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Tracy R. Butler
University of Kentucky

Lynda J. Sharrett-Field
University of Kentucky

Jennifer N. Berry
University of Kentucky

Mark A. Prendergast
University of Kentucky

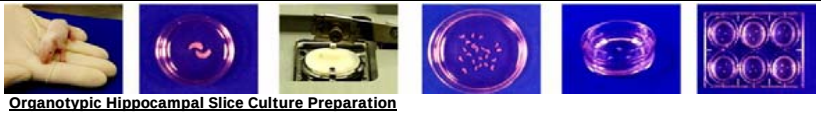
SEX DIFFERENCES IN CA1 HIPPOCAMPAL REGION CELL DEATH FOLLOWING ANTAGONISM OF GIRK CHANNELS DURING ETHANOL WITHDRAWAL

Tracy R. Butler, Lynda J. Sharrett-Field, Jennifer N. Berry, & Mark A. Prendergast
University of Kentucky, Department of Psychology,
Spinal Cord and Brain Injury Research Center, Lexington KY 40536

INTRODUCTION

Adenosine A1 receptor (A1r) activation results in neuronal inhibition via pre-synaptic inhibition of neurotransmitter release and post-synaptic hyperpolarization. The data presented shows sex-dependent cell death with adenosine A1r antagonism and caffeine during ethanol withdrawal (EWD) in vitro. However, no sex difference was detected in A1r immunoreactivity after chronic EtOH exposure in vitro, suggesting that the sex difference in cell death was not due to A1r expression, but may be downstream of A1rs. These studies also demonstrate that the selective NMDAr antagonist, APV, was able to attenuate the cell death observed in the CA1 region of female cultures, suggesting a role for NMDA receptor activation downstream of A1rs mediating the sex-dependent cell death. However, additional cellular mechanisms that may contribute to the sex-selective injury have yet to be investigated. Reduced activation of G-protein inwardly rectifying potassium channels (GIRKs) during EWD represents one potential mechanism the explain the sex-selective cell death that occurs with A1 receptor antagonism during EWD. Upon A1 receptor activation, GIRKs are directly activated by the beta/gamma dimer of the G-protein, resulting in potassium efflux and membrane hyperpolarization. Further, GIRKs are directly activated by acute ethanol (EtOH) in vitro. To further understand the mechanisms of the observed sex difference in cell injury with selective and non-selective adenosine receptor antagonism, the GIRK1/4 channel inhibitor Tertiapin-Q (TPQ) was used in EtOH-naïve and EtOH-withdrawn organtypic hippocampal cultures. Additionally, the current studies investigated the effects of chronic EtOH exposure on GIRK channel abundance in hippocampal cultures. As cell death in female cultures was most prominent in the CA1 region, data is also presented to explain greater sensitivity of the CA1 region as compared to the CA3 region and DG. Propidium iodide (PI) uptake was used to measure cell death in the CA1, CA3, and DG hippocampal regions, and a variety of primary antibodies were used to describe specific protein abundance with or without EtOH pre-exposure. Cell death in the CA1 region of female cultures with exposure to TPQ during EWD parallels the cell death observed with A1r antagonism during EWD. As GIRKs are activated downstream of A1r activation and A1r immunoreactivity is not affected by prolonged EtOH exposure in vitro, these data suggest that sex differences in GIRK channel abundance and/or function may contribute to the sex difference in cell death with antagonism of A1rs or GIRKs during EWD.

METHODS



Organotypic Hippocampal Slice Culture Preparation

- Whole brains from 8-day old male and female Sprague Dawley rat pups were removed & bilateral hippocampi were dissected out
- Hippocampi were sectioned coronally (200 µm), yielding ~24 hippocampal slices per animal
- Slices were placed onto biopore membrane inserts in 6-well cultures plates with 1 ml of culture media beneath each insert
- Culture plates were incubated at 37°C, 95% air/5% CO_2 and allowed 5 days in vitro (DIV) prior to start of experiments.
- Experiments were replicated 3-5 times with different rat litters.

Ethanol Exposure and Withdrawal

- At 5 DIV, EtOH-exposed slice cultures were placed into media with a calculated EtOH concentration of 50 mM (actual EtOH concentration range over 5 days = 43.1-26.9 mM).
- At 10 DIV, Control and EtOH-containing media was refreshed.
- At 15 DIV, after 10 days of EtOH exposure, slices were transferred into fresh culture media with the addition of the fluorescent nucleic acid stain propidium iodide (PI) for 24hr EWD for visualization of dead/dying cells.

Drug Exposure in Ethanol-Naïve or Ethanol-Withdrawn Cultures

- At 15 DIV, separate EtOH-naïve and EtOH-exposed cultures were exposed for 24hrs to: CCPA (10 nM); DPCPX (10 nM); CCPA + DPCPX; DPCPX + APV (20 µM); Caffeine (5-100 µM); TPQ (0.01 – 1 µM)

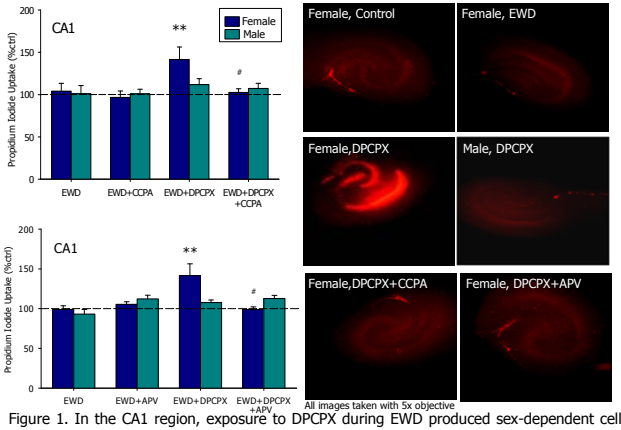
Assessment of Neurotoxicity

- Cell death was measured by quantification of PI fluorescence, visualized with a 5X objective on a Leica DMIRB microscope fitted for fluorescence detection (mercury-arc lamp) connected to personal computer via a SPOT 7.2 color mosaic camera
- Two-way ANOVAs (Sex x Treatment) were conducted within each hippocampal region (CA1, CA3, DG) with Fisher's LSD post-hoc tests when appropriate

Immunohistochemistry: A1r, GIRK1 channel, NeuN, MAP-2, NR1 & NR2B NMDAr subunits

- At 15 DIV, male and female control and 10 day EtOH-exposed (not withdrawn) cultures were fixed in 10% formalin for 30 min before exposure to permeabilization buffer for 45 min
- In separate studies, EtOH-exposed and/or EtOH-naïve cultures were exposed to the primary antibody in permeabilization buffer at 4°C for 24hrs: A1 receptor (1:200); GIRK1 channel (1:300); nuclei of mature neurons (NeuN; 1:200); microtubule associated protein-2 (MAP-2;1:200); NMDA NR1 receptor subunit (1:200); or the NMDA NR2B receptor subunit (1:200)
- Following 24hr incubation with primary antibody, cultures were washed twice in PBS & incubated for 24hrs at 4°C in permeabilization buffer with the addition of either tetramethylrhodamine isothiocynate (TRITC)-conjugated secondary antibody (1:200) or fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200) for fluorescent quantification

Figure 1. Sex-selective cell injury in the CA1 region with A1r antagonism during EWD



All images taken with 5x objective

Figure 1. In the CA1 region, exposure to DPCPX during EWD produced sex-dependent cell death, such that cell death was observed only in female cultures. This effect was prevented by co-exposure to CCPA or APV. $**P$ <0.01 vs. all other groups, including male EWD+DPCPX; $\#P$ <0.05 vs. female EWD+DPCPX. In additional ethanol-naïve cultures, neither CCPA, DPCPX, APV, or combinations of the two compounds (CCPA+DPCPX, or DPCPX+APV), produced toxicity in any hippocampal region.
Butler et al. (2008) *Alcoholism: Clinical and Experimental Research*

Figure 2. Caffeine exposure produces sex-selective hippocampal cell injury during EWD

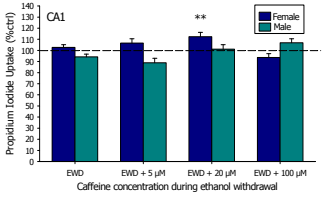


Figure 2. Effects of caffeine exposure during 24h EWD on cell injury in male and female hippocampal cultures. Cell death produced by caffeine exposure during EWD in the DG and CA1 regions was sex dependent, and independent of sex in the CA3 region. *P < 0.001 vs. control cultures (CA3); **P < 0.05 vs. male cultures, EWD female cultures, and female ethanol-naïve cultures exposed to caffeine.
Butler et al. (2009) *Alcohol and Alcoholism*

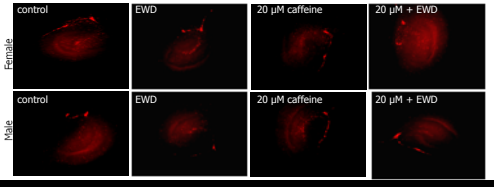


Figure 3. Sex-selective cell injury in the CA1 region with GIRK channel blockade during EWD

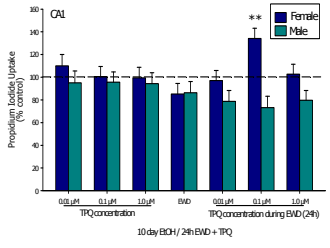


Figure 3. Effects of TPQ (.01-1 µM) exposure in EtOH-naïve and EtOH-exposed cultures. Cell death was only present in the CA1 pyramidal cell layer of female cultures. In parallel with previous studies, cell death was sex-dependent, such that only female cultures showed significant increases in PI uptake. **P < 0.05 vs. EWD and female EtOH-naïve cultures exposed to 100 nM TPQ.

Figure 4. A1 receptor & GIRK1 channel IR following 10 day EtOH exposure

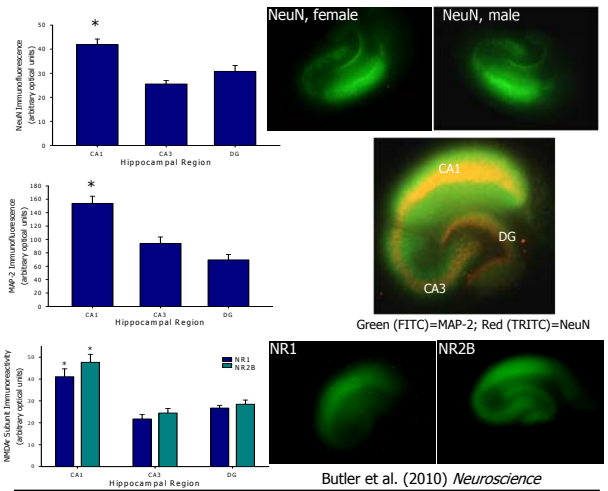
A1 Receptor Immunoreactivity (% control)

	CA1	CA3	DG
Female Cultures	108.6 (4.7)	103.4 (4.9)	110.4 (6.2)
Male Cultures	106.8 (6.7)	100.6 (6.7)	110.5 (7.8)

GIRK1 Channel Immunoreactivity (% control)

	CA1	CA3	DG
Female Cultures	98.7 (4.0)	102.2 (5.3)	93.6 (4.1)
Male Cultures	96.0 (4.1)	109.0 (5.5)	103.8 (4.2)

Figure 5. Proposed mechanisms of CA1 region cell injury



Green (FITC)=MAP-2; Red (TRITC)=NeuN

Butler et al. (2010) *Neuroscience*

CONCLUSIONS

- Initial studies demonstrated a sex difference in cell death with exposure to an A1 receptor antagonist (DPCPX) during EWD in CA1 pyramidal cells, such that female cultures were markedly more sensitive to injury compared to CA1 pyramidal cells of male cultures.
- Non-specific antagonism of adenosine receptors with caffeine during EWD produced results consistent with the previous studies of A1 receptor antagonism during EWD, again showing greater cell death in female cultures.
- Though results are still preliminary, inhibition of GIRK channels with TPQ during EWD resulted in sex-selective cell death in female cultures.
- The DPCPX-induced cell death in female cultures was attenuated by APV, a selective NMDA receptor antagonist. Additionally, neither immunohistochemical analysis of A1 receptor nor GIRK1 channel abundance showed alterations after 10 day EtOH exposure in either sex. *Together these data suggest that the sex-selective cell injury produced by adenosine receptor antagonism is due, at least in part, to signaling downstream from the A1 receptor, mediated in part by NMDA receptors, but not a consequence of A1 or GIRK1 abundance.*
- Also, these data suggest greater sensitivity of the female hippocampus to injury during EWD, independent of hormonal influences. The CA1 region in particular showed greater cell injury in all studies, perhaps due to greater abundance of mature neurons and NMDA NR2B-containing neurons.

ACKNOWLEDGEMENTS

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