

Estradiol Modulation of Kainic Acid-Induced Calcium Elevation in Neonatal Hippocampal Neurons

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The developing hippocampus of both males and females is exposed to high levels of the gonadal steroid estradiol. The impact of this estradiol exposure on developing hippocampal neurons is essentially unknown. In the rat, the newborn hippocampus is relatively insensitive to excitotoxic brain injury, which in adults is associated with the release of amino acids, in particular glutamate, resulting in a significant increase in intracellular calcium and eventual cell death. We have shown previously in the rat that administration of the glutamate agonist, kainic acid (KA), on the day of birth results in limited hippocampal damage, which is ameliorated by treatment with the gonadal steroid, estradiol. We now show that KA induces an increase in intracellular calcium through L-type voltage-sensitive calcium channels early in development and, later in

development, through polyamine-sensitive α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors with a modest increase through N-methyl-D-aspartate receptors. Pretreatment with the gonadal steroid, estradiol, decreases the percentage of neurons responding to KA and decreases the peak amplitude of the calcium transient early in development but has no effect later in development. Taken together, these data suggest that there is a developmental shift in the route of KA-induced intracellular calcium and estradiol modulates KA-induced intracellular calcium to a time restricted to early development, but whether this is the basis of the neuroprotective effect of estradiol remains to be determined. (*Endocrinology* 147: 1246–1255, 2006)

DURING THE PERINATAL period, the developing brain is exposed to high levels of gonadal steroids. In the diencephalon, this exposure is distinct in males *vs.* females, in particular neuronal aromatized estradiol, which is derived from androgen precursors originating in the males testis (1). Functionally, estradiol mediates sexual differentiation in the rodent brain (2), resulting in changes in behavior, volumetric differences, dimorphic synaptic patterning (2, 3), and alterations in neuronal differentiation (4). Estradiol exerts both trophic and maturational effects through modulation of excitatory amino acids (5) and apoptosis (6, 7) as well as regulation of trophic factors such as brain-derived neurotrophic factor (8). Estradiol also acts directly on N-methyl-D-aspartate (NMDA) receptors and L-type voltage-sensitive calcium channels (VSCCs) to regulate calcium influx (9, 10).

In the telencephalon, including the hippocampus, both males and female neurons are exposed to high levels of estradiol (11), and there is increasing evidence of *de novo* steroidogenesis by hippocampus neurons (12). The functional impact of elevated estradiol on the developing hippocampus is largely unknown. In the adult, there is considerable interest in the neuroprotective effects of estradiol after ischemic injury (13–17), and the hippocampus is particularly

susceptible to the deleterious effects of hypoxia as a consequence of excitotoxic glutamate (18, 19). The developing hippocampus is also susceptible to ischemic injury (20–22), but the cellular mechanisms of damage have not been well characterized, in part, because of the relatively low expression and functionality of glutamate receptors early in development (23–25).

Kainic acid (KA) is an α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor agonist that has been widely used to induce excitotoxic brain injury in adults (26–29) by inducing increased intracellular calcium and internucleosomal DNA fragmentation and cell loss (30, 31). We recently reported that KA administration causes significant damage to the dentate gyrus and moderate damage to the CA2/3 region of the hippocampus in female rat pups on the day of birth, whereas male pups are resistant to KA-induced damage. Damage in the female is ameliorated by pretreatment with the gonadal steroid, estradiol (32, 33). Thus, exploring the impact of estradiol on calcium influx induced by KA could provide insight into normal brain development as well as a potential mechanistic basis of neuroprotective effects of estradiol in the developing brain.

Using the calcium-sensitive dye, fura 2-AM, and cultured hippocampal neurons, we report here that KA induced an increase in intracellular calcium through AMPA receptor-mediated depolarization and subsequent opening of VSCCs around the day of birth. Approximately 4 d later, in contrast, KA led to calcium influx via polyamine-sensitive AMPA receptors and recruitment of NMDA receptors. Pretreatment with physiologic levels of estradiol decreased the percentage of cells responding to KA and decreased the peak amplitude of the KA-induced calcium transient during early develop-

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Abbreviations: AMPA, α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; DIV, days *in vitro*; DMSO, dimethylsulfoxide; KA, kainic acid; NAS, 1-naphthylacetylspermine trihydrochloride; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline; NMDA, N-methyl-D-aspartate; PSS, physiologic salt solution; VSCC, voltage-sensitive calcium channel.

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ment but had no effect on the KA-induced intracellular calcium response in more mature hippocampal neurons.

Materials and Methods

Animals

Subjects were first generation descendants of Sprague Dawley albino rats from Charles River Laboratory (Wilmington, MA). Female rats were bred at the University of Maryland School of Medicine (Baltimore, MD) animal facility. Animals were housed under a 12-h light, 12-h dark cycle with unlimited access to food and water. All animal procedures were approved by the University of Maryland Institutional Animal Care and Use Committee. Numbers of animals used was kept to a minimum, and procedures were performed in a manner that minimized suffering.

Hippocampal cell culture

A timed pregnant Sprague Dawley female was killed by CO₂ inhalation at gestational d 18. Fetal hippocampi were dissociated using trypsin digestion and triturated to give a single cell suspension. The suspension was incubated in a water bath for 15 min at 37 C to allow the cells to settle, and the supernatant was removed. Five milliliters of Hanks' balanced salt solution+ was added, and the cells were allowed to settle for 5 min. This process was repeated two more times. Cells were dissociated by pipetting and deoxyribonuclease treatment. Cell count and viability were determined using a hemacytometer and trypan blue staining before cells were plated at a density of 300,000 cells per coverslip on 25-mm poly-L-lysine-coated coverslips. These were then placed in 60-mm dishes containing 4 ml plating medium (86 ml MEM, 10 ml horse serum, 3 ml filter sterilized 20% glucose, and 1 ml pyruvic acid, 100 mM). Cells were placed in a 5% CO₂ incubator for 4 h at 37 C. Coverslips were transferred from the plating media to dishes containing 3 ml Neurobasal media (1 ml B-27 supplement, 1 ml antibiotic/antimycotic 100× liquid, and 125 μl L-glutamine to a volume of 50 ml with Neurobasal). Hippocampal neuronal cultures were treated with 1 μM estradiol dissolved in dimethylsulfoxide (DMSO) and added to 3 ml culture medium to achieve a final concentration of 1 nM. Vehicle-treated controls received the same volume of DMSO. This treatment results in a physiologic concentration of approximately 200 pg/ml estradiol (34). Hippocampal cell cultures intended for fluorescent imaging were again treated with estradiol or vehicle on days *in vitro* (DIV)2 and DIV6. On DIV3 and DIV7, 1 ml medium was removed from each culture dish and replaced with 1 ml fresh Neurobasal medium containing either estradiol or vehicle.

Fluorescent imaging

The day cultures were generated was designated as DIV0. To evaluate intracellular calcium responses developmentally, days *in vitro* were cor-

related to estimated postnatal days of life (Fig. 1). For example, DIV4 for neurons cultured from embryonic day 18 fetuses is considered roughly equivalent to postnatal d 0. On DIV4, DIV6, DIV8, and DIV10, calcium imaging using the cell-permeant fluorescent indicator fura 2-AM was performed on hippocampal cell cultures. On the day of imaging, hippocampal neurons were incubated with 3 μl fura 2-AM in DMSO with 20 μl pluronic acid for 30 min. Coverslips were then transferred to a tissue chamber on a microscope stage and were superfused for 30 min with a physiologic salt solution [PSS; 134 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 3 mM CaCl₂ salts, and 10 mM HEPES in 20% glucose solution (pH 7.4)] at room temperature. Superfusion removes extracellular dye and allows for the deesterification of the fura 2-AM.

At the beginning of every series of experiments, KCl or vehicle control was administered. A sharp rise in the calcium transient induced by KCl indicated that the cells were healthy and could be used for data collection, and baseline stability in response to vehicle control indicated reliability of the cellular response.

A Zeiss Axiovert 100 inverted microscope with illumination by a Til Photonics Polychrome II Monochromator was used (Applied Scientific Instrumentation, Eugene, OR), with excitation measured at 340 and 380 nm and emission measured at 520 nm. Images were obtained using a charge-coupled device video camera. Image acquisition and analysis was performed using the Metamorph/Metafluor 5.0 Imaging System (Universal Imaging Corp., Downingtown, PA). Temperature was maintained between 30 and 32 C throughout the experiment. Calcium calibration was performed using a calcium calibration buffer kit (Invitrogen, Carlsbad, CA), and all analysis was performed on the raw free calcium concentration data.

Baseline calcium fluorescence ratio was obtained with PSS superfusion followed by 3-min infusions of 10 μM KA. KA, at this concentration, elicited a calcium response in hippocampal cells, yet resulted in a low percentage of cell death. PSS was then superfused for 4–5 min to allow for complete clearance of the KA. Receptor antagonists at 100 μM concentrations, including 1) the NMDA receptor antagonist, MK801; 2) the AMPA/kainate antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX); 3) the L-type VSCC antagonist, diltiazem; and 4) the specific calcium-permeable AMPA receptor antagonist, 1-naphthylacetylspermine trihydrochloride (NAS), were then superfused for 4 min. NBQX, diltiazem, and MK801 were obtained from Sigma (St. Louis, MO), and NAS was a gift from Akira Isokawa (Daicel Chemical Industries, Ltd., Fort Lee, NJ). After this, a combination of 10 μM KA and 100 μM of each antagonist or combination of antagonists was applied for 3 min. All antagonists were administered at a dose (100 μM) determined to saturate all receptors and that was 10-fold greater than the concentration of the agonist. Each series of experiments with antagonists was performed on separate coverslips so that cells were exposed to only one antagonist to eliminate a potential confound due to the order of drug application. Ratiometric measures were obtained every 5–30 sec

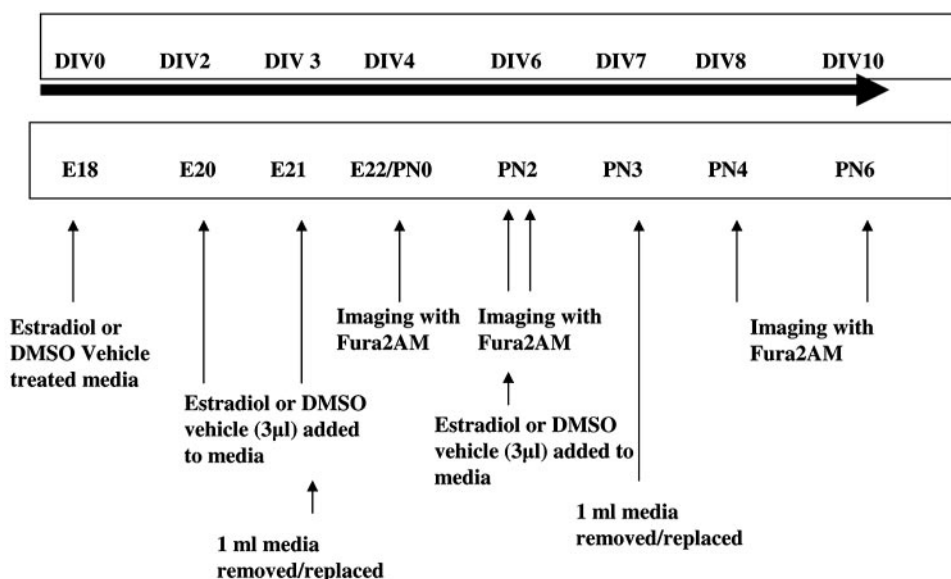


FIG. 1. Schematic representation of days *in vitro* examined and the theoretical correlation with postnatal day.

throughout the experimental paradigm. Cells were excluded from analysis if their baseline ratio was greater than 0.9, which might be indicative of an unhealthy cell, if the ratio did not return to baseline after either KA or antagonist perfusion, or if there was an indication of cell death with a precipitous drop in ratio to below baseline.

Cells were classified as responders or nonresponders depending on the size of the KA-induced rise in intracellular calcium. A more than 10% rise in intracellular calcium had been designated as threshold in prior imaging experiments because anything below that was deemed unreliable (35). Only cells responding with a more than 10% rise in intracellular calcium were analyzed further. Parameters analyzed were the percentage of cells that responded with an increase in intracellular calcium concentration more than 10% above baseline, the percent increase in calcium influx measured from baseline to peak amplitude, the rise time measured as the amount of time for the calcium transient to rise to 10% above baseline, the peak time measured as the amount of time for calcium to rise to its highest amplitude, and the recovery time measured by the amount of time for the calcium concentration to decline to 50% of the peak. Based on the heterogeneity of the observed KA-induced response, analysis was performed on the responses of individual cells ($n = 300\text{--}600$ neurons).

Statistics

A two-way ANOVA was performed on measures of peak KA-induced calcium concentration, rise time, peak time, and time to recovery with an *a priori* analysis of treatment by age, followed by *post hoc* Fisher's least significant difference comparisons with a level of $P < 0.05$ required to obtain statistical significance and multiple Student's *t* tests with a Bonferroni correction (36). A χ^2 test was performed on numbers of cells

responding to KA in each treatment group. Additional two-way ANOVAs were conducted on data obtained from antagonist experiments at two separate ages, DIV4 and DIV8, with analysis of calcium influx from KA alone *vs.* KA with antagonist. A separate ANOVA was conducted on data at each age to compare differences between antagonists. A linear regression analysis of baseline intracellular calcium concentration and Kolmogorov-Smirnov test on data from KA-induced change in intracellular calcium concentration at DIV4 were also performed.

Results

Baseline intracellular calcium concentration in hippocampal neurons

Heterogeneity in the response of cells to KA was observed, and a frequency histogram was generated to characterize the responses of cells (Fig. 2A). In a large percentage of cells, the rise in intracellular calcium after KA was less than 10%. These were considered nonresponders and were eliminated from further analyses. The responding cells were those with KA-induced rises in intracellular calcium of more than 10%. A representative pseudocolored micrograph of DIV4 neurons at baseline and in response to KA is presented in Fig. 2B.

Baseline differences in intracellular calcium concentrations may bias results obtained from fluorescent imaging. To determine whether there was a difference in baseline calcium between vehicle- and estradiol-treated hippocampal neu-

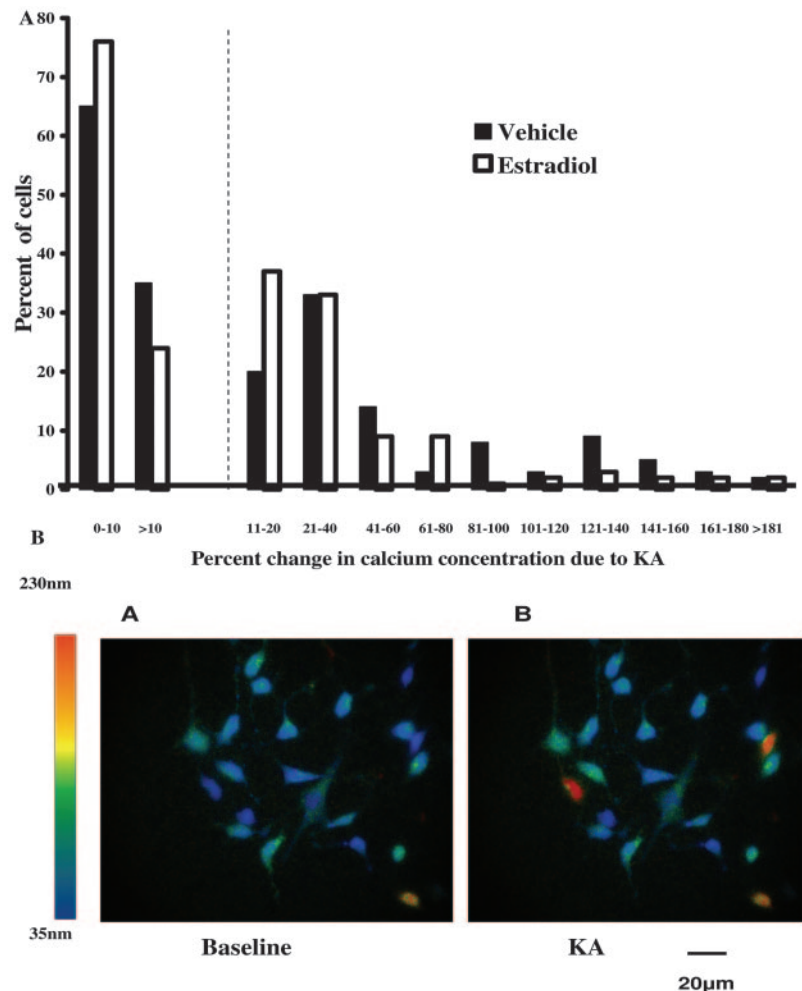


FIG. 2. A, Frequency histogram of the percent change in intracellular calcium concentration due to KA at E18DIV4. Cells that responded to KA with less than a 10% rise in intracellular calcium were considered nonresponders, and cells that responded to KA with greater than a 10% rise in intracellular calcium were considered responders. These are depicted to the left of the dotted line. To further demonstrate the heterogeneity in KA-induced response, the frequency distribution of responding cells only are depicted separately to the right of the dotted line, ranging from a 11–20% response to a maximal more than 181% response. B, Representative pseudocolored image of fluorescence emitted by DIV4 hippocampal neurons at baseline (A) and during exposure to KA (B). Scale bar, 20 μm .

rons, the baseline calcium concentrations of neurons from 10 experiments were obtained and analyzed using a one-way ANOVA for each age. There was no significant difference in baseline intracellular calcium levels between vehicle- and estradiol-treated neurons at DIV4 ($F_{1,257} = 0.121$, $P = 0.73$), DIV6 ($F_{1,302} = 0.132$, $P = 0.72$), DIV8 ($F_{1,246} = 1.61$, $P = 0.21$), or DIV10 ($F_{1,214} = 1.67$, $P = 0.21$; data not shown). Baseline mean intracellular calcium in vehicle-treated neurons ranged from 62.02 ± 7.8 to 84.76 ± 8.7 nM and in estradiol-treated neurons ranged from 59.25 ± 9.0 to 83.15 ± 9.5 nM.

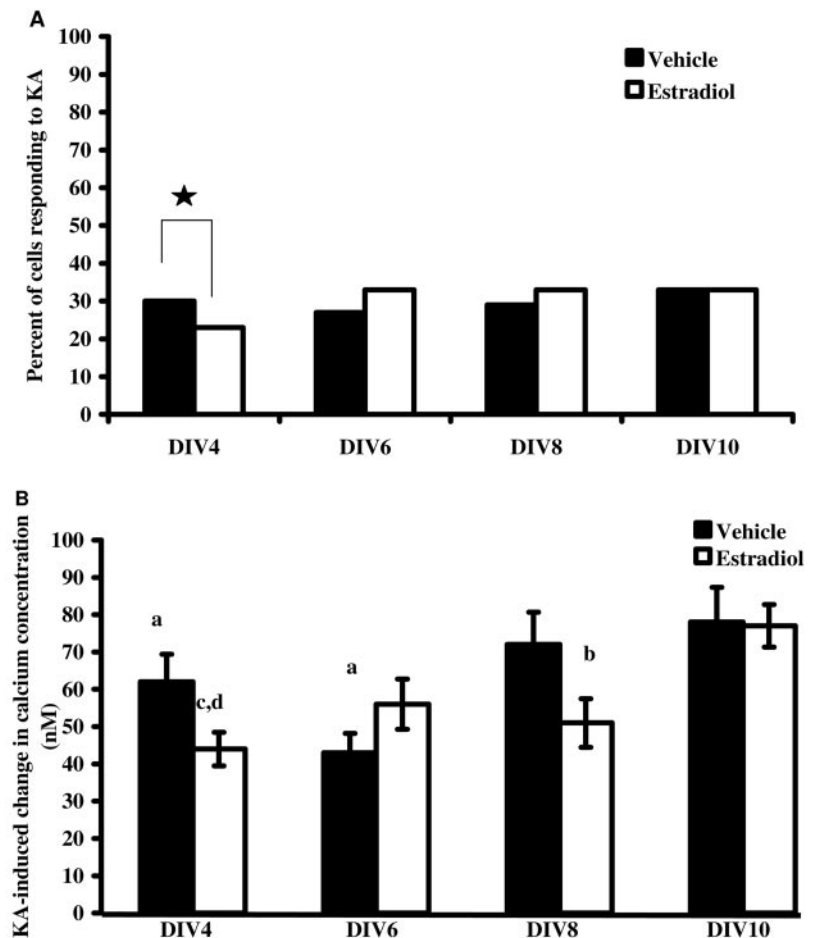
Variability in baseline intracellular calcium was noted in both estradiol- and vehicle-treated cells. Prior data suggests that hippocampal and cortical neurons and glial cells exhibit heterogeneity in baseline calcium levels (37–39). Baseline intracellular calcium varies according to cell type with cells in CA3 exhibiting higher baseline calcium concentration than those in CA1 and dentate gyrus and is higher in dendritic spines than in the soma (40, 41). Immature cortical and hippocampal pyramidal cells and interneurons also exhibit variability in baseline associated with spontaneous oscillatory activity (39, 42). Because our cultures were made from whole hippocampus, the mix of cell types may have contributed to the variability observed. To examine whether there was a correlation between baseline calcium concentration and the KA-induced change in calcium concentration, we performed a linear regression analysis on data from both vehicle- and estradiol-treated neurons (data not shown). There was a sig-

nificant correlation between baseline calcium concentration and the change in the calcium concentration due to KA in both vehicle-treated ($r^2 = 0.06$; $P < 0.005$) and estradiol-treated ($r^2 = 0.17$; $P < 0.0001$) neurons; however, the two regression lines did not differ ($P > 0.05$). Even though a statistical correlation was observed, it is important to note that variance in baseline accounted for only 5.5% of the variance in the peak amplitude of the transient in vehicle-treated cells and 16.9% in estradiol-treated cells. Nonetheless, to eliminate this confound, baseline calcium concentration was subtracted from the peak amplitude of the KA-induced calcium transient in all remaining analyses.

Developmental changes in neuronal response to KA

Between 300 and 500 neurons (12–20 cells per field from 13–18 coverslips spanning six to 14 cultures from six to 14 individual pregnant females) were imaged at each age; however, only those cells responding to KA were included in the analysis. Pretreatment of dissociated hippocampal cultures with estradiol *vs.* vehicle significantly decreased the percentage of cells responding to KA with a peak amplitude of greater than 10% at DIV4 ($n = 68$ –159, $\chi^2 = 5.86$, $P < 0.025$; Fig. 3A). There was no significant effect of estradiol at DIV6 ($\chi^2 = 3.06$; $P = 0.1$), DIV8 ($\chi^2 = 0.8$; $P = 0.1$), or DIV10 ($\chi^2 = 0.004$; $P = 0.1$). There was also no significant difference between estradiol treatment and vehicle-treated hippocam-

FIG. 3. A, Percentage of cells responding to KA from DIV4 through DIV10. Significantly more vehicle-treated cells responded to KA compared with cells pretreated with estradiol on DIV4 ($\chi^2 = 5.86$; $P < 0.025$). There was no significant difference in the percentage of cells responding at DIV6 ($\chi^2 = 3.06$; $P = 0.1$), DIV8 ($\chi^2 = 0.8$; $P = 0.1$), or DIV10 ($\chi^2 = 0.004$; $P = 0.1$). B, Developmental increase in KA-induced calcium transient in hippocampal neurons from DIV4 through DIV10. Calcium influx in vehicle-treated cells increased significantly from DIV4 to DIV10 and from DIV6 to DIV10 (a, significant difference from vehicle DIV10, $P < 0.05$). A significant increase in calcium influx in estradiol-treated cells occurred between DIV4 to DIV10 and from DIV8 through DIV10 (b, significant difference from estradiol DIV10, $P < 0.05$; c, $P < 0.01$). Separate Student's *t* tests for independent samples on data generated from the KA-induced calcium transient revealed an effect of steroid treatment on DIV4 only (d; $P < 0.01$).



pal cells with respect to the other parameters measured, including rise time of the calcium influx, time to peak intracellular calcium, and recovery time (data not shown).

A developmental increase in the magnitude of the calcium transient in both estradiol- and vehicle-treated hippocampal neurons in response to KA was observed between DIV4 and DIV10 (two-way ANOVA, $F_{3,725} = 7.96$; $P < 0.0001$; Fig. 3B), but there was no effect of estradiol treatment ($F_{3,725} = 0.53$, $P = 0.47$), nor was there an interaction ($F_{3,725} = 1.95$; $P = 0.12$). There was a 27% increase in the peak amplitude of the KA-induced calcium transient in vehicle-treated cells ($n = 77$ – 159) from DIV4 to DIV10 (Fisher's least significant difference test, $P < 0.05$) and a 76% increase from DIV6 to DIV10 ($P < 0.01$). A developmental increase in the peak amplitude of the calcium transient was also noted in estradiol-treated cells ($n = 68$ – 122) with a 73% increase from DIV4 to DIV10 ($P < 0.01$) and a 34% increase from DIV8 to DIV10 ($P < 0.05$).

The magnitude of the KA-induced calcium transient

To analyze the effect of estradiol treatment on the magnitude of the calcium transient, a Student's *t* test for independent samples was performed for each age group (36). A Bonferroni-Dunn correction for multiple Student's *t* tests was performed, and $P < 0.01$ was required for significance. A significant difference in the amplitude of the KA-induced calcium transient between vehicle- and estradiol-treated neurons was noted on DIV4 only ($t = 2.75$; $P < 0.01$, Fig. 3B). There was no difference in the amplitude of the KA-induced calcium transient between vehicle- and estradiol-treated neurons on DIV6, DIV8, or DIV10.

To more clearly delineate the difference in the peak amplitude of the KA-induced calcium transient between vehicle- and estradiol-treated cells, a cumulative probability curve for each age was developed. A significant leftward shift in the probability of low calcium influx responses to KA was observed in estradiol-treated hippocampal neurons compared with vehicle on DIV4 only (K-S; $P < 0.01$; Fig. 4).

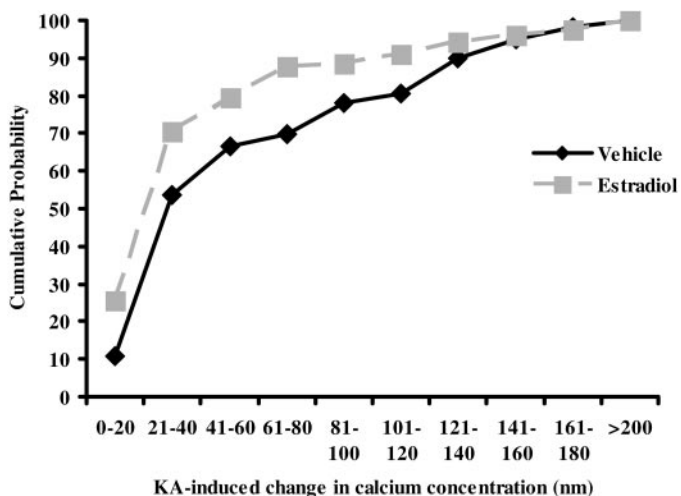


FIG. 4. Cumulative probability distribution for the change in peak amplitude of the KA-induced calcium concentration at DIV4. There is a leftward shift toward greater probability of smaller calcium transient responses in estradiol-treated cells compared with vehicle (K-S; $P < 0.01$).

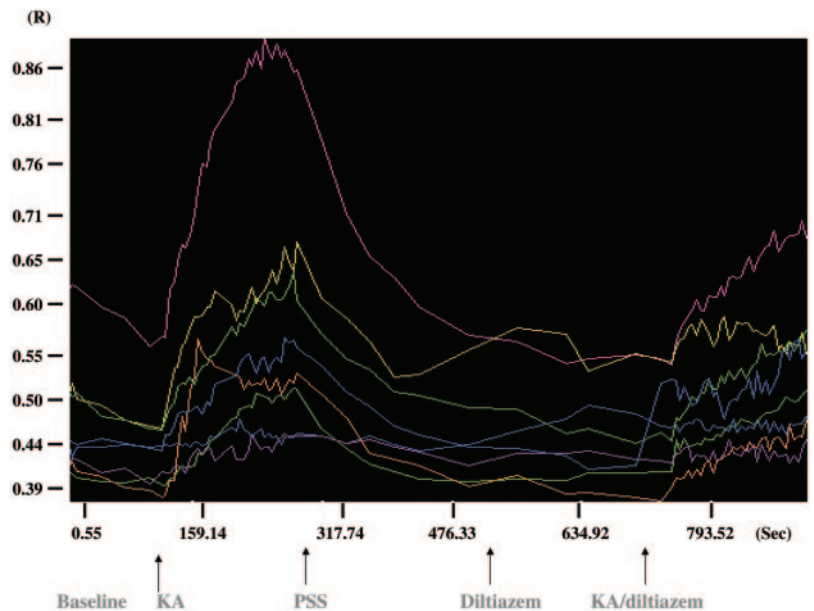
No difference was noted at any other age. This suggests that estradiol shifts the neuronal response to KA to include a higher percentage of cells responding with a smaller amplitude calcium influx compared with vehicle-treated neurons. In fact, 51% of neurons at DIV4 responded to KA with less than or equal to a 40-nm rise in the peak amplitude of the calcium transient. When cultures were pretreated with estradiol, the percentage of neurons responding to KA with less than or equal to a 40-nm rise in the peak amplitude of the calcium transient approached 70%. Taken together, these data indicate that estradiol decreased the percentage of cells responding to KA on DIV4, decreased the peak amplitude of the KA-induced calcium transient, and shifted the population response toward a smaller KA-induced calcium transient.

Source of KA-induced calcium influx in DIV4 neurons

To explore the possible sources of the KA-induced increase in intracellular calcium at DIV4, we performed fluorescent imaging experiments using the AMPA/kainate receptor antagonist, NBQX, the specific polyamine-sensitive AMPA receptor antagonist, NAS, the NMDA receptor antagonist, MK801, and the L-type VSCC blocker, diltiazem. Data were generated from 100–200 neurons imaged from six to 12 coverslips from three to five independent cultures per each separate experiment. A representative trace of fluorescence ratio signals from individual E18 DIV4 neurons to KA and diltiazem is provided in Fig. 5. Separate two-way ANOVAs were conducted on data generated from the intracellular calcium concentration for each responding neuron. The analysis for each antagonist and combination of antagonists administered on DIV4 revealed a significant effect of antagonism on KA-induced calcium influx but no effect of estradiol on the antagonism. Therefore, data for estradiol- and vehicle-treated hippocampal neuronal cultures were combined and are presented as percentage of peak amplitude of KA-induced intracellular calcium (Fig. 6A). Further analysis to compare the effect of each antagonist *vs.* the combination of antagonists was performed on the grouped data.

At DIV4, the AMPA/kainate receptor antagonist, NBQX ($n = 79$), reduced calcium influx by 61% compared with KA alone ($F_{1,158} = 17.71$; $P < 0.0001$). The polyamine-sensitive AMPA receptor antagonist, NAS ($n = 122$), produced a comparable 59% reduction in KA-induced calcium ($F_{1,243} = 28.02$; $P < 0.0001$). In contrast, the NMDA antagonist, MK801 ($n = 78$), did not significantly affect KA-induced calcium influx ($F_{1,156} = 1.54$; $P = 0.22$). The VSCC blocker, diltiazem, produced a 69% decrease in calcium influx ($F_{1,201} = 19.79$; $P < 0.0001$). The addition of MK801 to NBQX and diltiazem and NAS reduced KA-induced calcium concentration compared with KA alone ($n = 63$; $F_{1,127} = 11.67$; $P < 0.0001$) as did the combination of MK801, NBQX, and diltiazem (data not shown). When the antagonists were compared with one another, there was no significant difference in reduction in the peak amplitude of the KA-induced calcium concentration with the combination of NAS + NBQX + MK801 + diltiazem compared with NBQX, NAS, or diltiazem alone. These data, taken together, suggest that KA induced calcium influx through AMPA receptor-mediated membrane depolariza-

FIG. 5. Representative tracings of fluorescence ratio signals from individual neurons treated with KA and diltiazem. The x-axis represents the time in seconds and the y-axis represents the 340:380 ratio of fluorescence. The arrows indicate the approximate time when KA was administered, followed by washout with PSS; administration of diltiazem alone, followed by the combination of KA and diltiazem.



tion, leading to opening of the L-type VSCCs. The fact that NAS was equally potent as NBQX suggests this effect may be mediated by spermine-sensitive AMPA receptors. Nevertheless, NAS was not more effective than diltiazem, suggesting that KA induces minimal calcium influx directly through AMPA receptors in contrast to influx through the L-type channels resulting from AMPA receptor-mediated depolarization. This supports the hypothesis that the main mechanism of KA-induced intracellular calcium at DIV4 is through AMPA receptor-mediated depolarization, resulting in influx through VSCCs.

Source of KA-induced calcium influx in DIV8 neurons

To determine whether developmental age affects the route of KA-induced calcium influx, experiments were repeated on DIV8 hippocampal neurons. Data were generated from 100–200 neurons for each separate experiment from five to 11 coverslips from three to six independent cultures. Separate two-way ANOVAs were conducted on data generated from the intracellular calcium concentration of neurons in response to administration of each antagonist and combination of antagonists administered on DIV8. Analysis revealed a 66% reduction in calcium influx by NBQX ($n = 75$; $F_{1,149} = 22.76$; $P < 0.0001$), a comparable 67% reduction by the polyamine-sensitive AMPA receptor antagonist, NAS ($n = 123$; $F_{1,245} = 32.11$, $P < 0.0001$), and a 30% reduction by MK801 ($n = 139$; $F_{1,274} = 3.73$; $P < 0.05$). In striking contrast to DIV4, however, diltiazem did not significantly affect KA-induced calcium influx ($n = 127$; $F_{1,253} = 0.68$; $P = 0.41$). The combination of NAS, NBQX, MK801, and diltiazem resulted in a 73% reduction in KA-induced calcium compared with KA alone ($n = 67$; $F_{1,133} = 35.51$; $P < 0.0001$; Fig. 6B) as did the combination of NBQX, MK801, and diltiazem (data not shown). When the various antagonists were compared with one another, there was a significant difference in blockade by administration of the combination of NAS + NBQX + MK801 + diltiazem compared with MK801 or with diltiazem alone ($F_{4,421} = 3.77$; $P < 0.05$) but not compared with NBQX

or NAS alone ($P = 0.42$). Taken together, these data suggest a shift in the source of KA-induced calcium influx from voltage-gated calcium channels early in development to include NMDA receptors and calcium-permeable AMPA receptors later in development.

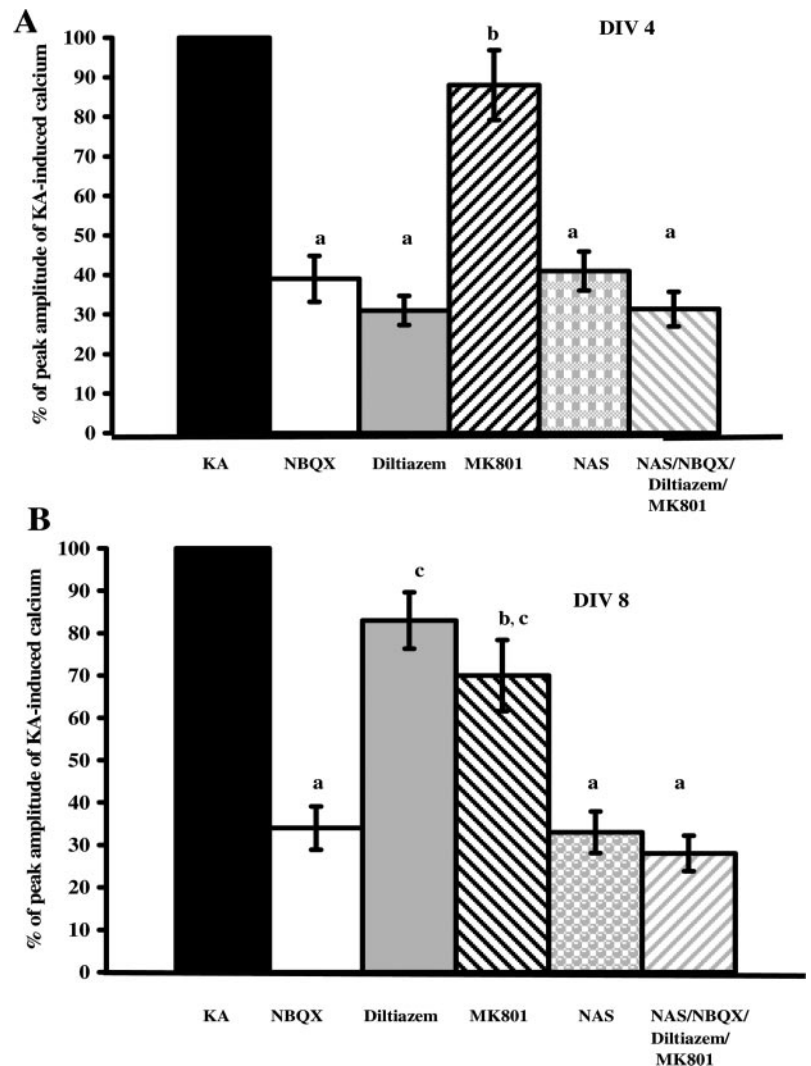
Discussion

We report here an increase in free intracellular calcium in rat hippocampal neurons *in vitro* in response to KA at ages that are roughly equivalent to the 1st week after birth, consistent with previous data demonstrating functional glutamate receptors early in development (43–45). KA-induced intracellular calcium increased with developmental age from DIV4 through DIV10, associated with previously reported developmental increases in glutamate binding and expression of glutamate receptor subunits, as well as increases in synaptic activity during the 1st postnatal week (46, 47). Pretreatment with estradiol for 48 h reduced the KA-induced calcium response by almost 29% and reduced the percentage of cells responding to KA by approximately 20% on DIV4 only.

Calcium influx after exposure to KA on DIV4 occurred predominantly through AMPA receptor-mediated depolarization and subsequent activation of diltiazem-sensitive, voltage-dependent calcium channels. These AMPA receptors could be blocked by NAS, a specific antagonist of GluR2-lacking calcium-permeable AMPA receptors. No significant contribution through the NMDA receptor could be detected based on the selective use of MK801 as an antagonist. The combination of antagonists to AMPA receptors, NMDA receptors, and L-type VSCCs did not fully block calcium influx, with approximately 20–30% of the KA-induced calcium influx persisting. Possible explanations for this residual KA-induced calcium include: 1) calcium influx through NBQX-resistant kainate receptors, 2) incomplete blockade of kainate receptors by NBQX, or 3) release of calcium through internal stores via activation of metabotropic receptors.

KA binds to both AMPA and kainate receptors. Neuronal

FIG. 6. A, Percent reduction in peak amplitude of the KA-induced calcium influx at DIV4. The AMPA/kainate receptor antagonist, NBQX, the specific calcium-permeable AMPA receptor antagonist, NAS, and the VSCC antagonist, diltiazem, significantly blocked calcium influx due to KA ($P < 0.0001$). Antagonism of the NMDA receptor alone with MK801 had no effect ($P = 0.22$), and there was no effect of hormone treatment and no interaction in any of the treatment groups. The combination of NAS, MK801, NBQX, and diltiazem reduced KA-induced calcium concentration compared with KA alone ($P < 0.0001$). When the antagonists were compared with one another, there was no significant difference in reduction in the peak amplitude of the KA-induced calcium concentration with the combination of NAS, NBQX, MK801, and diltiazem compared with NBQX, NAS, or diltiazem alone. a, Significant difference from KA ($P < 0.0001$); b, significant difference from the combination of NAS, NBQX, MK801, and diltiazem ($P < 0.05$). B, Percent reduction in peak amplitude of KA-induced calcium at DIV8. Separate two-way ANOVAs revealed a main effect of antagonism by NBQX, NAS ($P < 0.0001$), and MK801 ($P < 0.05$) but not diltiazem ($P = 0.41$). There was also a significant reduction in KA-induced calcium when NBQX, NAS, MK801, and diltiazem were coadministered ($P < 0.0001$). There was no effect of hormone treatment and no interaction in any of the experimental groups. When the various antagonists were compared with one another, there was a significant difference in blockade by administration of the combination of NAS, NBQX, MK801, and diltiazem compared with MK801 and diltiazem alone ($P < 0.05$) but not compared with NBQX or NAS alone ($P = 0.42$). a, Significant difference from KA alone ($P < 0.0001$); b, significant difference from KA alone ($P < 0.05$); c, significant difference from combination treatment ($P < 0.0001$).



AMPA receptors typically require $50 \mu\text{M}$ for saturation (48), whereas kainate receptors activate at a concentration as low as $0.5 \mu\text{M}$ (49, 50). The current dose of KA ($10 \mu\text{M}$) might preferentially bind to kainate receptors over AMPA receptors, resulting in influx of calcium through the kainate receptor (51). In addition, the competitive antagonist, NBQX, preferentially inhibits AMPA over kainate receptors (51, 52) with a 30-fold greater potency for AMPA *vs.* kainate binding sites (52). At the dose used ($100 \mu\text{M}$), NBQX blocks approximately 95% of the AMPA receptors but exerts only a partial blockade of the kainate receptor. The combination of preferential activation and partial antagonism of kainate receptors may explain the lack of complete inhibition of calcium influx by NBQX alone or all antagonists combined.

The residual calcium may also be due to release of calcium through internal stores. We have observed glutamate-induced release of calcium from internal stores via activation of metabotropic glutamate receptors (Hilton, G. D., J. L. Nuñez, L. L. Bambrick, S. M. Thompson, and M. M. McCarthy, unpublished data). In adults, KA-induced seizure activity has been shown to involve metabotropic receptors, resulting in neuronal damage (53, 54). Developmentally,

mRNA and protein for all metabotropic receptors are present in the hippocampus by the day of birth (55–57). NBQX does not interact with metabotropic receptors and would not be expected to block internal calcium release secondary to KA (58); therefore, any contribution to the KA-induced calcium transient by release of internal calcium might contribute to the residual calcium observed.

Comparison of the antagonism of KA-induced calcium influx by NBQX *vs.* NAS indicates the effects were essentially identical, suggesting that a large portion of AMPA receptors at this age lack GluR2 subunits and hence may be calcium permeable. If this were true, however, one would anticipate that antagonism of KA-induced calcium with NAS would be significantly greater compared with antagonism with diltiazem, but it was not. Two explanations are possible: 1) NAS, at the dose used, blocked some nonpolyamine-sensitive AMPA receptors; or 2) the actual amount of KA-induced calcium through the polyamine-sensitive AMPA receptor was negligible and hence not detected. In adults, a subpopulation of hippocampal neurons shows intermediate sensitivity to blockade by NAS, raising the possibility that sensitivity to NAS is determined not just by the presence or absence of

the GluR2 subunit but also by the ratio of subunits within the receptor complex (59, 60).

On DIV8, a developmental change in the neuronal response to KA was observed. KA-induced calcium influx was reduced by NMDA receptor and calcium-permeable AMPA receptor antagonists, with almost no contribution of the VSCCs. Once again, we were unable to completely block KA-induced calcium transients, but the magnitude of the residual was not different from DIV4.

In adults, estradiol is neuroprotective against excitotoxic and ischemic injury both *in vivo* and *in vitro* (13–15, 61, 62). Estradiol also protects against KA-induced injury, with the severity of damage negatively correlating to hormonal levels (63–65). In this study, hippocampal neurons were mixed male and female. We previously reported that KA-induced damage selectively in neonatal females and that estradiol prevented this damage (32, 33). Our goal here was to begin to understand how estradiol acts on immature hippocampal neurons to gain insight both into the mechanisms by which estradiol impacts normal hippocampal development and its neuroprotective actions. We observed a suppression of the neuronal responses to KA in estradiol-treated cells, with a decrease in the peak amplitude of the KA-induced calcium transient as well as an increase in the percentage of cells that fail to respond with calcium influx on roughly the equivalent of the day of birth. Estradiol had no effect on KA-induced intracellular calcium at any later point in development, highlighting a restricted effective period for estradiol on this parameter.

A number of mechanisms for the neuroprotective effect of estradiol have been proposed including regulation of members of the apoptotic cascade, resulting in modulation of cell death (62); increased expression of neurotrophins, resulting in increased cell survival and differentiation (8); regulation of the MAPK and phosphatidylinositol 3-kinase/protein kinase B signaling cascade (66, 67); and its action as a reactive oxygen species scavenger (68, 69). Estradiol has been shown to increase mitochondrial sequestration of calcium (9, 10, 67), attenuate glutamate-induced intracellular calcium influx in cultured hippocampal neurons (70), and decrease intracellular calcium induced by 3-nitropropionic acid in cerebral endothelial cells (71). The effects of estradiol on calcium influx differ according to age. Modulation of calcium-dependent processes is seen in juvenile tissue but not aged neurons (72). In older hippocampal slices, short-term, supraphysiologic levels of estradiol increase kainate-mediated currents in CA1 cells (73).

Estradiol also directly affects glutamatergic receptor function by down-regulating NMDA receptor-mediated excitatory amino acid binding (17, 74), altering NMDA subunit expression (75, 76), and reducing calcium influx through L-type VSCCs (69, 77). Variations in hormonal levels have also been associated with differences in expression of subunits of the AMPA receptor (78, 79) in an area-specific manner (76, 80, 81). Either of the latter two mechanisms could account for the observation that estradiol decreased the percentage of cells responding to KA and reduced the average size of the calcium transient. It is also possible that fewer cells respond to KA because of an estradiol-mediated decrease in neuronal viability. Previous data from our lab, however,

indicate estradiol does not decrease neuronal viability but instead increases survivability in dissociated hippocampal cultures (33, 34).

The mechanisms underlying hypoxic/ischemic injury in the newborn are complex and have not been clearly delineated. Consequently, therapeutic regimens focus on supportive care and do not address mechanisms of cell death and neuronal injury (82–85). Although we have shown that estradiol may reduce KA-induced intracellular calcium, we have not provided direct proof that these calcium changes result in the neuroprotection observed *in vivo* (32, 33), nor we were able to observe estradiol-mediated regulation of calcium influx specifically through ionotropic glutamate receptors or VSCCs. Instead, these results may speak to general mechanisms of calcium regulation during hippocampal development and the possible role of gonadal steroids in neuronal maturation.

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