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Androgens predispose males to GABA_A-mediated excitotoxicity in the developing hippocampus

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Abstract

Clinical evidence and animal models indicate greater brain damage in newborn males following injury. In adults, glutamate is the primary source of excitotoxic cell death and the steroid, estradiol, is neuroprotective. In neonatal brain, membrane depolarization following activation of GABA_A receptors is the major source of excitation. Consequent influx of calcium via L-type channels is normally trophic, but becomes excitotoxic during periods of excessive activation of GABA_A receptors, such as hypoxia-ischemia, alcohol exposure and seizures. The use of sex-specific hippocampal cultures revealed greater cell death induced by the GABA_A agonist, muscimol, in male-versus female-derived cultures. Pretreatment with the androgen, dihydrotestosterone (DHT) increased muscimol-induced death in both sexes. Exploration of calcium dynamics indicated that, counter to expectation, female neurons achieved higher $[Ca^{2+}]_i$ than male, but the calcium transient duration was shorter due to faster rise and decay. However, a second exposure to muscimol within minutes of the first, caused significant attenuation of $[Ca^{2+}]_i$ in female neurons. In contrast, while male neurons exposed to muscimol for the first time exhibited lower maximal $[Ca^{2+}]_i$, when exposed to muscimol again there was no attenuation in $[Ca^{2+}]_i$. The latter effect was induced in females by DHT, and inversely correlated with the amount of gamma2 subunit of the GABA_A receptor. This novel effect of androgen on GABA-mediated excitotoxicity suggests a unique opportunity for a sex-specific therapeutic approach involving antagonism of the androgen receptor in neonatal males at risk for brain injury.

Keywords

anesthesia; excitotoxicity; fetal alcohol; hypoxia-ischemia; seizure; sex difference

GABA_A receptor activation is a critical mechanism for excitation in the developing brain (Ben-Ari, 2002), but excessive activation is central to the pathogenesis of damage following hypoxia-ischemia (Andine et al., 1991; Nuñez et al., 2003) seizures (Dzhala et al., 2005; Khalilov et al., 2005), fetal alcohol (Galindo et al., 2005) and possibly early anesthetic exposure (Young et al., 2005). Immature hippocampal pyramidal neurons maintain high relative intracellular chloride, resulting in membrane depolarization following GABA_A receptor activation (Leinekugel et al., 1995; LoTurco et al., 1995; Obrietan and van den Pol, 1995;

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Ganguly et al., 2001; Ben-Ar, 2002). This leads to calcium influx via L-type voltage sensitive calcium channels (Obrietan and van den Pol, 1995; Nuñez et al., 2005). We have previously documented a sex difference in response to GABA_A receptor-mediated injury in the developing hippocampus (males>females), with exogenous application of the steroid hormone, estradiol, enhancing damage (Nuñez and McCarthy, 2003). However, we have also observed that endogenous estradiol concentrations are the same in neonatal male and female hippocampus (Amateau et al., 2004; McCarthy and Konkle, 2005). Therefore, estradiol alone cannot account for the enhanced sensitivity of males to GABA_A receptor mediated injury.

Circulating androgens, both testosterone and its primary metabolite, dihydrotestosterone (DHT), are synthesized and released from the testes during the perinatal period (Sokka and Huhtaniemi, 1995). A major function of elevated androgens in males is to serve as a precursor to neuronal estrogen, as it is locally aromatized within the neonatal brain (Naftolin et al., 1971; McEwen et al., 1977). However, in contrast to most sexually dimorphic regions of the brain, the hippocampus has low aromatase activity (McCarthy and Konkle, 2005), but high levels of androgen receptor, particularly in the principle cells (Xiao and Jordan, 2002). One beneficial effect of androgen sensitivity of the developing hippocampus may be to promote spatial learning ability later in life (Isgor and Sengelaub, 1998, 2003). However, such benefit comes at a cost, as we now report that androgen exacerbates cell death induced by GABA_A-mediated excitotoxicity. The enhanced cell death is not due to increased depolarizing GABA, which is an effect achieved by estradiol, but rather is correlated with decreased expression of the $\gamma 2$ subunit of the GABA_A receptor and a failure to desensitize the magnitude of the calcium transient in response to repeated activation of the GABA_A receptor. Thus, the increased vulnerability in males appears to be due to the cumulative effects of elevated intracellular calcium that can occur during conditions associated with injury. This novel observation raises the possibility of sex-specific therapeutic approaches to pediatric brain injury.

Materials and Methods

Tissue Preparation and Treatment

Newborn (postnatal day 0) male and female rats (Sprague-Dawley, Charles River Labs, Wilmington, MA, USA) were obtained from breeder females at the University of Maryland, Baltimore. Animal use procedures were approved by the University of Maryland, Baltimore IACUC, and followed NIH guidelines. In all procedures, tissue from male and female rats remained separate. Hippocampal neurons were cultured according to previously established procedures (Nuñez et al., 2005). Briefly, hippocampi were dissected into HBSS- [88ml sterile H₂O, 10 ml Hank's balanced salt solution (Ca²⁺ and Mg²⁺-free) 10X, 1 ml HEPES buffer, 1.0 M, pH 7.3, 1 ml antibiotic/antimycotic 100X liquid], then additional HBSS- was added to the tube to a volume of 4.5 ml, with 0.5ml trypsin (2.5%), and incubated 15 minutes at 37°C. Supernatant was discarded and tissue washed twice with HBSS-, dissociated by trituration, plated on 25mm Poly-L-lysine coated cover slips at a density of 300,000 cells per coverslip, and placed in 100mm dishes containing 4ml plating medium [86ml MEM, 10 ml horse serum, 3 ml glucose (filter sterilized, 20%) 1ml pyruvic acid, 100mM]. Cell number and viability were determined by trypan blue exclusion and cells allowed 4 hours to adhere to the coverslips in a 37°C, 5% CO₂ incubator after which coverslips were removed from the plating dishes and placed into 35mm dishes filled with Neurobasal- [1ml B-27 supplement, 1ml Antibiotic/Antimycotic 100X, 125 μ l L-Glutamine and filled to 50ml with Neurobasal (phenol red free)]. All cell culture chemicals and solutions were obtained from Invitrogen (Carlsbad, CA, USA). Treatments were on days *in vitro* (DIV) 0 and 1 and included one of the following four regiments: 1) DMSO (vehicle < 0.5% by volume), 2) 1nM dihydrotestosterone (DHT - androgen receptor agonist), or 3) 1 μ M flutamide (Fluandrogen receptor antagonist), followed 30 minutes later by 1nM dihydrotestosterone (Flu + DHT).

TUNEL Labeling

On day *in vitro* 2, cultured cells were administered either saline or 10 μ M muscimol, with the treatments repeated 4 hours later. The treatment paradigm is hypothesized to mimic the waves of depolarization that occur following many forms of early brain injury including hypoxia-ischemia and seizures (Dirnagl et al., 1999; Hartings et al., 2003; Allen and Attwell, 2004). Twenty-four hours following the last muscimol or saline administration, cultures were processed for TUNEL immunoreactivity. Briefly, cells were fixed in 4% paraformaldehyde for 25 min at RT, washed 3x PBS for 5min, permeabilized by immersion in 0.2% Triton X-100 solution in PBS, and stained using the Promega DeadEnd Colorimetric TUNEL system (Madison, WI, USA) and TUNEL+ cells visualized by diaminobenzidine (DAB). Cells were lightly counter-stained with cresyl violet, dehydrated and mounted on slides. TUNEL+ and living neurons were counted using the fractionator technique and the MicrobrightField NeuroLucida program (version 2.01, Colchester, VT, USA). An unbiased disector frame was randomly placed throughout each coverslip, with a total of 100 fields sampled to obtain an estimated # of TUNEL+ cells and # of living cells/ coverslip. A coefficient of error (CE) value of 0.1 to 0.06 from each coverslip was obtained for each individual estimate. A total of 5 coverslips were sampled/group from 2 culture runs.

Calcium Imaging in Cultured Hippocampal Neurons

Cells underwent calcium imaging on day *in vitro* (DIV) 2 according to previously established protocol (Nuñez et al., 2005; Nuñez et al., 2007). Briefly, hippocampal neurons were incubated for 30 min at RT with the cell permeant fluorescent indicator Fura-2-AM (3 μ M; Molecular Probes, Eugene, OR, USA) in DMSO (<0.5%) before transfer to a tissue chamber mounted on a microscope stage, and superfusion with physiological salt solution (PSS) at 32-34°C to remove extracellular dye and allow for esterification of Fura-2-AM. All drugs (see below) were administered in the superfusate. The imaging system consisted of a Zeiss Axiovert 100 inverted microscope with Till Photonics Polychrome II Monochromator (Applied Scientific Instrumentation, Eugene, OR, USA), a Hamamatsu CCD video camera and image intensifier. Image acquisition and analysis was performed with Metamorph/Metafluor Imaging System, version 5.0 (Universal Imaging Corporation, Downingtown, PA, USA). Cells in the field of view were characterized morphologically using a 60X objective, making a distinction between neurons and glia. Individual cells were chosen for analysis 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, glial cells are amorphous in shape, with flat and nondistinct edges and no distinct processes. Only data obtained from neurons was included. Baseline measurement of resting $[Ca^{2+}]_i$ for individual cells was obtained over a 5-minute period while the cells were superfused with PSS. This was followed by a 50 sec pulse of muscimol (10 μ M), with data acquired for a further 5 to 7 min until re-establishment of baseline. In some experiments, this was followed by a second 50 sec pulse of muscimol.

The following parameters were documented during calcium imaging: 1) average baseline $[Ca^{2+}]$, 2) peak $[Ca^{2+}]_i$ following muscimol application, 3) time to attain peak $[Ca^{2+}]_i$ (rise time), 4) time following muscimol application to re-attain one-half baseline $[Ca^{2+}]_i$ (decay time), and 5) decrement in the peak magnitude of a 2nd muscimol-induced calcium transient. This value was determined by the formula $\{([Ca^{2+}] - [Ca^{2+}]_{if}) / [Ca^{2+}]_{if}\} * 100$, where $[Ca^{2+}]_{if}$ is the peak $[Ca^{2+}]_i$ following the first muscimol application and $[Ca^{2+}]_{is}$ is the peak $[Ca^{2+}]_i$ following the second muscimol application. Data from a total of 40-55 hippocampal neurons was collected in each group, obtained from 3 to 4 culture runs. The $[Ca^{2+}]_i$ was calculated from the ratio of the background corrected Fura-2 emission (520nm) at two excitation wavelengths (340 nm/380 nm) by *in situ* solution calibration (Nuñez et al., 2005),

performed using a calcium calibration buffer concentration kit (Molecular Probes, Eugene, OR, USA).

Protein Quantification by Western Blot

Tissue for western blot analysis was obtained from culture dishes containing day *in vitro* 2 hippocampal cells, and in a separate experiment from the hippocampi of postnatal day 2 rats treated with steroids (DHT - 50ug; flutamide - 150ug) for 2 days prior. Hippocampal cell-containing culture dishes were placed on ice and 75µl of icecold lysis buffer containing 50mM Tris-HCl, 1% Na-deoxycholate, 0.25% NP-40, 150 mM NaCl, 1mM EDTA and protease inhibitors (1µg/ml of aprotinin, leupeptin, and pepstatin; 1mM phenylmethylsulfonyl fluoride) was added to each dish. Cells were scraped off of the coverslips and collected along with the liquid. Cells were collected from 3 separate culture runs, with 6 samples from the control, DHT, DHT + flutamide treated male and female cultures. For the *in vivo* tissue, hippocampi were microdissected out onto a chilled surface and flash frozen using isopentane and stored at -80° C until homogenization with ice-cold lysis buffer. Tissue was collected from 3 separate litters, with 6 samples from the control, DHT, flutamide, and DHT + flutamide treated males and females. Tissue samples were centrifuged at 3,000X g for 30 min at 4°C. The supernatant fraction was collected, and protein determined by Bradford assay. Samples of 20µg protein from each animal were electrophoresed on a SDS-polyacrylamide gel (8-16% Tris glycine) and transferred to a polyvinylidenedi-fluoride membrane, washed with 0.1M TBS and incubated in 0.1M TBS containing 5% nonfat dry milk for 1 hr at RT. Membranes were then incubated with 1) a rabbit polyclonal antibody generated against the rat gamma2 subunit of the GABA_A receptor, at a dilution of 1:1000 (Affinity Bioreagents, Golden, CO, USA) or, 2) a mouse monoclonal antibody generated against the housekeeping gene, GAPDH (1:10,000; Chemicon, Temecula, CA). All antibodies were diluted in TBS containing 0.05% Tween-20 (TBS-T), and incubated for 3 hours at room temperature, followed by incubation mouse anti-rabbit HRP-linked secondary antibody (1:2500, Cell Signaling Technology, Beverly, MA) for 30 min at RT. Immunoreactive bands were detected using an enhanced chemiluminescence kit (ECL kit, New England Biolabs, Beverly, MA, USA) and membranes were exposed to film (Hyperfilm-EC; Amersham, Arlington Heights, IL, USA). The proteins were detected as bands of specific molecular masses (gamma2 subunit = 44-46 kDa; GAPDH=37 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 U.S. National Institutes of Health and available at <http://rsb.info.nih.gov/nih-image/>). In order to control for gel loading, iad values for the protein levels of the gamma2 subunit were divided by GAPDH.

Statistical Analysis

Three-way analyses of variance (sex, treatment, muscimol application) were performed on the number of TUNEL-positive nuclei per coverslip. Two-way analyses of variance (sex and treatment as factors) were performed on peak intracellular calcium concentration, rise time, decay time, decrement in the magnitude of muscimol-induced calcium response and integrative area density from western blots. For all ANOVAs and post-hoc (Tukey) tests, a level of $p < 0.05$ was required to obtain statistical significance. For all calcium imaging values, data was averaged across a given field of view, therefore taking into account potential biases of region of the coverslip and over-representation of a given region of the coverslip.

Results

DHT exposure or being male increases vulnerability of hippocampal neurons to muscimol-induced excitotoxicity

In the present experiment, we used TUNEL immunoreactivity (ir) as an indicator of dead and dying cells (Figure 1). Repeated muscimol application significantly increased the number of TUNEL-ir cells in all hippocampal cultures, ($F_{1,48}=768.61$; $p<0.0001$), but the increase was larger in male-derived (125% more than vehicle) versus female-derived cultures (83% more than vehicle; $F_{1,48}=8.55$; $p<0.032$). Pre-treatment with DHT had no effect on cell death unless combined with muscimol application in which case it significantly increased the proportion of TUNEL-ir cells in both male-and female-derived hippocampal cultures ($F_{2,48}=64.43$; $p<0.0001$; post-hoc Tukey's; $p<0.01$), and this increase was greater than the proportion of TUNEL-ir cells observed in muscimol alone treated cultures ($F_{2,48}=79.82$; $p<0.0001$). The DHT-mediated enhancement in the number of TUNEL-ir cells was completely blocked by pretreatment with flutamide, indicating the effect occurs via the androgen receptor.

In contrast, there was no effect of treatment on the number of living cells in either male (Vehicle = 60117.7 ± 692.2 ; Musc = 59269.6 ± 634.3 ; DHT+Musc = 60356.2 ± 710.4 ; Flu+DHT+Musc = 60243.7 ± 684.7) or female hippocampal cultures (Vehicle = 61125.5 ± 441.6 ; Musc = 59261.6 ± 668.8 ; DHT+Musc = 60858.1 ± 725.3 ; Flu+DHT+Musc = 61001.3 ± 702.4).

The magnitude of a muscimol-induced calcium transient is greater in females and increased by treatment with estradiol, but not DHT

Because there was greater muscimol-induced cell death in hippocampal neurons derived from males or following pretreatment with DHT, we hypothesized that both these variables would increase the magnitude of a calcium transient induced by muscimol. Three parameters of the calcium response to a 50 second application of muscimol were quantified: 1) peak $[Ca^{2+}]_i$, 2) rise time to peak $[Ca^{2+}]_i$ and 3) decay time (duration of elevated $[Ca^{2+}]_i$ to 50% of baseline;).

Female hippocampal neurons achieved significantly higher $[Ca^{2+}]_i$ in response to muscimol application as compared to male hippocampal neurons ($F_{1,19}=12.450$; $p<0.005$; Tukey's; $p<0.01$, Figure 2A). In male and female hippocampal cultures, neither pretreatment with DHT nor the combination of flutamide and DHT had any effect on the maximal increase in $[Ca^{2+}]_i$.

The time to reach maximal calcium after muscimol is faster in females and unaffected by DHT

The amount of time required to attain the maximal intracellular calcium concentration is reflective of the magnitude of GABA_A receptor-mediated membrane depolarization, the number of VSCCs open and the kinetics of VSCCs. Even though the peak was higher, female neurons achieved it faster than male neurons ($F_{1,20}=10.36$; $p<0.002$, Figure 2B). Pretreatment with the androgen DHT or DHT plus flutamide had no significant effect on the rise time to peak $[Ca^{2+}]_i$.

The time to clear calcium after a muscimol-induced transient is faster in females and altered by pretreatment with DHT

The decay time, a measure of how quickly a cell reallocates calcium out of the cytoplasm, was significantly faster in female than male neurons ($F_{1,20}=6.70$; $p<0.011$). Muscimol application lasted for 50 seconds and male hippocampal neurons maintained elevated $[Ca^{2+}]_i$ for 91 ± 3 seconds post-muscimol application while female hippocampal neurons cleared elevated $[Ca^{2+}]_i$ by 54 ± 4 seconds. There was a significant sex by treatment interaction on decay time ($F_{4,20}=52.64$; $p<0.0001$). Pretreatment with DHT increased the decay time in female neurons

to that of males, and this effect was blocked by flutamide (Tukey's, $p < 0.01$). There was no effect of DHT on decay time in male neurons (Figure 2C).

Male and DHT-treated neurons do not attenuate their response to a second muscimol application

We investigated the impact of sex and hormone manipulation on repeated GABA_A receptor activation as reflected in $[Ca^{2+}]_i$. A first application of muscimol (50 second pulse) was followed 5-7 minutes later by a second application of the same dose and duration (Figure 3). We quantified the peak $[Ca^{2+}]_i$ to each muscimol application and noted the change in response to the second application. Female hippocampal neurons exhibited a significant 50% drop in $[Ca^{2+}]_i$ to a second muscimol application ($F_{1,14}=18.62$; $p<0.0001$; Tukey's, $p<0.01$), whereas male hippocampal neurons showed no significant change, reducing their response by less than 10%. Pretreatment with the androgen, DHT, altered the response of female neurons such that they no longer exhibited an attenuation in response to the second muscimol application ($F_{3,14}=12.14$; $p<0.0001$). The effect of DHT was blocked by the antagonist, flutamide, again indicating a receptor-mediated effect.

The $\gamma 2$ subunit of the GABA_A receptor is lower in male hippocampus and reduced by pretreatment with DHT

We investigated the effect of sex and exogenous steroid hormone treatment on protein levels of the $\gamma 2$ subunit in hippocampal neurons in animals and in cultured hippocampal neurons using Western blot analysis

In vivo—Newborn female hippocampi had more $\gamma 2$ protein than male hippocampi ($F_{1,30}=52.21$; $p<0.0001$; Figure 4A). DHT treatment reduced $\gamma 2$ levels in females to that of vehicle treated males, and further reduced levels in males as revealed by a significant sex by treatment interaction ($F_{4,30}=8.98$; $p<0.0008$; Tukey's, $p<0.05$). Pretreatment with flutamide blocked the effect of DHT in both males and females. Importantly, flutamide alone had no effect in but elevated $\gamma 2$ levels in males to that of females (Tukey's, $p<0.05$), demonstrating that endogenous androgen in males maintained $\gamma 2$ at low levels. Data are expressed as grayscale integrative area density (mean $\pm$ SEM value, $n = 6$ /sex/treatment).

In vitro—Consistent with the *in vivo* findings, cultured hippocampal neurons from females had significantly higher protein levels of $\gamma 2$ relative to males $F_{1,30}=85.67$; $p<0.0001$; Tukey's, $p < 0.05$; Figure 4B) and DHT treatment reduced $\gamma 2$ levels in both male and female hippocampi to below that of vehicle-treated males ($F_{4,30}=21.01$; $p<0.0001$; Tukey's, $p < 0.05$). Data are expressed as grayscale integrative area density (mean $\pm$ SEM value, $n = 6$ /sex/treatment).

Discussion

Both clinically and in animal models, newborn males are more sensitivity to brain injury. They suffer greater damage to the same magnitude insult and experience more deleterious outcomes (Hindmarsh et al., 2000; Lauterbach et al., 20001; Reiss et al., 2004; Johnston and Hagberg, 2007). There has been little if any mechanistic rationale for this sex difference. In the present study we used muscimol, a selective GABA_A receptor agonist, which when applied to immature primary hippocampal neurons results in membrane depolarization and increases free $[Ca^{2+}]_i$ via influx through L-type VSCCs (Nuñez et al., 2005). Repeated muscimol exposure induces excitotoxic cell death of hippocampal neurons *in vivo* and *in vitro* due to excessive calcium influx (Nuñez et al., 2003; Nuñez and McCarthy, 2004). We previously reported that males are more sensitive than females to depolarizing GABA-mediated injury using an *in vivo* model (Nuñez et al., 2003; Nuñez et al., 2003; Nuñez and McCarthy, 2004), and confirm that here with an *in vitro* model. Moreover, we provide evidence that androgens, not estrogens,

mediate this sex difference and that contrary to expectation, the increased vulnerability in males is not due to increased amplitude of calcium transients, but rather to the rate of calcium clearance and a lack of attenuation in calcium response to a second depolarizing event. The duration of elevated intracellular calcium following muscimol application is carefully regulated by the cell. Following influx of calcium, cells re-establish homeostasis by calcium extrusion, or sequestration into organelles (Siesjo, 1990; Dayanithi et al., 2006). Calcium-induced calcium release from internal stores and buffering by calcium binding proteins can further affect the duration of elevated intracellular calcium (Mattson et al., 1991).

Sex and neonatal exposure to steroid hormones both affected intracellular calcium responses following activation of GABA_A receptors. Female hippocampal neurons exhibited significantly greater peak $[Ca^{2+}]_i$ following muscimol, along with a more rapid time to peak and a faster rate of decay of $[Ca^{2+}]_i$. This resulted in a higher-intensity but shorter duration calcium transient in females compared to males. Treating either male or female hippocampal neurons with estradiol enhances the peak magnitude of muscimol-induced intracellular calcium (Nuñez et al., 2005; Nuñez et al., 2007). The concordance in the effect of estradiol on both faster rise time and increased peak is consistent with an estradiol-induced increase in the number of VSCCs and/or the magnitude of the depolarizing response to muscimol. Estradiol increases both the Cav1.2 ($\alpha 1C$) isoform of the L-type VSCC and activation of NKCC2, the co-transporter that maintains high $[Cl]_i$ in neonatal neurons, thereby increasing the depolarizing GABA response (Nuñez et al., 2005). Androgen treatment had no effect on the peak $[Ca^{2+}]_i$ response to muscimol, or the rise time, suggesting no effect on calcium channels or depolarization. However, DHT treatment significantly extended the duration of calcium clearance in female hippocampal neurons to that of males.

Early in brain development, repetitive stimulation of GABA_A receptors occurs during epileptic seizures, trauma, hypoxia-ischemia or secondary to drug or alcohol abuse (Dirgnal et al., 1999; Villeneuve et al., 2000; Harting et al., 2003; Wang, 2003). Repeated release of GABA and enhanced GABA_A receptor activation occurs during hypoxia-ischemia (Cataltepe et al., 1996; Allen and Attwell, 2004) and experimentally manipulated repetitive exposure to high doses of muscimol can accelerate the rate of the normal developmental conversion of depolarizing to hyperpolarizing GABA (Ganguly et al., 2001). To mimic these conditions, we exposed male and female hippocampal neurons to a second bolus of muscimol 5-7 minutes after the first. In cultures derived from female hippocampi, there was on average a 50% reduction in the magnitude of the calcium transient in response to the second depolarization, an effect that is presumed to be neuroprotective. In male neurons, however, there was no attenuation in response to the second depolarization, and treatment of female neurons with DHT increased the amplitude of the second response to that of the first. All of the other characteristics regarding rise time, peak and decay time were maintained, thus males or neurons exposed to DHT have a cumulative exposure to calcium that is far greater than that of females neurons experiencing the same insult.

The GABA_A receptor is a heteromeric pentamer that is rapidly desensitized following ligand binding. The kinetics of desensitization are influenced by the subunit conformation (Hutcheon et al., 2000; Bianchi and Macdonald, 2002). The decrement observed in response to the second application of muscimol can be considered reflective of decreased activation of the GABA_A receptor. In immature pyramidal cells, GABA_A receptor desensitization occurs more slowly than in adult neurons and this property promotes maintained responsiveness to repetitive GABA_A receptor activation (Hutcheon et al. 2000). Early in development, GABA_A receptors in hippocampal neurons have low levels of $\gamma 2$ subunit, a subunit that promotes rapid, longer-lasting inactivation (Hutcheon et al., 2000; Bianchi and Macdonald, 2002). As development progresses, there is an increase in $\gamma 2$ subunit levels. A reduction in response to a second exposure to ligand is traditionally termed desensitization. For the GABA_A receptor,

desensitization can occur in milliseconds and be complete within 100s of seconds (Bianchi et al., 2001). In hippocampal neurons, desensitization is multi-phasic and the subunits that compose the GABA_A receptor contribute to variability in the number and duration of distinct phases (Hutcheon et al., 2000; Bianchi and Macdonald, 2002; Chang et al., 2002).

Desensitization plays an important and complex role in shaping inhibitory synaptic currents (Jones and Westbrook, 1995; Bianchi and Macdonald, 2002) and varies across the lifespan. In the current study, desensitization applies to the decrement in calcium response seen in immature neurons following a second application of muscimol with an inter-stimulus interval of minutes. We found that female hippocampal neurons reduced by half the amplitude of the calcium transient upon second exposure to muscimol, whereas male neurons did not. The hormonal control of this desensitization is determined by androgens. DHT treatment essentially eliminated desensitization in both male and female neurons. Recall that DHT had no impact on the size of the calcium transient, indicating that the control of desensitization is via a distinct and separate mechanism.

In mature neurons, desensitization of synaptic GABA_A receptors requires the $\gamma 2$ subunit, and neither this subunit nor desensitization is prevalent in immature neurons (Gambarana et al., 1991; Killisch et al., 1991; Roberts and Kellogg, 2000; Bianchi and Macdonald, 2002). When comparing $\gamma 2$ levels by Western blot analysis of hippocampi collected *in vivo* on the day of birth and on postnatal day 2, as well as in sex-specific hippocampal neuronal cultures, females always had significantly higher levels of the $\gamma 2$ subunit compared to males. Consistent with the findings on desensitization, $\gamma 2$ protein levels were significantly reduced in both males and females by exogenous DHT treatment. Moreover, the androgen receptor antagonist, flutamide increased $\gamma 2$ levels in newborn males to that of females, but had no effect in females, implicating endogenous androgens as the determinant of the observed sex difference. Higher levels of $\gamma 2$ in females is consistent with the occurrence of desensitization but is nonetheless difficult to reconcile with the currently held view that desensitization of the GABA_A receptor is an event measured in seconds, not minutes. Important to bear in mind is that in males, which *in vivo* would be exposed to DHT, there would be only very rapid desensitization and it would not be expected to influence calcium dynamics. It is only in females that have no DHT but do have estradiol, that longer lasting desensitization would be expected. Previous lack of attendance to the sex of the preparation could have precluded the detection of such effects.

We propose that the lack of desensitization in males contributes to their enhanced sensitivity to various forms of early brain trauma (Hindmarsh et al., 2000; Lauterbach et al., 2000; Reiss et al., 2004; Johnston and Hagberg, 2007). Conversely, the greater degree of desensitization in female hippocampal neurons may protect against injury which results from repetitive activation of the GABAergic system and the accompanying excessive calcium influx. Consistent with these findings, male hippocampal neurons were more likely than female neurons to die following muscimol treatment and exogenous DHT greatly increased the likelihood of death following muscimol-induced excitation in both sexes.

All of the observed effects of DHT were blocked by the androgen receptor antagonist, flutamide, suggesting traditional nuclear steroid receptor mediated transcriptional mechanisms. The primary gene target appears to be the $\gamma 2$ subunit of the GABA_A receptor. Moreover, all of the observed actions of androgens appear to be distinct from the effects of estradiol, implicating distinct cellular mechanisms of these two gonadal steroids to determine hippocampal neuronal vulnerability. Estradiol has a potent neuroprotective effect against glutamate-mediated excitotoxicity (Singer et al., 1996; Wilson et al., 2000; Mize et al., 2003; Hilton et al., 2006), but can exacerbate GABA-mediated excitotoxicity via enhancing the magnitude of the calcium transient (Nuñez et al., 2005). Here we report that androgen also increases the excitotoxic capacity of depolarizing GABA, but via a distinct mechanism

involving the management of intracellular calcium. We postulate that the elevated circulating levels of DHT during the late embryonic/early postnatal period in males may be a predisposing factor affecting the response to deleterious early events (e.g. fetal alcohol exposure, seizures, hypoxia-ischemia). This novel observation may explain the increased vulnerability of newborn males to brain damage and could provide novel sex-specific therapeutic approaches via temporary antagonism of the androgen receptor.

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Figure 1. Exposure to androgen increases cell death induced by GABA_A receptor activation in neonatal hippocampal neurons

The number of TUNEL-positive nuclei per coverslip was assessed in male- and female-derived primary hippocampal cultures. Repeated muscimol application significantly increased the number of TUNEL-ir cells in both male and female hippocampal cultures, (2-way ANOVA * $p < 0.01$) but the amount of cell death was greater in males (# $p < 0.05$). Pre-treatment with the non-aromatizable androgen, DHT, had no effect on its own but significantly increased the proportion of TUNEL-ir cells induced by muscimol administration in both male- and female-derived hippocampal cultures (^ $p < 0.01$). The exacerbating effect of pretreatment with DHT was completely blocked by the androgen receptor antagonist, flutamide. Data was collected

from two separate culture experiments and a mean was calculated for each coverslip, with $n = 5$ /group. Representative photomicrographs of cresyl violet counterstained primary hippocampal cultures, with arrows denoting location of TUNEL+ cells. Scale bar = 100 μ m.

Figure. 2. Sex influences calcium response to GABA_A receptor activation

(A) Female-derived immature hippocampal neurons achieved a higher peak $[Ca^{2+}]_i$ following muscimol ($10\mu M$) application compared to male-derived neurons (2-way ANOVA, Tukey's, $\#p < 0.05$). There was no effect of pretreatment with DHT or DHT + flutamide (androgen receptor antagonist) on peak $[Ca^{2+}]_i$ response to muscimol in either male or female-derived hippocampal neurons.

(B) The rise time to peak $[Ca^{2+}]_i$ after onset of muscimol application was significantly shorter in female-derived hippocampal neurons compared to all other groups (2-way ANOVA; Tukey's, $\#p < 0.05$), despite the higher peak $[Ca^{2+}]_i$. There was no effect of pretreatment with DHT or DHT + flutamide.

(C) The duration of the decay of the calcium transient induced by muscimol application was significantly shorter in female-derived hippocampal neurons compared to all other groups except females + DHT + flutamide (2-way ANOVA; Tukey's $\#p < 0.01$). Pretreatment with DHT increased the decay time in female hippocampal neurons ($*p < 0.05$ compared to females). Pretreatment with DHT or DHT + flutamide had no effect on decay time in male-derived

hippocampal neurons and flutamide blocked the effect of DHT in female neurons. For all experiments, data represent mean $\pm$ SEM, with a total of 40-55 hippocampal neurons investigated in each group with neurons obtained across 3-4 culture runs.

Figure 3. Both male-derived and DHT-treated neurons do not attenuate the response to a second muscimol application

A bolus application of muscimol was followed by a second application of muscimol 5-7 minutes later.

(A) Mean ($\pm$ SEM) traces of muscimol-induced calcium responses in male-derived control neurons pretreated with dihydrotestosterone (DHT) or DHT + flutamide. Traces were standardized following re-establishment of baseline so that the second calcium transient initiates at 400 secs in all cases. There was no change in the peak $[Ca^{2+}]_i$ achieved following the second application of muscimol in any male neurons, regardless of treatment.

(B) The same procedure using female-derived hippocampal neurons resulted in a significant attenuation in peak $[Ca^{2+}]_i$ following the second bolus of muscimol, unless the female neurons were pretreated with DHT, in which case they resembled male neurons.

(C) Percent change between magnitude of first and second muscimol-induced calcium peak. Female hippocampal neurons displayed significantly greater decrement than males (Tukey's, $\#p<0.05$), which was completely abolished by DHT pretreatment (Tukey's, $*p<0.05$).

Figure. 4. Androgen treatment eliminates the female bias toward elevated $\gamma 2$ subunit of the GABA_A receptor

(A) Hippocampi collected from postnatal day 2 pups were analyzed by quantitative western blot for protein levels of the $\gamma 2$ subunit of the GABA_A receptor. Females had significantly higher $\gamma 2$ levels than males (2-way ANOVA, Tukey's $\#p < 0.05$). There was no effect of pretreatment with estradiol on postnatal days 0 and 1, but pretreatment with the androgen, DHT, significantly reduced levels in females as well as males, so that both were below control males ($*p < 0.05$). The combination of DHT and the androgen receptor antagonist flutamide, completely prevented the downregulation of $\gamma 2$ by DHT.

Importantly, flutamide alone had no effect in females but elevated $\gamma 2$ levels in males to that of females (* $p < 0.05$). Data are expressed as grayscale integrative area density (mean $\pm$ SEM value, $n = 6$ /sex/treatment). Photomicrographs above the histograms show one representative lane (equivalent of one animal) for the corresponding group below.

(B) In a complementary experiment, western blot analysis of the level of $\gamma 2$ protein was also conducted on cultured hippocampal neurons derived from newborn males or females. As *in vivo*, female-derived tissue had significantly higher $\gamma 2$ protein than male (2-way ANOVA, Tukey's $\#p < 0.05$), with elimination of the sex difference by DHT pretreatment of females (Tukey: $*p < 0.05$ compared to control females). DHT treatment in males again significantly reduced $\gamma 2$ protein levels below those in control males (Tukey: $*p < 0.05$) and there was no effect of treatment with estradiol in either sex. Data are expressed as grayscale integrative area density (mean $\pm$ SEM value). Hippocampal cells were collected from 3 culture runs, with each sample ($n = 6$ for each group) obtained from two individual coverslips. Photomicrographs above the histograms show one representative lane (equivalent of two coverslips combined) for the corresponding group below.