phasic and tonic components of GABAergic currents in neurons developing in vitro but the molecular mechanism of this modulation remains to be elucidated.

Acknowledgement

This work was supported by grant from Polish Ministry of Education and Science (No. PBZ-MIN-001/P05/28). Maria Pytel is indebted to the Foundation for Polish Science (FNP) for the scholarship for young scientist (2006).

The authors are thankful to Mrs. Ewa Pindel and Mr. Piotr Brzeznicki for their help in preparing and performing a part of experiments.

References

Andjus, P.R., Stevic-Marinkovic, Z., Cherubini, E., 1997. Immunoglobulins from motoneurone disease patients enhance glutamate release from rat hippocampal neurones in culture. Journal of Physiology 504, 103–112.

Asimiadou, S., Bittigau, P., Felderhoff-Mueser, U., Manthey, D., Sifringer, M., Pesditschek, S., Dzietko, M., Kaindl, A.M., Pytel, M., Studniarczyk, D., Mozrzymas, J.W., Ikonomidou, C., 2005. Protection with estradiol in developmental models of apoptotic neurodegeneration. Annals of Neurology 58, 266–276.

Backstrom, T., 1976. Epileptic seizures in women related to plasma estrogen and progesterone during the menstrual cycle. Acta Neurologica Scandinavica 54, 321–347.

Barberis, A., Lu, C., Vicini, S., Mozrzymas, J.W., 2005. Developmental changes of GABA synaptic transient in cerebellar granule cells. Molecular Pharmacology 67 (4), 1221–1228.

Bencsits, E., Ebert, V., Tretter, V., Sieghart, W., 1999. A significant part of native γ-aminobutyric acid A receptors containing α_4 subunits does not contain γ or δ subunits. Journal of Biological Chemistry 274, 19613–19616.

Cherubini, E., Conti, F., 2001. Generating diversity at GABAergic synapses. Trends in Neurosciences 24 (3), 155–162.

Cherubini, E., Gaiarsa, J.L., Ben-Ari, Y., 1991. GABA: an excitatory transmitter in early postnatal life. Trends in Neurosciences 14 (12), 515–519.

Clements, J.D., 1996. Transmitter timecourse in the synaptic cleft: its role in central synaptic function. Trends in Neurosciences 19 (5), 163–171.

Ebert, V., Scholze, P., Fuchs, K., Sieghart, W., 1999. Identification of subunits mediating clustering of $GABA_A$ receptors by rapsyn. Neurochemistry International 34, 453–463.

Farrant, M., Nusser, Z., 2005. Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. Nature Reviews Neuroscience 6 (3), 215–229.

Hollrigel, G.S., Soltesz, I., 1997. Slow kinetics of miniature IPSCs during early postnatal development in granule cells of the dentate gyrus. Journal of Neuroscience 17 (13), 5119–5128.

Jonas, P., 1995. Fast application of agonists to isolated membrane patches. In: Sakmann, B., Neher, E., (Eds.), Single-channel Recording. Plenum Press, New York and London, pp. 231–243.

Khazipov, R., Khalilov, I., Tyzio, R., Morozova, E., Ben-Ari, Y., Holmes, G.L., 2004. Developmental changes in GABAergic actions and seizure susceptibility in the rat hippocampus. European Journal of Neuroscience 19, 590–600.

Lason, W., 2000. Effects of estrogens on seizures and neurotoxicity. Polish Journal of Pharmacology 52 (1), 59–62.

Mody, I., Pearce, R.A., 2004. Diversity of inhibitory neurotransmission through $GABA_A$ receptors. Trends in Neurosciences 27 (9), 569–575.

Montano, M.M., Welshons, W.V., Saal, F.S., 1995. Free estradiol in serum and brain uptake of estradiol during fetal and neonatal sexual differentiation in female rats. Biology of Reproduction 53, 1198–1207.

Mossier, B., Tögel, M., Fuchs, K., Sieghart, W., 1994. Immunoaffinity purification of γ-aminobutyric $acid_A$ ($GABA_A$) receptors containing γ_1-subunits. Evidence for the presence of a single type of γ-subunit in $GABA_A$ receptors. Journal of Biological Chemistry 269, 25777–25782.

Mozrzymas, J.W., 2004. Dynamism of $GABA_A$ receptor activation shapes the "personality" of inhibitory synapses. Neuropharmacology 47 (7), 945–960.

Murphy, D.D., Segal, M., 1996. Regulation of dendritic spine density in cultured rat hippocampal neurons by steroid hormones. Journal of Neuroscience 16, 4059–4068.

Murphy, D.D., Cole, N.B., Greenberger, V., Segal, M., 1998a. Estradiol increases dendritic spine density by reducing GABA neurotransmission in hippocampal neurons. Journal of Neuroscience 18 (7), 2550–2559.

Murphy, D.D., Cole, N.B., Segal, M., 1998b. Brain-derived neurotrophic factor mediates estradiol-induced dendritic spine formation in hippocampal neurons. Proceedings of the National Academy of Sciences of the United States of America 95 (19), 11412–11417.

Neville Jr., D.M., Glossmann, H., 1974. Molecular weight determination of membrane protein and glycoprotein subunits by discontinuous gel electrophoresis in dodecyl sulfate. Methods in Enzymology 32, 92–102.

Nusser, Z., Mody, I., 2002. Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. Journal of Neurophysiology 87 (5), 2624–2628.

Ogris, W., Lehner, R., Fuchs, K., Furtmüller, B., Höger, H., Homanics, G.E., Sieghart, W., 2006. Investigation of the abundance and subunit composition of $GABA_A$ receptor subtypes in the cerebellum of α1-subunit-deficient mice. Journal of Neurochemistry 96, 136–147.

O'Neill, K., Chen, S., Brinton, R.D., 2004. Impact of the selective estrogen receptor modulator, tamoxifen, on neuronal outgrowth and survival following toxic insults associated with aging and Alzheimer's disease. Experimental Neurology 188, 268–278.

Ortinski, P.I., Lu, C., Takagaki, K., Fu, Z., Vicini, S., 2004. Expression of distinct alpha subunits of $GABA_A$ receptor regulates inhibitory synaptic strength. Journal of Neurophysiology 92 (3), 1718–1727.

Overstreet, L.S., Wadiche, L., Bromberg, D.A., Bensen, A.L., Westbrook, G.L., 2005. GABAergic signaling to newborn neurons in dentate gyrus. Journal of Neurophysiology 94 (6), 4528–4532.

Perrot-Sinal, T.S., Davis, A.M., Gregerson, K.A., Kao, J.P., McCarthy, M.M., 2001. Estradiol enhances excitatory gamma-aminobutyric acid-mediated calcium signaling in neonatal hypothalamic neurons. Endocrinology 142 (6), 2238–2243.

Pierson, R.C., Lyons, A.M., Greenfield Jr., L.J., 2005. Gonadal steroids regulate $GABA_A$ receptor subunit mRNA expression in NT2-N neurons. Brain Research. Molecular Brain Research 18 138 (2), 105–115.

Pöltl, A., Hauer, B., Fuchs, K., Tretter, V., Sieghart, W., 2003. Subunit composition and quantitative importance of $GABA_A$ receptor subtypes in the cerebellum of mouse and rat. Journal of Neurochemistry 87, 1444–1455.

Rudick, C.N., Woolley, C.S., 2001. Estrogen regulates functional inhibition of hippocampal CA1 pyramidal cells in the adult female rat. Journal of Neuroscience 21 (17), 6532–6543.

Rudick, C.N., Woolley, C.S., 2003. Selective estrogen receptor modulators regulate phasic activation of hippocampal CA1 pyramidal cells by estrogen. Endocrinology 144 (1), 179–187.

Rudick, C.N., Gibbs, R.B., Woolley, C.S., 2003. A role for the basal forebrain cholinergic system in estrogen-induced disinhibition of hippocampal pyramidal cells. Journal of Neuroscience 23 (11), 4479–4490.

Rune, G.M., Lohse, C., Prange-Kiel, J., Fester, L., Frotscher, M., 2006. Synaptic plasticity in the hippocampus: effects of estrogen from the gonads or hippocampus? Neurochemical Research 31, 145–155.

Sarto-Jackson, I., Ramerstorfer, J., Ernst, M., Sieghart, W., 2006. Identification of amino acid residues important for assembly of GABA receptor α_1 and γ_2 subunits. Journal of Neurochemistry 96, 983–995.

Semyanov, A., Walker, M.C., Kullmann, D.M., Silver, R.A., 2004. Tonically active $GABA_A$ receptors: modulating gain and maintaining the tone. Trends in Neurosciences 27 (5), 262–269.

Shen, H., Gong, Q.H., Yuan, M., Smith, S.S., 2005. Short-term steroid treatment increases delta $GABA_A$ receptor subunit expression in rat CA1 hippocampus: pharmacological and behavioral effects. Neuropharmacology 49 (5), 573–586.

Sinkkonen, S.T., Hanna, M.C., Kirkness, E.F., Korpi, E.R., 2000. $GABA_A$ receptor epsilon and theta subunits display unusual structural variation between species and are enriched in the rat locus ceruleus. Journal of Neuroscience 20 (10), 3588–3595.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Analytical Biochemistry 150, 76–85.

Stell, B.M., Brickley, S.G., Tang, C.Y., Farrant, M., Mody, I., 2003. Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by delta subunit-containing $GABA_A$ receptors. Proceedings of the National Academy of Sciences of the United States of America 100 (24), 14439–14444.

Wagner, D.A., Goldschen-Ohm, M.P., Hales, T.G., Jones, M.V., 2005. Kinetics and spontaneous open probability conferred by the epsilon subunit of the $GABA_A$ receptor. Journal of Neuroscience 25 (45), 10462–10468.

Wang, L.H., Tsai, C.L., 1999. Effects of gonadal steroids on the GABA and glutamate contents of the early developing tilapia brain. Brain Research. Developmental Brain Research 114 (2), 273–627.

Weiland, N.G., Orchinik, M., 1995. Specific subunit mRNAs of the $GABA_A$ receptor are regulated by progesterone in subfields of the hippocampus. Brain Research. Molecular Brain Research 32, 271–278.

Zezula, J., Fuchs, K., Sieghart, W., 1991. Separation of α_1, α_2 and α_3 subunits of the $GABA_A$-benzodiazepine receptor complex by immunoaffinity chromatography. Brain Research 563, 325–328.