

Prolongation and enhancement of γ -aminobutyric acid_A receptor mediated excitation by chronic treatment with estradiol in developing rat hippocampal neurons

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Keywords: calcium influx, development, excitatory amino acids, GABA, Sprague–Dawley rats, steroid hormones

Abstract

GABA_A receptor activation during brain development is a critical source of excitation. This is due to the positive equilibrium potential for chloride relative to resting membrane potential, resulting in membrane depolarization sufficient to open voltage sensitive calcium channels. The gonadal steroid estradiol has pronounced trophic effects on the developing hippocampus, promoting cell survival and synaptogenesis. In the current study, we investigated the effect of estradiol on GABA_A receptor-mediated calcium transients in cultured neonatal hippocampal neurons, from Sprague–Dawley rats, using the calcium sensitive dye, Fura-2-AM. Treatment of hippocampal neurons with physiological levels of estradiol significantly increased the peak amplitude of calcium transients, increased the number of cells responding to the GABA_A agonist muscimol with membrane depolarization, and delayed the rate of clearance of free intracellular calcium. These effects were significantly attenuated by pretreatment with the oestrogen receptor antagonist ICI-182,780. This suggests that estradiol, via its action on the oestrogen receptor, prolongs the developmental duration of depolarizing GABA. Estradiol likely maintains GABA-mediated excitation by promoting increased protein levels of the active/phosphorylated form of the chloride cotransporter Na⁺K⁺2CL⁻ and L-type voltage sensitive calcium channels containing the α 1C subunit. We propose that a component of the trophic effects of estradiol on hippocampal development results from enhanced calcium influx subsequent to GABA_A receptor activation.

Introduction

GABA is the predominant inhibitory neurotransmitter in the adult brain but is also an important source of excitatory drive in immature neurons. Excitation is achieved via GABA_A receptor activation and opening of voltage sensitive calcium channels following membrane depolarization (Cherubini *et al.*, 1991; Obrietan & van den Pol, 1995; Leinekugel *et al.*, 1999). The resultant increase in intracellular calcium subsequent to GABA_A receptor activation confers trophic effects on the developing brain (Ben-Ari *et al.*, 1994; Obata, 1997; Fiszman & Schousboe, 2004). Developmentally, the excitatory vs. inhibitory action of GABA is a function of the transmembrane chloride gradient. During the neonatal period, the equilibrium potential for chloride (E_{Cl}^-) is positive relative to the resting membrane potential (Rivera *et al.*, 1999; Wang *et al.*, 2001; Rivera *et al.*, 2005). Therefore, there is a net outward driving force upon chloride when GABA_A receptors are open, resulting in membrane depolarization sufficient to open voltage sensitive calcium channels (Leinekugel *et al.*, 1995; Obrietan & van den Pol, 1995; Liljelund *et al.*, 2000; Owens & Kriegstein, 2002; Ikeda *et al.*, 2003). As development progresses, E_{Cl}^- becomes negative

relative to the resting membrane potential, shifting the driving force on chloride inward, and leading to GABA_A receptor-mediated hyperpolarization, the primary basis for synaptic inhibition in the mature brain.

Placing depolarizing GABA in the context of brain development requires incorporation of physiologically relevant modulators that exert functional influences on maturation. We have recently implicated depolarizing GABA action in the establishment of sex differences in the hypothalamus that are determined by neonatal exposure to estradiol (McCarthy *et al.*, 2002). The hippocampus may likewise be affected by the interplay between estradiol and GABA (Murphy *et al.*, 1998b; Segal & Murphy, 2001), given that sex differences in hippocampal morphometry are established within the first week of life (Hilton *et al.*, 2003; Nuñez *et al.*, 2003a) and persist into adulthood (Madeira & Lieberman, 1995; Isgor & Sengelaub, 1998). Intriguingly, we have observed that the immature hippocampus is exposed to high levels of estradiol in both males and females during normal brain development (Amateau *et al.*, 2004). Estradiol exerts trophic effects on hippocampal development such as enhancing cell survival (Hilton *et al.*, 2003), promoting neurite extension (Brinton *et al.*, 1997) and increasing dendritic spine density (Murphy *et al.*, 1998b), endpoints that may be related to the marked induction of brain derived neurotrophic factor (BDNF) by estradiol in the neonatal hippocampus (Murphy *et al.*, 1998a; Solum & Handa, 2002). BDNF levels increase in response to depolarizing GABA, which in turn promotes additional

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Received 14 September 2004, accepted 19 April 2005

GABA release in a positive feed-forward enhancement of excitation (Obrietan *et al.*, 2002; Rivera *et al.*, 2004). Taken together, these observations have led us to rethink the role of estradiol in hippocampal development and postulate that it may act as a trophic factor during normal hippocampal development, as well as play a role in the establishment of sex differences in the hippocampus. In order to elucidate the roles for estradiol in hippocampal development, we have investigated the effect of estradiol on calcium transients evoked by GABA_A receptor-mediated depolarization in cultured hippocampal neurons and potential mechanisms via which estradiol modulates GABA_A receptor-mediated excitation.

Materials and methods

Preparation of primary hippocampal neuron cultures

Primary cultures of hippocampal neurons were prepared based on Banker & Goslin (1998). Timed pregnant Sprague–Dawley female rats purchased from Charles River Laboratories (Wilmington, MA, USA) were killed on gestational day 18 or 22. In order to minimize stress and discomfort perceived by the dams, animals were overdosed with carbon monoxide prior to killing. All animal procedures were approved by the University of Maryland, Baltimore Institutional Animal Care and Use Committee, and followed National Institute of Health guidelines.

Hippocampi were dissected into HBSS+ [88 mL sterile H₂O, 10 mL Hank's balanced salt solution (Ca²⁺ and Mg²⁺-free) 10×, 1 mL HEPES buffer, 1.0 M, pH 7.3, 1 mL antibiotic/antimycotic 100× liquid], then additional HBSS+ was added to the tube to a volume of 4.5 mL, with 0.5 mL trypsin (2.5%), and incubated in a 37 °C water bath for 15 min. The supernatant was discarded and the tissue washed with HBSS+. Cells were dissociated by trituration, with cell number and viability determined by trypan blue exclusion. Cells were plated on 25 mm Poly L-lysine (Sigma, St. Louis, MO, USA) coated coverslips at a density of 300 000 cells per coverslip, and placed in 60 mm dishes containing 4 mL plating medium [86 mL MEM, 10 mL horse serum, 3 mL glucose (filter sterilized, 20%) 1 mL pyruvic acid, 100 mM]. Cells were allowed 4 h to adhere to the coverslips in a 37 °C, 5% CO₂ incubator. The coverslips were removed from the plating dishes and placed into 60 mm dishes filled with Neurobasal+ [1 mL B-27 supplement, 1 mL antibiotic/antimycotic 100×, 125 µL L-glutamine and filled to 50 mL with Neurobasal (phenol red free and serum free)]. All cell culture chemicals and solutions were obtained from Invitrogen (Carlsbad, CA, USA).

Drug treatment

Hippocampal cultures were treated on days *in vitro* (DIV) 0, 2 and 4 with 1 nM 17β-estradiol (dissolved in DMSO), 1 µM ICI-182,780 (dissolved in DMSO), 1 µM ICI-182,780, followed 30 min later by 1 nM 17β-estradiol, or vehicle alone (DMSO). The estradiol treatment regimen was chosen because it mimics the physiological exposure to estradiol as occurs in neonatal male rats. We have previously determined that 1 nM 17β-estradiol administered every other day is sufficient to maintain culture medium estradiol levels at around 200 pg/mL – at the high end of the physiological range for the neonatal brain (Nuñez *et al.*, 2003a). To investigate the effect of prolonged estradiol on calcium transients induced by muscimol, a separate set of hippocampal cultures was treated on DIV 0, 2, 4, 6 and 8 with 1 nM 17β-estradiol dissolved in DMSO or vehicle. To investigate the effect of prolonged muscimol exposure (extended GABA_A receptor activation), a subset of the culture dishes was treated

on DIV 4 with 10 µM muscimol or sterile saline. The treatment was repeated four hours later, and the entire process repeated on DIV 5, for a total of four muscimol treatments. This component of the experiment is based in a previously published *in vivo* paradigm (Nuñez *et al.*, 2003a, b) where we gave multiple muscimol administrations to induce excitotoxic brain damage. For proper interpretation of those results, it is important to determine if short-term, repeated muscimol administration would affect muscimol-induced calcium transients by advancing the normal developmental progression towards hyperpolarizing GABA, as previously demonstrated (Ganguly *et al.*, 2001).

Calcium imaging of cultured hippocampal neurons

Cells were used for calcium imaging on DIV 5, 6, 8 and 10. These DIV were chosen because they cover ages roughly equivalent to the day of birth (DIV 5 for neurons cultured from embryonic day 18 fetuses) to postnatal day 9 (DIV 10 for neurons cultured from embryonic day 22 fetuses). Rats in our vivarium typically give birth on embryonic day 22–23. This time course allowed for assessment of the impact of DIV as well as fetal age at time of culturing, and controlled for parameters such as plating density and viability. Given that neurogenesis in the dentate gyrus continues into postnatal life, dentate granule cells would be relatively under-represented in our cultures compared to other cell types.

On the day of calcium imaging, hippocampal neurons were incubated with the cell permeant fluorescent indicator Fura-2-AM (3 µM; Molecular Probes, Eugene, OR, USA) in DMSO (< 0.5%) at room temperature for 30 min before the coverslips were transferred to a tissue chamber mounted on a microscope stage, and superfused with physiological salt solution (PSS 140 mM NaCl, 5 mM KCl, 1.2 mM Na₂PO₄, 1.4 mM MgCl₂, 1.8 mM CaCl₂, 11.5 mM glucose, 10 mM HEPES; pH 7.4) at 32–34 °C to remove extracellular dye and allow for esterification of Fura-2-AM. All drugs were administered in the superfusate. The imaging system uses a Zeiss Axiovert 100 inverted microscope, with illumination provided by a Til Photonics Polychrome II Monochromator (Applied Scientific Instrumentation, Eugene, OR, USA). Fluorescent images were obtained using a CCD video camera and image intensifier. Image acquisition was performed with the Metafluor 5.0 Imaging System (Universal Imaging Corporation, Downingtown, PA, USA). Cells in the field of view were characterized morphologically using a 60× objective, making a distinction between neurons and glia. Individual cells were chosen for analyses by the investigator and traced using the Metafluor program. Numerous criteria were used to distinguish neurons from glia, but the most important was shape – the somas of pyramidal neurons are triangular in appearance with rounded and clearly distinct edges. Neurons usually possess at least two primary processes. In contrast, glia are amorphous in shape, with flat and nondistinct edges and no distinct processes. We have included a photomicrograph of our cell cultures visualized without counter stain to elucidate this point (Fig. 1). In the current experiments, only data obtained from neurons was analysed. Baseline measurements of resting calcium concentrations for individual cells were obtained over a 5-min period while the cells were superfused with PSS. This was followed by a 50-s pulse of the test solution (10 µM muscimol or 10 µM glutamate), with data acquired for a further 5 min (allowing for re-establishment of baseline calcium levels). As a positive control, a 50-s pulse of 50 mM KCl was applied at the end of each imaging session. Some coverslips were treated with glutamatergic (100 µM MK-801, 100 µM NBQX), GABAergic (100 µM bicuculline) or voltage sensitive calcium channel antagonists (1 µM diltiazem, 1 µM nimodipine) for 40 s prior to the introduction of the test solution, followed by concurrent application

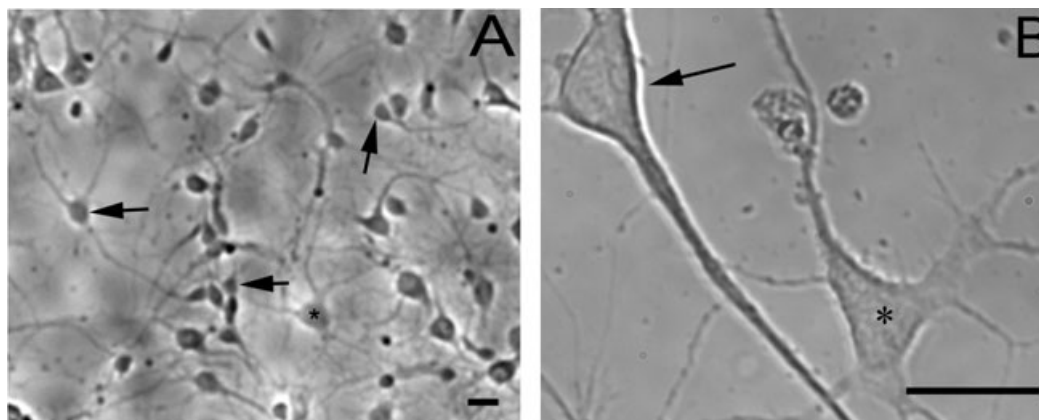


FIG. 1. Photomicrographs of primary hippocampal neurons and glia taken using a (A) 40 \times objective and a (B) 100 \times objective on DIV 5 from E18 cultures. Numerous criteria were used to distinguish neurons from glia, but the most important was shape – the somas of pyramidal neurons are triangular in shape with rounded and clearly distinct edges (large arrow), while glia are amorphous in shape, with flat and nondistinct edges and no distinct processes (asterisks). Scale bar, 10 μ m.

with the test solution. For preparations treated with blockers of the voltage sensitive calcium channels, diltiazem or nimodipine was applied 30 min prior to loading with Fura-2-AM. Both of these drugs, when used at 1 μ M concentration specifically block L-type voltage sensitive calcium channels (Dobrev *et al.*, 1999). The following parameters were documented during calcium imaging: percentage of cells that responded with muscimol-induced calcium transients [the criteria for response (either yes or no) was performed on a cell-to-cell basis, with 'yes' indicated as an increase in intracellular calcium concentration to at least 10% above baseline for that individual cell], peak intracellular calcium concentration of each 'responding' cell (value of the highest response collected over the sampling period), and $t_{1/2}$, the time post-maximal intracellular calcium concentration at which the intracellular calcium concentration for a given cell was at a value exactly between the maximal (t_0) and initial intracellular calcium concentration (t_{initial}), $t_{1/2} = (t_{\text{initial}} - t_0)/2$. For comparative purposes, the data was normalized, with the peak intracellular calcium concentration representing 100%. Intracellular calcium concentration was calculated from the ratio of the background corrected Fura-2 emission (520 nm) at two excitation wavelengths (340 nm/380 nm) by *in situ* solution calibration (Grynkiewicz *et al.*, 1985), performed using a calcium calibration buffer concentration kit (Molecular Probes, Eugene, OR, USA). There were a total of 75–120 neurons analysed for each treatment group.

Collection of cultured hippocampal cells for Western blot analysis

To investigate the effect of estradiol treatment on proteins associated with GABA-mediated excitation and GABA_A receptor-mediated membrane depolarization and calcium influx, Western blot analysis of the cultured hippocampal neurons was performed. Culture dishes containing DIV 5 hippocampal cells cultured on E18 were placed on ice and 75 μ L of ice-cold lysis buffer containing 50 mM Tris-HCl, 1% Na-deoxycholate, 0.25% NP-40, 150 mM NaCl, 1 mM EDTA and protease inhibitors (1 μ g/mL of aprotinin, leupeptin, and pepstatin; 1 mM phenylmethylsulphonyl fluoride) was added to each dish. Cells were scraped off of the coverslips and collected along with the liquid. Cells were collected from two separate culture runs, with 12 samples from the estradiol and control treated groups. Following cell collection, samples were centrifuged at 3000 \times g for 30 min at 4 $^{\circ}$ C. The supernatant fraction was collected, and the protein

concentration determined by Bradford assay. Twenty micrograms of total protein from each animal were electrophoresed on a SDS-polyacrylamide gel (8–16% Tris glycine) and transferred to a polyvinylidene-difluoride membrane. Membranes were washed with 0.1 M TBS and blocked for 1 h at room temperature in 0.1 M TBS containing 5% nonfat dry milk. Membranes were then incubated with either a rabbit polyclonal antibody generated against the phosphorylated form of the human Na⁺K⁺2 Cl⁻ (pNKCC1) protein, at a dilution of 1 : 1000 (generous gift of Dr B. Forbush, Yale University); a rabbit polyclonal antibody generated against the rat K⁺Cl⁻ (KCC2) protein at a dilution of 1 : 2500 (obtained from Upstate, Lake Placid, NY, USA); a rabbit polyclonal antibody generated against the rat α 1C subunit of the voltage-sensitive calcium channel protein (Ca_v1.2) at a dilution of 1 : 1000 (obtained from Chemicon International, Temecula, CA, USA) or a rabbit polyclonal antibody generated against the rat α 1D subunit of the voltage-sensitive calcium channel protein (Ca_v1.3) at a dilution of 1 : 500 (obtained from Chemicon International, Temecula, CA, USA). All antibodies were diluted in TBS containing 0.05% Tween-20 (TBS-T), and incubated for 3 h at room temperature. Membranes were incubated in mouse anti-rabbit HRP-linked secondary antibody (1 : 2500, Cell Signalling Technology, Beverly, MA) for 30 min at room temperature, and then washed with TBS-T. Immunoreactive bands were detected using an enhanced chemiluminescence kit (ECL kit, New England Biolabs, Beverly, MA) and membranes were exposed to film (Hyperfilm-ECL, Amersham Pharmacia Biotech, Arlington Heights, IL). To allow for across group comparisons, each membrane was exposed to all four antibodies (on consecutive days). The proteins under investigation were detected as bands of specific molecular masses (pNKCC1, 158 kDa; KCC2, 140 kDa; Ca_v1.2, 200 kDa; Ca_v1.3, 200 kDa), and the integrative grayscale pixel area-density (iad) was captured with a CCD camera and analysis performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available at <http://www.rsbl.info.nih.gov/nih-image/>).

Statistical analysis

One-way analyses of variance on peak intracellular calcium concentration, normalized $t_{1/2}$, and integrative area density of Western blots were performed, followed by *posthoc* Newman-Keuls comparisons, with a level of $P < 0.05$ required to obtain statistical significance. The

nonparametric χ^2 test was performed on data for the percentage of cells that responded with calcium influx. A large number of statistical comparisons were made and for clarity in the text, the F -value and P -level for each are presented only in the figure legends.

Results

Muscimol-induced calcium influx in developing hippocampal neurons

Acute (10 μ M) muscimol application to developing cultured hippocampal neurons led to elevated intracellular calcium (Fig. 2), consistent with previous reports (maximal intracellular calcium concentration of 138–148 nM over DIV 5–6 from E18 cultured hippocampal neurons as compared to 150–175 nM over the same time points observed by LoTurco *et al.*, 1995; Ma *et al.*, 2001). Baseline intracellular calcium concentration ranged from 33 to 39 nM. Peak amplitude of muscimol-induced calcium transients was 227% greater than baseline calcium levels on DIV 5 from E18 cultured neurons (147.5 ± 13.62 nM), falling to 92% above baseline levels by DIV 8 from E22 cultured neurons (67.5 ± 8.12 nM). Acute (50 mM) KCl application produced 350–400% larger calcium transients than baseline calcium levels at all ages examined and was used to confirm the viability of cells selected for analysis. KCl-induced calcium transients were not significantly affected by age or pretreatment (estradiol or vehicle).

Calcium entry through voltage sensitive calcium channels

In order to determine the route of calcium entry following muscimol application and GABA_A receptor activation, hippocampal cultures were treated with diltiazem [voltage sensitive calcium channel (VSCC) blocker specific to the L-type VSCC when used at concentrations of 1 μ M and lower], nimodipine (L-type specific VSCC blocker), 100 μ M bicuculline (selective GABA_A receptor antagonist), and 100 μ M MK-801 + NBQX (antagonists of the NMDA and AMPA/kainic acid receptors) prior to and concurrent with acute muscimol application. Bicuculline, diltiazem and nimodi-

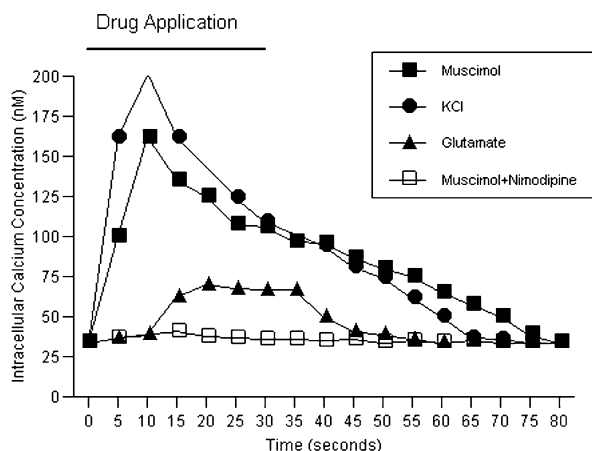


FIG. 2. Representative traces of calcium transients in individual hippocampal neurons on DIV 6 from E18 cultures responding to either 10 μ M muscimol, 10 μ M glutamate, 50 mM KCl, or 10 μ M muscimol + 1 μ M nimodipine. Note the larger muscimol-induced calcium transient as compared to glutamate, and the appreciable antagonism of the muscimol-induced calcium transient by concurrent nimodipine application. Baseline intracellular calcium concentration is ≈ 34 nM at this time point.

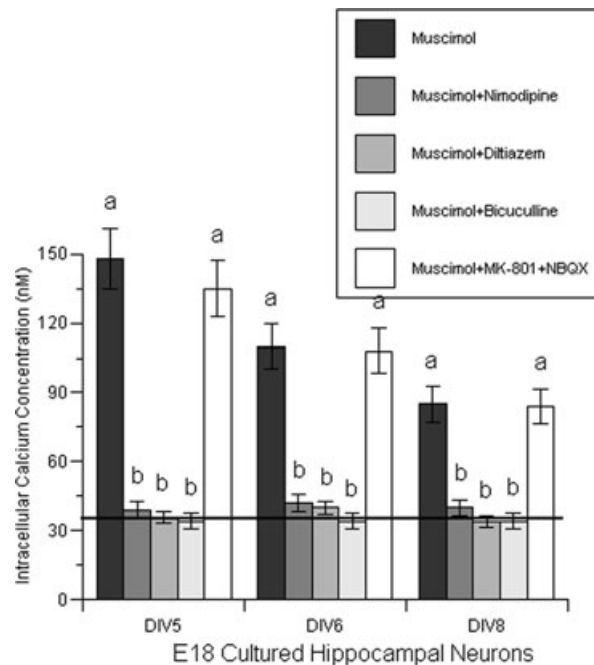


FIG. 3. Quantification of the effect of blockade of muscimol-induced calcium transients with both the L-type voltage sensitive calcium channel antagonists diltiazem (1 μ M) and nimodipine (1 μ M) or the GABA_A receptor antagonist bicuculline (100 μ M). There is a main effect of GABA_A and L-type voltage sensitive calcium channel antagonism on muscimol induced calcium transients on DIV 5 ($F_{3,91} = 13.40$; $P < 0.001$), DIV 6 ($F_{3,95} = 12.76$; $P < 0.001$) and DIV 8 in E18 cultured hippocampal neurons ($F_{3,98} = 14.22$; $P < 0.001$). *Posthoc* Newman–Keuls tests indicated significant differences between bicuculline + muscimol, diltiazem + muscimol and nimodipine + muscimol and muscimol alone treated hippocampal neurons at all three time points ($P < 0.01$). The muscimol-induced calcium response was unaffected by treatment with 100 μ M MK-801 (NMDA receptor antagonist) + 100 μ M NBQX (AMPA receptor antagonist). MK-801 plus NBQX did not significantly attenuate muscimol induced calcium transients at any age examined. *Posthoc* Newman–Keuls tests indicated significant differences between MK-801 + NBQX + muscimol and baseline intracellular calcium levels at all three time points ($P < 0.01$). The letter 'b' denotes a significant difference from muscimol alone treatment (ANOVA; $P < 0.01$), and 'a' denotes a significant difference from baseline intracellular calcium concentration ($P < 0.01$). The solid line represents mean baseline intracellular calcium concentration ≈ 35 nM. Data were acquired from three separate culture runs, with 50–75 neurons analysed in each group at each time point.

pine completely blocked muscimol-induced calcium transients in cultured hippocampal neurons, while there was no effect of MK-801 + NBQX (Fig. 3), indicating that L-type voltage sensitive calcium channels mediate muscimol-induced calcium entry in young hippocampal neurons, with no significant calcium influx through NMDA or AMPA/kainate receptors. Data were acquired from three separate culture runs, with 50–75 neurons analysed in each group at each time point.

Estradiol enhances muscimol-induced calcium transients

Significant effects of pretreatment with physiological levels of 17 β -estradiol were observed on three parameters: (i) the percentage of cells responding with measurable muscimol-induced calcium transients; (ii) the maximal amplitude of muscimol-induced calcium transients in responding cells and (iii) the amount of time required for the intracellular calcium concentration subsequent to acute muscimol application to return to baseline intracellular calcium levels (Fig. 4).

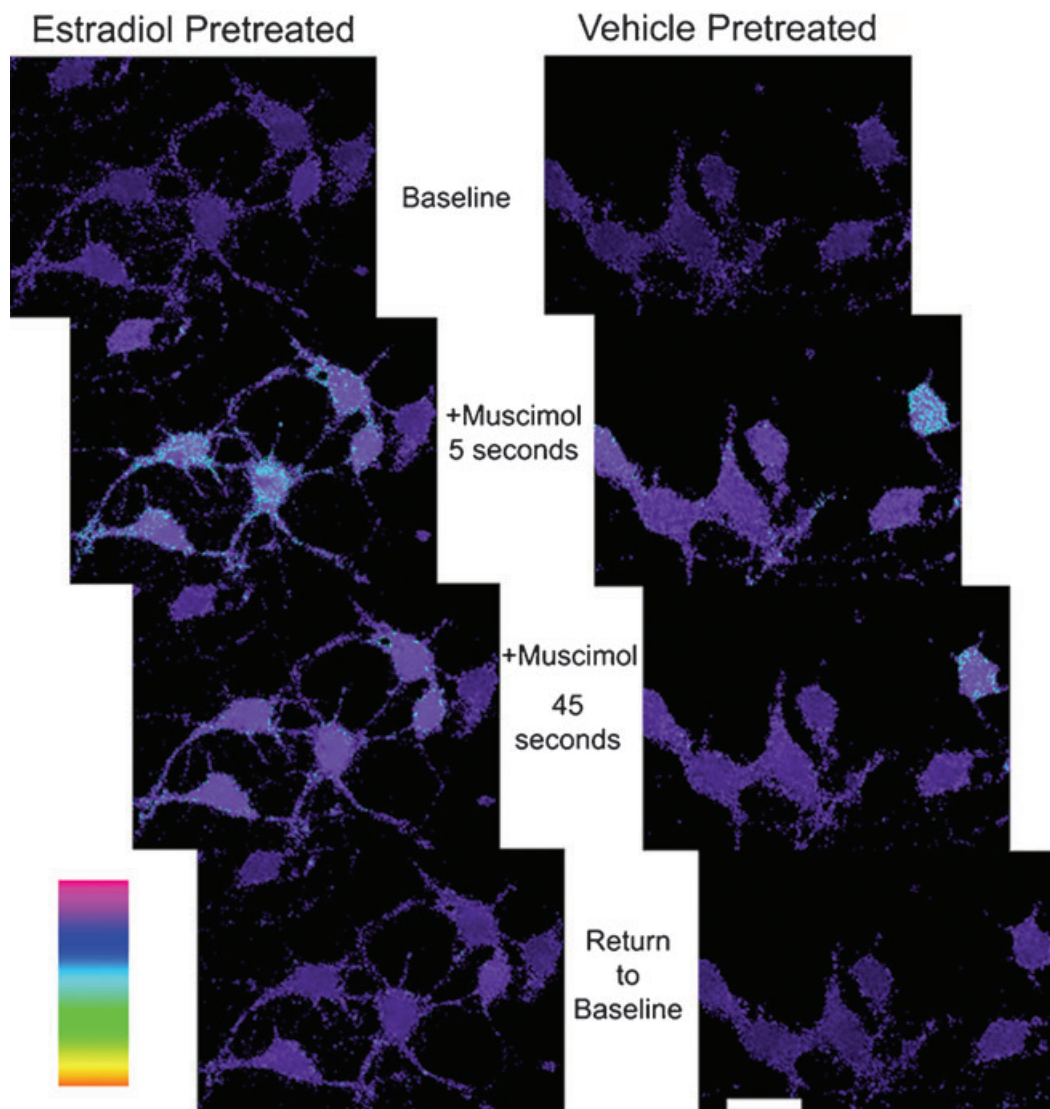


FIG. 4. Representative pseudocolor images of hippocampal neurons on DIV 8 from E18 cultures. The pseudocolor corresponds to the fluorescence associated with Fura-2-AM binding to free calcium, with higher fluorescence (brighter colour) associated with greater levels of free calcium. Estradiol pretreatment enhances the peak intracellular calcium concentration, the percentage of cells responding to muscimol with calcium transients and prolongs the amount of time required to reduce the intracellular calcium concentration to half of the peak concentration. Scale bar, 10 μ m.

Estradiol increases the percentage of cells exhibiting muscimol-induced calcium transients

Pretreatment of cultured primary hippocampal neurons with physiological levels of 17β -estradiol led to an increase in the percentage of cells responding with muscimol-induced calcium transients (Fig. 5A). In both the vehicle and estradiol pretreated groups, the highest percentage of cells responding with muscimol-induced calcium transients were observed on DIV 5 from E18 cultured hippocampal neurons, the earliest time point examined. During this period, the developing hippocampus *in vivo* experiences high levels of estradiol (Amateau *et al.*, 2004). With an increase in the age of the cultured hippocampal neurons there was a concomitant decline in the percentage of cells that responded with muscimol-induced calcium transients regardless of pretreatment (vehicle or estradiol). At DIV 5 in E18 cultures, up to 75% of neurons from vehicle-pretreated cultures and 87% from estradiol-pretreated cultures responded to muscimol with appreciable calcium transients. By DIV 5 in E18 cultures, the percentage of cells responding to muscimol with a calcium transient dropped to 51% in vehicle-pretreated hippocampal cultures but remained elevated at 73% in

estradiol-pretreated cultures. By DIV 8 in E22 cultures, the percentage of cells responding with muscimol-induced calcium transients dropped to 41% and was unaffected by estradiol pretreatment.

An important caveat to this observation is that estradiol pretreatment occurred 1–4 days prior to calcium imaging. Therefore, an effect of estradiol on the percentage of cells responding with muscimol-induced calcium transients might not persist through DIV 8 in E18 cultures or DIV 8 in E22 cultures. To investigate if the drop in the percentage of cells responding to muscimol across days *in vitro* may be related to the drop in the levels of estradiol in the culture medium, and not a maturational phenomenon, a separate group of cultured hippocampal neurons were investigated on DIV 10 from E22 cultures. Half of these cultured hippocampal cells were treated with vehicle, while the other half received 1 nM 17β -estradiol treatment on DIV 0, 2, 4, 6 and 8 (elevated estradiol levels were maintained for a longer period of time as compared to the other estradiol pretreated cultures). Consistent with the muscimol-induced calcium responses on DIV 8 in E22 vehicle-treated cultures (40.6%), only 41.5% of DIV 10 hippocampal neurons from vehicle-treated hippocampal neurons

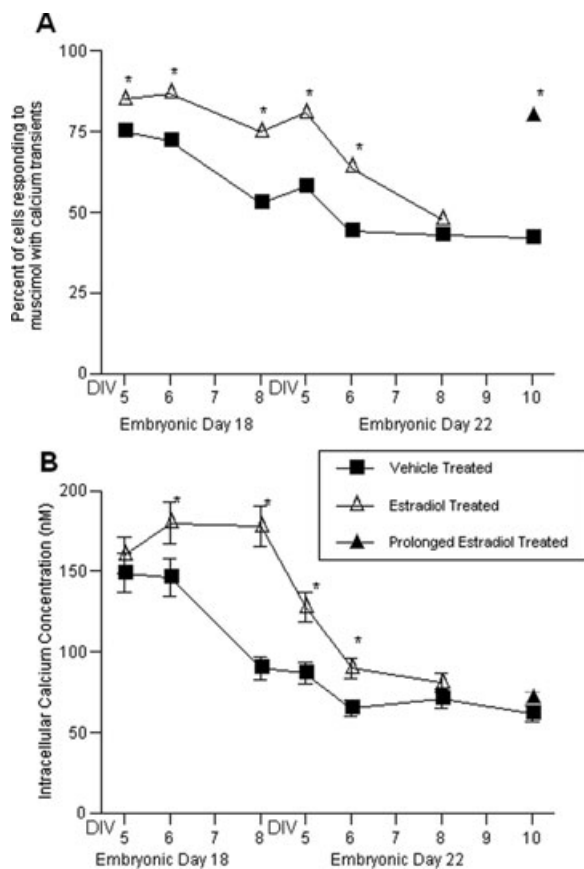


FIG. 5. Estradiol pretreatment enhances muscimol-induced calcium responses. (A) Estradiol pretreatment increases the percentage of cells that respond with muscimol-induced calcium transients. There is a significant effect on DIV 5 ($\chi^2 = 6.07$; $P = 0.014$), DIV 6 ($\chi^2 = 6.05$; $P = 0.014$) and DIV 8 ($\chi^2 = 5.29$; $P = 0.027$) in E18 cultured hippocampal neurons, a significant effect on DIV 5 ($\chi^2 = 22.37$; $P < 0.0001$), DIV 6 in E22 cultured hippocampal neurons ($\chi^2 = 13.54$; $P = 0.0002$), and no effect by DIV 8 in E22 cultured hippocampal neurons (χ^2 , $\star P < 0.05$). There was a significant effect of prolonged estradiol treatment on the percentage of cells that responded with muscimol-induced calcium transients on DIV 10 in E22 cultured hippocampal neurons ($\chi^2 = 23.58$; $P < 0.0001$). Data were obtained from four separate culture runs, with 100–125 neurons analysed in each group at each time point. For the prolonged estradiol data, 25–50 neurons from two separate culture runs were analysed. (B) Estradiol pretreatment increases the maximal muscimol-induced intracellular calcium concentration. There is a significant effect of muscimol application on the magnitude of intracellular calcium responses on DIV 5 ($F_{2,257} = 23.56$; $P < 0.0001$), DIV 6 ($F_{2,125} = 33.66$; $P < 0.0001$), and DIV 8 in E18 cultured hippocampal neurons ($F_{2,137} = 67.28$; $P < 0.0001$), DIV 5 ($F_{2,177} = 21.83$; $P < 0.0001$), DIV 6 ($F_{2,117} = 347.57$; $P < 0.0001$), and DIV 8 in E22 cultured hippocampal neurons ($F_{2,121} = 32.73$; $P < 0.0001$). There was a significant effect of muscimol application on the magnitude of intracellular calcium responses on DIV 10 in E22 cultured hippocampal neurons ($F_{2,78} = 22.17$; $P < 0.0001$). *Posthoc* Newman–Keuls tests indicate significant differences in the magnitude of muscimol-induced calcium transients between estradiol and vehicle pretreated hippocampal neurons on DIV 6 and 8 in E18 cultures, and DIV 5, 6 and 8 in E22 cultures ($P < 0.05$ for all measures). Data were obtained from four separate culture runs, with 100–125 neurons analysed in each group at each time point. For the prolonged estradiol data, 25–50 neurons from two separate culture runs were analysed. The lack of contiguous graph lines indicates that data came from three distinct culture sets, one cultured on embryonic day 18 and imaged on DIV 5, 6 and 8 – one cultured on embryonic day 22 and imaged on DIV 5, 6 and 8, and one cultured on embryonic day 22 and imaged on DIV 10.

responded with muscimol-induced calcium transients. However, 80% of the prolonged estradiol treated hippocampal neurons responded with muscimol-induced calcium transients on DIV 10 ($\chi^2 = 6.25$;

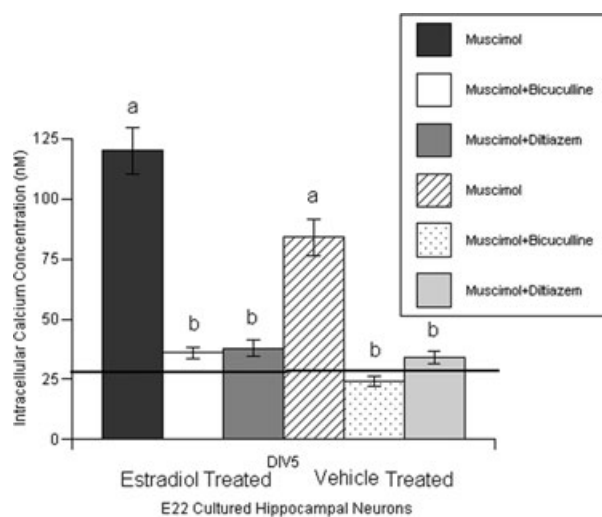


FIG. 6. Bicuculline (100 μM) and diltiazem (1 μM) block muscimol-induced calcium transients in both estradiol pretreated ($F_{2,71} = 13.40$; $P < 0.0001$) and vehicle pretreated ($F_{2,75} = 12.76$; $P < 0.0001$) hippocampal neurons on DIV 5 in E22 cultures. Data were obtained from three separate culture runs, with 75–100 cells analysed in all estradiol and vehicle pretreated groups. The letter ‘b’ denotes a significant difference from muscimol treatment (ANOVA; $P < 0.01$), and ‘a’ denotes a significant difference from baseline intracellular calcium concentration ($P < 0.01$). The black line represents the mean baseline intracellular calcium concentration (≈ 33 nM).

$P = 0.014$) (Fig. 5A). Data were obtained from two separate culture runs, with 25–50 neurons analysed in each group at each time point. Thus, in the presence of prolonged exposure to high physiological levels of estradiol, large numbers of hippocampal neurons maintain a depolarizing response to GABA_A receptor activation.

Estradiol significantly increases the magnitude of muscimol-induced calcium transients

Pretreatment with physiological levels of estradiol led to a 50–120% increase in the maximal amplitude of the muscimol-induced calcium transients in responding cells on DIV 6 and 8 from E18 cultures, and DIV 5 and 6 from E22 cultures (Fig. 5B). Data were obtained from four separate culture runs, with 100–125 neurons analysed in each group at each time point. Consistent with effects in vehicle-pretreated hippocampal cultures, bicuculline and diltiazem blocked muscimol-induced calcium transients in estradiol pretreated hippocampal neurons (Fig. 6). Data were obtained from three separate culture runs, with 75–100 cells analysed in all estradiol and vehicle pretreated groups. As with the effect on the percentage of neurons responding with muscimol-induced calcium transients, the developmental decline in the magnitude of muscimol-induced calcium transients was greatly attenuated by pretreatment with estradiol. In estradiol-pretreated neurons, the average magnitude of the calcium transients was maintained at ≈ 180 nM from DIV 5 through DIV 8 in E18 cultures. This is significantly greater than vehicle pretreated cultures, in which the average magnitude rapidly fell from 147.5 ± 13.62 nM on DIV 5 to 90.2 ± 7.25 nM by DIV 8 from E18 cultures, which was half the average amplitude of estradiol pretreated cells. There was no difference in the baseline intracellular calcium concentration between vehicle pretreated and estradiol pretreated cultured hippocampal neurons at any time point investigated (Table 1). In contrast to the sustaining effect of chronic estradiol pretreatment on the number of neurons responding

TABLE 1. Estradiol pretreatment has no effect on baseline intracellular calcium concentration in developing hippocampal neurons

DIV/Culture Age	Baseline $[Ca^{2+}]_i$ (nM)	
	Vehicle Pretreated	Estradiol Pretreated
5/E18	32.84 ± 1.07	33.88 ± 0.68
6/E18	34.17 ± 0.89	32.73 ± 0.82
8/E18	38.91 ± 0.76	38.67 ± 2.66
5/E22	33.64 ± 0.79	33.54 ± 0.76
6/E22	36.29 ± 1.52	36.21 ± 1.03
8/E22	35.17 ± 0.68	35.68 ± 1.46

Data were obtained from 150 to 200 cells from each group at each time point.

(Fig. 5A), there was no statistically significant effect of sustained levels of estradiol on the magnitude of muscimol-induced calcium transients in these older hippocampal neurons.

Antagonism of the oestrogen receptor significantly attenuates the enhancing effects of estradiol on muscimol-induced calcium transients

A separate set of hippocampal cultures were used to investigate the effect of oestrogen receptor antagonism on the magnitude and percentage of cells responding with muscimol-induced calcium transients. The two experimental groups investigated were ICI-182,780 (oestrogen receptor antagonist) alone treated cultures, and ICI-182,780+ estradiol treated cultures, with data compared to a new cohort of estradiol alone and vehicle treated hippocampal neurons. A total of 45–70 cells were assayed at two different time points (DIV 5 from both the E18 and E22 cultures). We have determined that the effects of estradiol are mediated via oestrogen receptors, based on the observation that pretreatment with the oestrogen receptor antagonist ICI-182,780 blocked the effects of estradiol treatment on the magnitude of the calcium influx induced by muscimol and the percentage of cells that responded to muscimol with calcium influx (Fig. 7A and B). Importantly, treatment with ICI-182,780 in the absence of estradiol had no effect, indicating a lack of nonspecific or toxic effects of the oestrogen receptor antagonist.

Estradiol delays the clearance of free intracellular calcium

Estradiol pretreatment also prolonged the amount of time required by hippocampal neurons to lower the intracellular calcium concentration to 50% of the peak amplitude ($t_{1/2}$) subsequent to acute muscimol application (Fig. 8). While the difference in $t_{1/2}$ was subtle but significant on DIV 5 and 6 in E18 cultures (29.43 ± 2.54 s in estradiol pretreated cultures vs. 21.56 ± 1.41 s in vehicle treated cultures), this effect was very pronounced on DIV 5 and 6 in E22 cultures (37.90 ± 2.59 s in estradiol pretreated cultures vs. 14.63 ± 1.26 s in vehicle pretreated cultures). Data were obtained from three separate culture runs, with 60–80 neurons analysed in each group at each time point. As development progressed, hippocampal neurons from both vehicle and estradiol pretreated groups cleared intracellular calcium and regained homeostatic calcium levels more quickly (approximately 15 s for both groups on DIV 8 from E22 cultures).

Estradiol enhances inward-directed chloride transporter protein levels

Estradiol treated hippocampal neurons had elevated protein levels of the phosphorylated (active) form of the chloride cotransporter

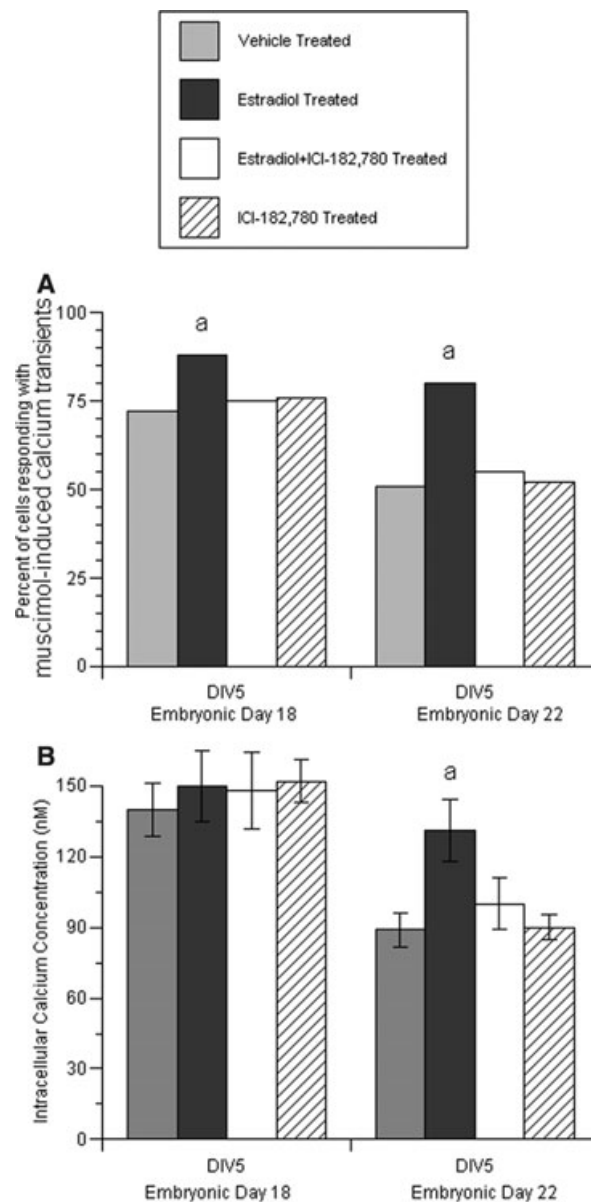


FIG. 7. The oestrogen receptor antagonist ICI-182,780 significantly attenuates the effect of estradiol on muscimol-induced calcium responses. (A) There is a significant effect of estradiol alone treatment on the percentage of cells responding with muscimol-induced calcium transients on DIV 5 in both E18 and E22 cultured hippocampal neurons ($\chi^2 > 4.50$; $P < 0.05$), with the effect completely abolished in the ICI-182,780 plus estradiol treated cultures. Consistent with the action of ICI-182,780 as an oestrogen receptor antagonist, there is no effect of ICI-182,780 alone on the percent of cells responding with muscimol induced calcium transients. Data were obtained from two separate culture runs, with 45–70 neurons analysed in each group at each time point. (B) Estradiol treatment increases the maximal muscimol-induced intracellular calcium concentration, and this effect was significantly attenuated by pretreatment with ICI-182,780. There was no effect of ICI-182,780 alone. There was a significant effect of muscimol application on the magnitude of intracellular calcium responses on both DIV 5 hippocampal neurons cultured on embryonic day 18 ($F_{3,192} = 23.21$; $P < 0.0001$) and 22 ($F_{3,186} = 19.23$; $P < 0.0001$). *Posthoc* Newman–Keuls tests indicate significant differences in the magnitude of muscimol-induced calcium transients between estradiol and all other groups of pretreated hippocampal neurons on DIV 5 in E22 cultures, but not in E18 cultures ($P < 0.01$ for all measures). Data were obtained from two separate culture runs, with 45–70 neurons analysed in each group at each time point. The letter ‘a’ denotes a significant difference from vehicle treatment (ANOVA; $P < 0.01$).

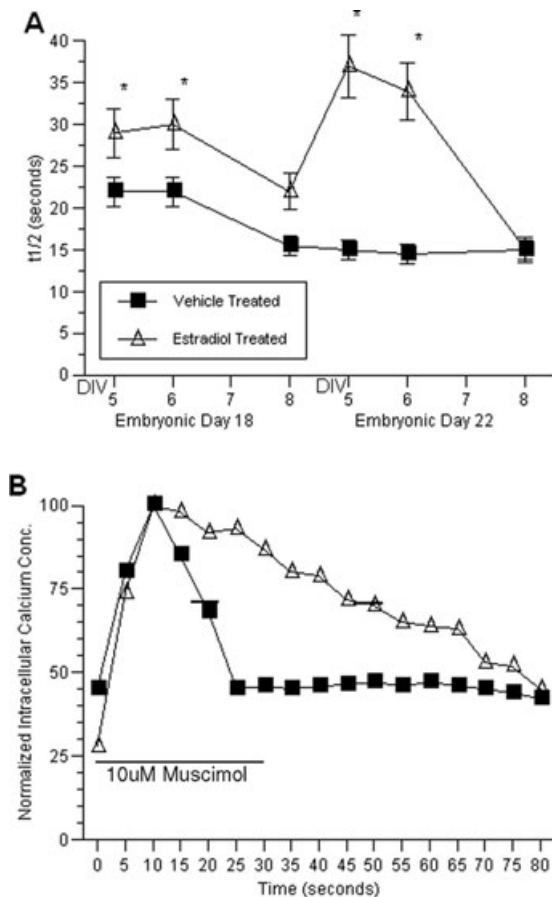


FIG. 8. Estradiol prolongs the amount of time to reduce intracellular calcium to 50% of the peak amplitude ($t_{1/2}$) following muscimol application. There is a main effect of estradiol pretreatment on $t_{1/2}$ on DIV 5 ($F_{1,49} = 8.43$; $P < 0.006$), and DIV 6 in E18 hippocampal cultures ($F_{1,22} = 6.81$; $P < 0.021$), DIV 5 ($F_{1,37} = 59.96$; $P < 0.0001$), and DIV 6 in E22 hippocampal cultures ($F_{1,25} = 36.24$; $P < 0.0001$), but not DIV 8 in either E18 or E22 hippocampal cultures. (A) Effect of estradiol across all time points (ANOVA, $\star P < 0.01$). Data were obtained from three separate culture runs, with 60–80 neurons analysed in each group at each time point. (B) Representative traces from DIV 5 from E22 cultured hippocampal neurons. The mean baseline intracellular calcium concentration is ≈ 34 nM at this time point. The lack of contiguous graph lines indicates that data came from two distinct culture sets – one cultured on embryonic day 18 and imaged on DIV 5, 6 and 8, and one cultured on embryonic day 22 and imaged on DIV 5, 6 and 8. The dash in B represents the $t_{1/2}$ point for this particular trace.

$\text{Na}^+\text{K}^+2\text{Cl}^-$ (pNKCC1) but equivalent protein levels of the chloride cotransporter K^+Cl^- (KCC2) as compared to vehicle treated cultures (Fig. 9). Increased protein levels of pNKCC1 are indicative of enhanced chloride concentration inside of the cells. While the increase in pNKCC1 (28.5%) is modest, the effect may partially be responsible for enhanced membrane depolarization (and significantly more calcium influx) following GABA_A receptor activation, along with a greater percentage of hippocampal neurons responding with GABA_A-mediated excitation.

Estradiol increases protein levels of $\alpha 1C$ subunit of the L-type voltage sensitive calcium channel

Estradiol treated hippocampal neurons had significantly elevated protein levels of the L-type voltage sensitive calcium channel (L-type VSCC) containing the $\alpha 1C$ subunit (2.08 times greater than protein

levels in control cultures), with no effect on the L-type VSCC containing the $\alpha 1D$ subunit as compared to vehicle treated controls (Fig. 10). The L-VSCC containing the $\alpha 1C$ subunit is preferentially expressed in pyramidal cells, while the L-type VSCC containing the $\alpha 1D$ subunit is preferentially expressed in granule cells. Overall, there were significantly higher protein levels of L-type VSCC containing the $\alpha 1C$ subunit as compared to L-type VSCC containing the $\alpha 1D$ subunit (36 times greater protein levels across estradiol treated cultures, and 15 times greater protein levels across control cultures), most likely due to the greater number of pyramidal cells than granule cells in our embryonic hippocampal cultures. The increase in the amount of L-type VSCC containing the $\alpha 1C$ subunit protein following estradiol treatment could be hypothesized to result in greater calcium entry into the cell subsequent to membrane depolarization, consistent with estradiol enhancement of GABA_A-mediated calcium currents.

No effect of repeated muscimol exposure on muscimol-induced calcium transients

Activation of the GABA_A receptor by muscimol or GABA itself is thought to promote the developmental switch from GABA mediated excitation to inhibition (Ganguly *et al.*, 2001). However, we recently reported that twice daily muscimol treatments on postnatal days 0 and 1 leads to hippocampal neuron loss that is blocked by pretreatment with the L-type voltage sensitive calcium channel blocker diltiazem, thus damage is due to GABA-mediated excitation and the resultant increase of intracellular calcium (Nuñez *et al.*, 2003a). Therefore, two potential concerns in the current and previous findings are: (i) that the muscimol treatment paradigm may hasten the onset of GABA-mediated inhibition, thereby diminishing the probability of GABA_A receptor-induced calcium transients and (ii) that repeated muscimol treatment may lead to cell death resulting from attenuated excitation and calcium influx. In light of Ganguly *et al.* (2001), we investigated whether repeated muscimol application affected the probability or magnitude of muscimol-induced calcium transients. Cultured hippocampal neurons from two separate culture runs were administered muscimol two times daily on DIV 4 and 5, followed by calcium imaging using acute muscimol application on DIV 5 in E18 hippocampal cultures. Hippocampal neurons administered muscimol twice daily on DIV 4 and 5 continued to exhibit significant increases in intracellular calcium in response to an acute muscimol application on DIV 5 ($F_{2,137} = 35.04$; $P < 0.0001$), and this response did not differ from that of neurons that only received one acute muscimol application on DIV 5 (137.14 ± 3.19 nM and 132.51 ± 4.51 nM, respectively). Data were obtained from 40 to 60 neurons from both the chronic muscimol and vehicle treated groups. Thus, the muscimol treatment paradigm employed in the current experiment does not hasten the shift of GABA_A responses from depolarizing to hyperpolarizing.

Discussion

Membrane depolarization induced by GABA_A receptor activation in immature neurons is becoming increasingly recognized as an influential force in brain development. Calcium influx through voltage sensitive calcium channels (VSCCs) subsequent to excitatory GABA action can be both trophic (Obata, 1997; Ganguly *et al.*, 2001) and excitotoxic (Nuñez *et al.*, 2003a,b; Nuñez & McCarthy, 2003), depending upon the magnitude, timing and cellular phenotype in which such influx occurs. We now report that in developing hippocampal neurons, exposure to physiological levels of estradiol

FIG. 9. Western blot analysis of the effect of estradiol on protein levels of two chloride cotransporters (A) phosphorylated $\text{Na}^+\text{K}^+\text{2Cl}^-$ (pNKCC1) and (B) K^+Cl^- (KCC2), in DIV 5 hippocampal neurons cultured on E18. pNKCC1 is the active form of NKCC1, a chloride cotransporter involved in chloride influx, with KCC2 a chloride cotransporter involved in chloride efflux. Estradiol treated cultures had significantly increased levels of pNKCC1 ($F_{1,22} = 4.955$; $P < 0.01$) and no effect on the levels of KCC2 compared to control cultures. Hippocampal cells for Western blot analysis were collected from two separate culture runs, with each sample ($n = 12$ for each group) obtained from two individual coverslips. Western blot analysis of both KCC2 and pNKCC1 were performed on the same samples.

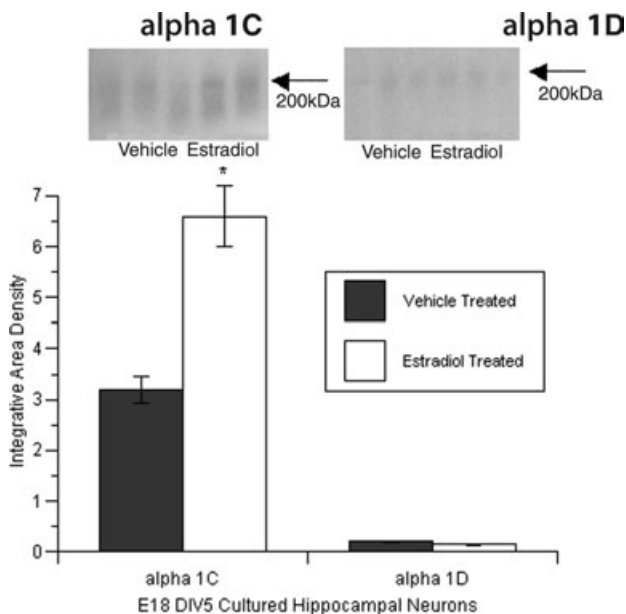
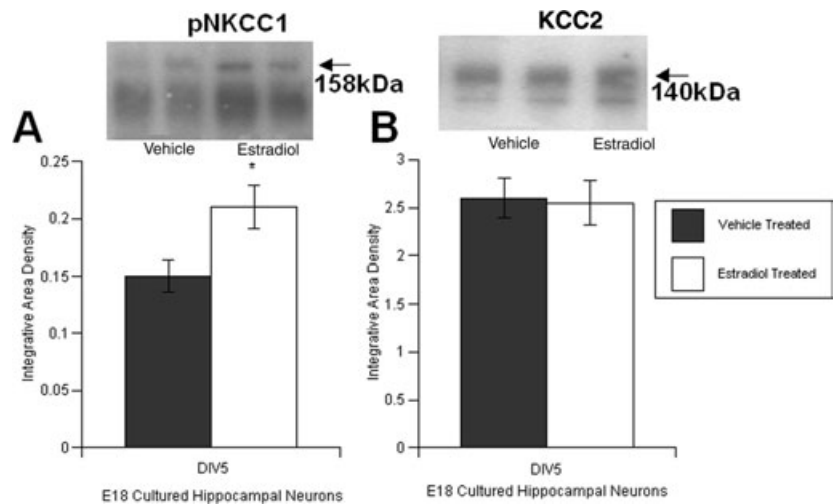


FIG. 10. Western blot analysis of the effect of estradiol on two subunits of the L-type voltage sensitive calcium channel, $\alpha 1C$ ($\text{Ca}_v1.2$) and $\alpha 1D$ ($\text{Ca}_v1.3$), in DIV 5 hippocampal neurons cultured on E18. The $\alpha 1C$ subunit of the L-type voltage sensitive calcium channel is found predominantly in pyramidal cells in the hippocampus, while the $\alpha 1D$ subunit is found predominantly in granule cells in the hippocampus. Estradiol treated cultures had significantly increased levels of $\alpha 1C$ protein ($F_{1,12} = 44.46$; $P < 0.0001$) compared to control cultures. Hippocampal cells for Western blot analysis were collected from two separate culture runs, with samples ($n = 7$ for each group) obtained from two individual coverslips. Western blot analysis of both $\alpha 1C$ and $\alpha 1D$ were performed on the same samples.

prior to application of the GABA_A receptor agonist, muscimol, has profound effects on the depolarizing GABA response, resulting in up to twice the magnitude of L-type VSCC-mediated calcium transients. Moreover, the percentage of cells responding to muscimol with calcium transients is increased across several developmental time points in hippocampal cultures pretreated with physiological levels of estradiol compared to untreated controls, and the amount of time required to clear intracellular calcium is significantly lengthened. These events appear to be dependent upon estradiol action through the oestrogen receptor, given that oestrogen receptor antagonism using ICI-182,780 significantly attenuates the effects of estradiol. The

estradiol-mediated enhancement in the magnitude of the muscimol-induced calcium transients and the percentage of cells responding with muscimol-induced calcium transients appears to be due to an increase in the phosphorylation of NKCC1, along with an increase in the L-type VSCC containing the $\alpha 1C$ subunit. These data support the hypothesis that in the developing hippocampus, estradiol, via oestrogen receptor activation, delays the maturational change from GABA mediated excitation to inhibition.

GABA-mediated excitation and calcium influx

The current report corroborates and extends the elegant work of Ben-Ari and colleagues (Leinekugel *et al.*, 1995; Ben-Ari *et al.*, 1997), showing that in the developing hippocampus, GABA-mediated excitation is observed on the day of birth and persists at least through the end of the first postnatal week. We found that GABA_A receptor activation significantly enhanced intracellular calcium levels via L-type VSCCs, and up to 75% of all cultured hippocampal neurons displayed muscimol-induced calcium transients, further supporting the claim that GABA is an important excitatory neurotransmitter during the late embryonic and early postnatal period (Cherubini *et al.*, 1991; Leinekugel *et al.*, 1999). Activation of the GABA_A receptor and opening of L-type VSCCs is solely responsible for muscimol-induced calcium influx in young cultured hippocampal neurons as muscimol-induced calcium transients were completely blocked by bicuculline, the selective GABA_A receptor antagonist, and the L-type VSCC antagonists nimodipine and diltiazem, with no effect of the NMDA receptor antagonist, MK-801, or the specific AMPA/kainate receptor antagonist, NBQX. Importantly, diltiazem and bicuculline also blocked muscimol-induced calcium entry in estradiol-pretreated cultures, indicating that estradiol does not invoke a novel route of calcium entry, but rather enhances calcium transients through L-type VSCCs. The consistency of action of both nimodipine and diltiazem is important given that diltiazem, which is easily tolerated for consumption by adults and children (Ramoska *et al.*, 1990; Flynn & Pasko, 2000), may be a potential therapeutic agent in the treatment of brain injury involving over-activation of the GABA_A receptor and excessive calcium influx (see below).

Mechanism of estradiol action on GABA-mediated excitation

During development, there is a gradual shift from GABA-mediated excitation to inhibition that is controlled by the balance of chloride

cotransporter expression, including NKCC1 and KCC2 (Schwartz-Bloom & Sah, 2001). NKCC1 promotes chloride, sodium and potassium transport into the cell, and its expression is high in neonatal brain but declines with advancing age. Conversely, KCC2 promotes chloride and potassium exit from the cell and its expression is low neonatally but increases as development progresses such that by the end of the second postnatal week, KCC2 levels are elevated, while NKCC1 levels are significantly decreased (Plotkin *et al.*, 1997; Delpire, 2000; Rivera *et al.*, 2004). Our data indicate estradiol increases pNKCC1, the phosphorylated/active form of NKCC1, with no effect on KCC2. These estradiol-induced changes in the chloride cotransporters would be predicted to result in higher relative chloride concentration inside the cell. We speculate this mechanism is responsible for the increase in the percentage of cells responding with muscimol-induced calcium transients and the prolonged developmental period of GABA-mediated excitation induced by estradiol. Changes in chloride cotransporter protein levels may also affect the amount of calcium entering the cell by increasing the magnitude of membrane depolarization and hence the number and/or duration of L-type VSCC openings.

Additional mechanisms by which estradiol could affect the magnitude of muscimol-induced calcium transients would be via directly increasing the number of L-type VSCCs, prolonging the channel open time subsequent to membrane depolarization or altering the sensitivity of L-type VSCCs to muscimol-induced membrane depolarization, thereby allowing more calcium to enter the cell. Estradiol pretreatment of hippocampal cultures significantly increased (greater than two-fold) the level of the L-type VSCC containing the $\alpha 1C$ subunit, a first demonstration of physiological levels of estradiol increasing L-type VSCC. These data are consistent with previous work demonstrating that estradiol potentiates voltage-sensitive calcium currents in prepubertal hippocampal neurons (Joels & Karst, 1995). However, estradiol has also been reported to inhibit calcium currents through L-type VSCCs in developing striatal neurons (Mermelstein *et al.*, 1996).

An increase in the number of GABA_A receptors by estradiol would also be predicted to enhance membrane depolarization and therefore muscimol-induced calcium transients. We have previously observed that males have elevated levels of both the neurotransmitter GABA and GAD mRNA in the neonatal hippocampus relative to females. However, there is no effect of exogenous estradiol treatment on the GABA_A receptor subunit composition or the amount of GABA_A receptor binding in the perinatal rat hippocampus (Davis *et al.*, 1996a; Davis *et al.*, 1999a,b; Davis & McCarthy, 2000). Therefore, it seems unlikely that an action of estradiol on the GABA_A receptor contributes to the enhancement in the magnitude or proportion of cells displaying muscimol-induced calcium currents.

Finally, estradiol may promote the selective survival of the population of neurons that demonstrate muscimol-induced calcium transients. Cell death is a naturally occurring process during postnatal brain development and is profoundly influenced by estradiol (Davis *et al.*, 1996b; Nuñez *et al.*, 2001; Simerly, 2002). Estradiol not only inhibits cell death in the developing brain, but also stimulates hippocampal neurogenesis (Tanapat *et al.*, 1999) and synapse formation and stabilization in the adult (Woolley, 1999). Estradiol also increases brain-derived neurotrophic factor mRNA, a survival promoting factor, in the neonatal rat hippocampus (Solum & Handa, 2002). However, in our hands estradiol pretreatment does not selectively promote the survival of cultured hippocampal neurons in the absence of an insult (Hilton *et al.*, 2003; Nuñez & McCarthy, 2003), suggesting the currently observed effects were not due to differential cell survival.

Prolonged estradiol maintains GABA-mediated excitation

Estradiol treatment over a prolonged period of time (8 days *in vitro*) maintained the depolarizing GABA response in hippocampal neurons for a longer period compared to vehicle-treated hippocampal neurons, and neurons that received estradiol pretreatment for only 4 days *in vitro*. Therefore, estradiol has the ability to delay the shift from GABA_A mediated excitation to inhibition. On DIV 10 in E22 cultured hippocampal neurons, 80% of chronic estradiol treated neurons responded with muscimol-induced calcium transients – double the percentage in vehicle treated cultures. However, the magnitude of the muscimol-induced transients in the chronic estradiol treated neurons was only marginally greater (14%) than in vehicle treated cultures. The disconnect between the effect of estradiol on the number of neurons responding with muscimol-induced calcium transients and the maximal value of the calcium transient induced by muscimol suggests two separate cellular mechanisms may underlie estradiol's modulation of depolarizing GABA in developing hippocampal neurons.

Potential role for estradiol in early brain injury

Excessive activation of the GABA_A receptor during the first few days of life leads to hippocampal neuron loss and deficits in hippocampus-dependent behavioural performance (Nuñez *et al.*, 2003a,b). This damage is prevented by pretreatment with diltiazem, and exacerbated by pretreatment with high physiological levels of estradiol (Nuñez & McCarthy, 2003; Nuñez *et al.*, 2003a). Differences in the normal physiological levels of estradiol, while mediating sex-specific brain development, may also influence the sensitivity of the immature brain to injury. The effect of elevated estradiol concentration may contribute to the increased susceptibility of the immature male brain over females to insults involving excessive calcium influx (Naeye *et al.*, 1971; Lauterbach *et al.*, 2001).

Acknowledgements

This work was supported by National Institutes of Health Grant MH 52716 to MMM and Society for Neuroscience Minority Postdoctoral Fellowship to JLN. We thank Jesse Alt for expert technical assistance.

Abbreviations

BDNF, brain derived neurotrophic factor; DIV, days *in vitro*; VSCC, voltage sensitive calcium channel.

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