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SEX- AND AGE-DEPENDENT EFFECTS OF ANDROGENS ON GLUTAMATE-INDUCED CELL DEATH AND INTRACELLULAR CALCIUM REGULATION IN THE DEVELOPING HIPPOCAMPUS

S. L. ZUP, [a,b]* **N. S. EDWARDS** [c] **AND M. M. MCCARTHY** [c]

[a] *Program in Developmental and Brain Sciences, University of Massachusetts Boston, Boston, MA 02125, United States*

[b] *Department of Psychology, University of Massachusetts Boston, Boston, MA 02125, United States*

[c] *Department of Pharmacology, Program in Neuroscience, University of Maryland School of Medicine, Baltimore, MD 21201, United States*

Abstract—Hippocampal neurons must maintain control over cytosolic calcium levels, especially during development, as excitation and calcium flux are necessary for proper growth and function. But excessive calcium can lead to excitotoxic cell death. Previous work suggests that neonatal male and female hippocampal neurons regulate cytosolic calcium differently, thereby leading to differential susceptibility to excitotoxic damage. Hippocampal neurons are also exposed to gonadal hormones during development and express high levels of androgen receptors. Androgens have both neuroprotective and neurotoxic effects in adults and developing animals. The present study sought to examine the effect of androgen on cell survival after an excitatory stimulus in the developing hippocampus, and whether androgen-mediated calcium regulation was the governing mechanism. We observed that glutamate did not induce robust or sexually dimorphic apoptosis in cultured hippocampal neurons at an early neonatal time point, but did 5 days later – only in males. Further, pretreatment with the androgen dihydrotestosterone (DHT) protected males from apoptosis during this time, but had no effect on females. Calcium imaging of sex-specific cultures revealed that DHT decreased the peak of intracellular calcium induced by glutamate, but only in males. To determine a possible mechanism for this androgen neuroprotection and calcium regulation, we quantified three calcium regulatory proteins, plasma membrane calcium ATPase1 (PMCA1), sodium/calcium exchanger1 (NCX1), and the sarco/endoplasmic reticulum calcium ATPase 2 (SERCA2). Surprisingly, there was no sex difference in the level of any of the three proteins. Treatment with DHT significantly decreased PMCA1 and NCX1, but increased SERCA2 protein levels in very young animals but not at a later timepoint. Taken together, these data suggest a complex interaction of sex, hormones, calcium regulation and developmental age; however androgens acting during the first week of life are implicated in regulation of hippocampal cell death and may be an underlying mechanism for sexually dimorphic apoptosis.

Key words: sex difference, dihydrotestosterone, development, glutamate, calcium imaging, neuroprotection.

INTRODUCTION

Gonadal hormones, acting during a critical perinatal period, can harness the developmental process of apoptosis to sculpt areas of the brain in a sexually dimorphic pattern (reviewed in Forger, 2006). Both the sexually dimorphic nucleus (SDN) and the anteroventral periventricular nucleus (AVPV) are larger in one sex than the other due mainly to hormonally-driven cell death (Davis et al., 1996; Krishnan et al., 2009; Waters and Simerly, 2009). Interestingly, the hormone estradiol drives apoptosis in opposite directions depending on the nucleus: decreasing cell death in the SDN but increasing it in the AVPV, suggesting that estradiol is not intrinsically neuroprotective or neurotoxic during development but instead is capable of both actions, though presumably via different mechanisms.

Research on the neuroprotective effects of gonadal hormones during normal development as well as during adult pathological states has focused mainly on estradiol, leaving neuroprotection due to androgens such as testosterone and dihydrotestosterone (DHT) underexplored. However, the same dichotomy of neuroprotection versus exacerbation of damage observed with estradiol has been demonstrated with androgens under a variety of conditions. Activation of the androgen receptor (AR) protects neurons against insults such as β-amyloid (Pike, 2001; Nguyen et al., 2005, 2010), oxidative stress (Ahlbom et al., 1999), serum

*Correspondence to: S. L. Zup, Psychology Department, University of Massachusetts Boston, 100 Morrissey Boulevard, Boston, MA 02125, United States. Tel: +1-617-287-6319; fax: +1-617-287-6336.

E-mail address: susan.zup@umb.edu (S. L. Zup).

Abbreviations: ANOVA, analysis of variance; AR, androgen receptor; AVPV, anteroventral periventricular nucleus; CCD, charge-coupled device; DAB, diaminobenzidine; DHT, dihydrotestosterone; DIV, day *in vitro*; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; H/I, hypoxic/ischemic birth injury; HBSS, Hank's balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IAD, integrative grayscale pixel area density; NCX1, sodium/calcium exchanger 1; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; PMCA1, plasma membrane calcium ATPase 1; PND, postnatal day; PSS, physiological salt solution; RT, room temperature; SDN, sexually dimorphic nucleus; SERCA2, sarco/endoplasmic reticulum calcium ATPase 2; TBS, Tris-buffered saline; TBS-T, TBS containing 0.05% Tween-20; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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deprivation (Hammond et al., 2001), and glutamate or kainate-induced death (Cardounel et al., 1999; Ramsden et al., 2003), but exacerbates neuron loss due to cerebral ischemia (Hawk et al., 1998; Cheng et al., 2007). Interestingly, in the adult ischemia model, androgens can be both neuroprotective and neurotoxic depending on hormone dose and other variables (Uchida et al., 2009; Fanaei et al., 2013). In the developing nervous system, androgens likewise protect against cell death in the spinal nucleus of the bulbocavernosus (Nordeen et al., 1985; Forger, 2006), but also exacerbate neuronal death induced by excitatory GABA in the hippocampus (Nunez and McCarthy, 2008). Although not classically considered a sexually dimorphic structure, the hippocampus is slightly but significantly larger in males, has sex-specific hormonal regulation of dendritic spine density and regulates behaviors such as spatial navigation and stress responding which differ in males and females (Madeira and Lieberman, 1995; Isgor and Sengelaub, 1998, 2003; Shors et al., 2001; Leranth et al., 2003). Importantly, the hippocampus also expresses ARs at very high levels throughout life (Sar et al., 1990; Kerr et al., 1995; Xiao and Jordan, 2002; Tabori et al., 2005) and is exquisitely sensitive to excitatory stimuli such as glutamate and excitatory GABA.

GABA is the major excitatory neurotransmitter during early postnatal development (Ben-Ari et al., 2012) but cedes that role to glutamate around the end of the first week of life (Ganguly et al., 2001), which is still a time of normal developmental cell death in the hippocampus (Ganguly et al., 2001; Nunez and McCarthy, 2007; Ben-Ari et al., 2012). Previous work from our lab on gonadal hormone neuroprotective/neurotoxic effects following excitotoxic insults found interactions of sex and hormones at different ages: estradiol is protective against glutamate excitotoxicity around the seventh day of life (Hilton et al., 2004, 2006), and DHT exacerbates excitatory GABA-induced cell death right after birth (Nunez and McCarthy, 2008). Further, sex alone seems to predetermine a neuron's reaction to excitatory GABA right after birth, with males suffering more cell death than females (Nunez et al., 2003a,b). However, the effect of androgens on neuronal survival following glutamate stimulation, and whether that effect is sex specific, is unknown at either developmental time point.

An important signal common to developmental cell death, excitotoxic cell death, and also androgen neuroprotection, is regulation of intracellular calcium. The developing brain is especially sensitive to calcium influx after an excitatory stimulus, so that only the appropriate level of neural/electrical activity allows for neuronal survival – a desirable outcome since only those neurons with appropriate connections are allowed to survive and thrive while others die off. In response to an excitatory stimulus, calcium can enter the cytosol from the extracellular milieu and/or be released from internal stores such as the endoplasmic reticulum (ER). Homeostatic mechanisms such as the plasma membrane calcium ATPase1 (PMCA1) and the sodium/calcium exchanger1 (NCX1) can help regulate cytosolic calcium by expelling it into the extracellular space, and the sarco/endoplasmic reticulum calcium ATPase 2 (SERCA2) can sequester cytosolic calcium within the neuron's ER (Blaustein and Lederer, 1999; Bading, 2013). Calcium release from these internal stores appears to be especially important in regulating glutamate-induced cell death during early neonatal development (Hilton et al., 2006), with external calcium influx becoming more important later in life (Nunez and McCarthy, 2009). Interestingly, activation of the AR via DHT has been shown to increase the amount of calcium influx after glutamate application in cultured hippocampal neurons by altering levels of SERCA2 mRNA (Foradori et al., 2007; Foradori and Handa, 2008). Therefore, the current study examined the effects of the androgen DHT on cell death and intracellular calcium levels following a mild glutamate stimulus in hippocampal cultures, as well as expression of the SERCA2, NCX1 and PMCA1 proteins, during early and late neonatal development – the time when glutamate starts to supersede GABA as the major excitatory neurotransmitter.

EXPERIMENTAL PROCEDURES

Primary hippocampal cultures

Animal use and care procedures were approved by the University of Maryland, Baltimore Institutional Animal Care and Use Committee and followed National Institutes of Health Guidelines. All cell culture chemicals and solutions were purchased from Invitrogen (Carlsbad, CA, USA) unless otherwise noted. Newborn rat pups (postnatal day 0; PND0) were obtained from Sprague–Dawley breeding pairs (Harlan, Frederick, MD, USA) at the University of Maryland, Baltimore breeding colony and hippocampi were dissected and cultured as described previously (Nunez et al., 2005; Hilton et al., 2006). Briefly, equal numbers of PND0 male and female hippocampi were dissected into a single sterile tube for mixed-sex cultures, or into separate sterile tubes for sex specific cultures, that contained HBSS+ (88-ml sterile H20, 10-ml Hank's balanced salt solution (Ca^{++}- and Mg^{++}-free) 10×, 1-ml HEPES buffer, 1.0 M, pH 7.3, 1-ml antibiotic/antimytotic 100× liquid), then subjected to trypsin digestion and incubated for 20–30 min at 37 °C. Supernatant was discarded and tissue washed twice with HBSS+, dissociated by titration, plated on 25-mm poly-L-lysine-coated coverslips at a density of 400,000 cells per coverslip, and then placed in 100-mm dishes containing 5-ml plating medium (86-ml MEM, 10-ml horse serum, 3-ml glucose (filter sterilized, 20%), 1-ml pyruvic acid, 100 mM). Cell number and viability were determined by Trypan Blue exclusion and cells were allowed 4 h to adhere to the coverslips in a 37 °C, 5% CO_2 incubator, then coverslips were removed from the plating dishes and placed into 35-mm petri dishes filled with 3 ml of Neurobasal + media (1-ml B-27 supplement, 1-ml antibiotic/antimycotic 100× liquid, 125 µl L-glutamine and filled to 50 ml with Neurobasal [glutamine and phenol red – free]). One-third of media was removed from each culture dish and replaced with fresh media on day *in vitro* 4 (DIV4). For all experiments, cultures were