INTRODUCTION

Effects of chronic ethanol exposure and withdrawal on the brain are heterogeneous and continue to be more fully characterized, though it is generally accepted that chronic ethanol exposure produces adaptive changes in function, affinity and/or density of multiple receptor proteins. These adaptive changes make the brain vulnerable to excitotoxic insult during ethanol withdrawal (Prendergast et al., 2004), contribute to ethanol-withdrawal-related behaviors and seizures (Hoffman et al., 1990) and may contribute to alcohol craving in humans (Koob, 2003; De Witte, 2004). Notable cellular alterations include up-regulation, increased sensitivity and synaptic reorganization of NMDA receptors (Hu and Ticku, 1995; Kalluri et al., 1998; Carpenter-Hyland et al., 2004; Suvarna et al., 2005), subunit-dependent alterations of GABA_\text{A} receptor expression (Mahtre et al., 1993; Mahtre and Ticku, 1994; Devaud et al., 1997), up-regulation of L-type Ca^{2+} channels (Guppy and Littleton, 1994) and subtype-dependent inhibition of P2X receptors (Davies et al., 2002, 2006).

The adenosine receptor system has received relatively little attention with regard to adaptive changes that occur during prolonged ethanol exposure. However, it has been shown that ethanol increases extracellular adenosine levels (Clark and Dar, 1989) and produces increases in adenosine A_1 receptor density in male rats (Daly et al., 1994; Concasa et al., 1996; Jarvis and Becker, 1998). Behavioral data suggest a role for adenosinergic systems in mediating behaviors during acute ethanol intoxication and withdrawal. For example, A_1 receptor antagonism attenuates ethanol-induced motor incoordination (Connole et al., 2004; Dar, 2006), while both A_1 antagonism and non-specific adenosine receptor antagonist with caffeine reduces the anxiolytic effects of acute ethanol in rodents (Prediger et al., 2004).

Specific A_1 receptor agonism also reduces anxiogenic behaviors and seizures during acute ethanol withdrawal, whereas A_2A receptor agonism does not (Concas et al., 1996; Prediger et al., 2006).

Adenosine is an endogenous inhibitory neuromodulator that binds with its greatest affinity to the G_{\text{ion}} protein-coupled A_1 receptor subtype and the G_{\text{protein}} protein-coupled A_2A receptor subtype under basal conditions (Fredholm et al., 1999). Adenosine levels rise to nearly 10 times basal levels under pathological conditions hallmarked by neuronal hyperexcitability, such as hypoxia/ischemia with reperfusion (Dux et al., 1990; Lynch III et al., 1998), which produces an excitatable neuronal state similar to that produced by ethanol withdrawal. Other identified adenosine receptor subtypes (A_2B and A_3) require greater adenosine concentrations to be fully activated, such as under these pathological conditions (Fredholm et al., 1999).

In vivo and in vitro rodent models of stroke, hypoglycemia and global or focal ischemia-induced excitotoxicity have demonstrated that pharmacological agonism of A_1 receptors (Hsu et al., 1994; Rudolph and Schubert, 1997), acute antagonism of A_2A receptors (in vivo: von Lubitz et al. (1995)), inhibition of
It was demonstrated that female hippocampi, particularly pyramidal cells of the CA1 region, showed significantly greater neurotoxicity than male hippocampi in response to treatment with the selective $A_1$ receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) for 24 h of ethanol withdrawal. This neurotoxicity was attenuated by the NMDA receptor antagonism, suggesting relief of tonic inhibition of NMDA receptor-mediated signaling (Butler et al., 2008). It is not clear, however, if non-selective adenosine receptor antagonism with use of caffeine would produce effects similar to those of DPCPX during ethanol withdrawal.

Caffeine is a non-selective competitive adenosine receptor antagonist at concentrations equivalent to plasma levels typically achieved in humans (ranging from ~80 to 400 mg/dL/day or ~5 to 20 $\mu$M; Fredholm et al., 1999). The primary behavioral effects of caffeine are mediated by the $A_1$ and $A_2A$ adenosine receptor subtypes with reported $K_d$ values of 20 $\mu$M and 8.1 $\mu$M, in rat brain, and 12 and 2.4 $\mu$M in human brain, respectively (Fredholm et al., 1999, 2001). Caffeine fails to cause neurotoxicity at behaviorally relevant concentrations (50 and 150 $\mu$M) in cultured cerebellar granule cells, but is able to produce toxicity at concentrations ranging from 250 $\mu$M to 1000 $\mu$M in cultured cerebellar granule cell, auditory cortical neurons and fetal cortical cell cultures (Gepdiremen et al., 1998; Kang et al., 2002; Bakuridze et al., 2005; Ucuncu et al., 2005). Toxicity at these high concentrations can be attenuated by co-administration with nimodipine, a voltage-dependent calcium channel blocker; dantrolene, an inhibitor of calcium release from the endoplasmic reticulum; and ATP receptor antagonism (Gepdiremen et al., 1998; Bakuridze et al., 2005; Ucuncu et al., 2005). Caffeine has been used extensively in models of intracellular calcium signaling, as caffeine is able to reliably mediate calcium flux via ryanodine receptors and inositol triphosphate receptors (IP3R) in neuronal tissue, though at concentrations that would be toxic to humans (Nagarkatti et al., 2008). At behaviorally relevant concentrations of caffeine, however, neurotoxicity has been reported in only one in vivo model of caffeine exposure (IP administration of 50 mg/kg caffeine at 5-h intervals) in which cell death was apparent in many brain regions, including the dentate gyrus of the hippocampus, but not the CA1 and CA3 hippocampal regions (Kang et al., 2002).

The current studies were designed to examine the effect of non-specific adenosine receptor antagonism with caffeine during ethanol withdrawal on neuronal viability. As a previous series of studies revealed a sex difference in neurotoxicity produced by specific $A_2$ receptor antagonism using the same ethanol exposure regimen, cell death in male and female organotypic hippocampal slice cultures was compared after exposure to caffeine during ethanol withdrawal. It was hypothesized that exposure to behaviorally relevant concentrations of caffeine during ethanol withdrawal would produce toxicity in a potentially sex-dependent manner. The model used in the current studies was modified from Stoppini et al. (1991) who cultured hippocampi from 2- to 23-day-old Wistar rat pups. These authors observed that the morphological aspects of the hippocampus remained intact, with the highest level of morphologic integrity obtained with brains taken from 8- to 15-day-old pups. Gutierrez and Heinemann (1999) have shown that cultured hippocampi (from 6- to 9-day old rat pups) maintain their trisynaptic circuitry, though the dentate gyrus is devoid of af- ferent input. Additionally, Martens and Wree (2001) compared distribution of hippocampal glutamate receptors and found that hippocampi taken from 6-day-old rat pups did not differ in NMDAr or AMPAr distribution as compared to in situ preparation of hippocampi from PND 30 rats. These findings provide support for use of the organotypic model in studying ethanol withdrawal phenomena.

**METHODS**

**Organotypic hippocampal slice culture preparation**

Eight-day-old male and female Sprague-Dawley rat pups (Harlan Laboratories; Indianapolis, IN, USA) were humanely euthanized for aseptic whole brain removal. Brains were immediately transferred to dishes containing a frozen dissecting medium made of the minimum essential medium (MEM), 25 mM HEPES and 50 $\mu$M streptomyein/penicillin (adapted from Stopini et al. (1991)). Bilateral hippocampi were removed, cleaned of extra tissue under a dissecting microscope and placed into a culture medium composed of the dissecting medium with the addition of 36 mM glucose, 25% (v/v) Hanks’ balanced salt solution, 25% heat-inactivated horse serum (HIHS) and 0.05% streptomycin/penicillin. Hippocampi were sectioned coronally at 200 $\mu$m using the McIlwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd, Gomshall, UK) and placed into a fresh culture medium. Approximately 12 slices were obtained from each hippocampus, yielding ~24 slices per animal that were varied among treatment groups. Slices with intact hippocampal cell layers were selected and placed onto Millicell-CM 0.4 $\mu$m biopore membrane inserts pre-incubated in 1 mL of the culture medium at 37°C in a 35-mm 6-well culture plate. Three slices were placed on each insert, yielding 18 slices per plate. Excess medium from the top of the membrane insert was aspirated to allow the slices exposure to the incubator atmosphere of 5% CO$_2$/95% air. Slices were allowed 5 days to attach to the insert membrane before experiments were initiated, and all experiments were replicated —four to five times with different rat litters. Culture medium supplies were obtained from Gibco BRL (Gaithersburg, MD, USA), with the exception of HIHS (Sigma-Aldrich Co., St. Louis, MO, USA). Care of animals was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), as well as the University of Kentucky’s Institutional Animal Care and Use Committee.

**Caffeine exposure in ethanol-naивé cultures**

At 5 and 10 days in vitro (DIV), all cultures were transferred into a fresh pre-incubated culture medium in new 6-well culture
plates. At 15 DIV, a portion of the male and female cultures was again put into a fresh culture medium (control groups). The remaining male and female cultures were exposed to 5, 20 or 100 μM caffeine (Sigma-Aldrich Co.) dissolved in the culture medium. The concentrations of caffeine were chosen to be near the dissociation constants of caffeine for A1 and A2A receptors, reported at 20 and 8.1 μM, respectively, in the rat brain (in Fredholm et al. 1999)). Additionally, the concentrations were chosen for their human-use relevance, as per person consumption ranges from 80 to 400 mg daily, approximating plasma levels of 5–20 μM (Daly and Fredholm, 1998). Further, the amount of caffeine in a typical cup of coffee (plasma concentration >10 μM) is sufficient to block ~15% of A1 and A2A receptors, and a concentration of 100 μM blocks ~80% of A1 and A2A receptors while remaining below a toxic dose (~500 μM) (reviewed in Fredholm et al. 1999)). Also at 15 DIV, the culture medium contained the fluorescent nucleic acid stain propidium iodide (PI; 3.74 μM), which penetrates dying or compromised cells with damaged membranes (Zimmer et al., 2000). Uptake of PI allowed visualization and quantification of cell death using densitometry 24 h after the onset of caffeine exposure (16 DIV).

Ethanol withdrawal and caffeine exposure during ethanol withdrawal

For studies involving caffeine exposure during withdrawal from chronic ethanol exposure, male and female cultures were placed in a culture medium with an ethanol concentration of 50 mM at 5 and 10 DIV (for 10 consecutive days) before beginning a 24 h withdrawal period. Measures taken to minimize ethanol evaporation included surrounding ethanol-treated cultures with double-distilled water containing an ethanol concentration of 50 mM in topless polypropylene containers before enclosing the culture plates in a plastic bag filled to capacity with incubator-equivalent air mixture (5% CO₂, 95% air). Despite these precautions, the calculated concentration of 50 mM initially approximates ~43.1 mM and decreases to nearly half that value (~29 mM) after 5 consecutive days before being refreshed at the calculated concentration of 50 mM for the remaining 5 days of ethanol exposure (Butler et al., 2008). Similar ethanol loss has been noted with a calculated starting concentration of 100 mM, despite taking the same precautions noted above (Prendergast et al., 2004). At 15 DIV, after 10 consecutive days of ethanol exposure, some male and female cultures underwent ethanol withdrawal in the absence of caffeine (EWD group), while the remaining cultures were transferred into the culture medium with the addition of 5, 20 or 100 μM caffeine for the duration of the 24 h ethanol withdrawal period. As in the experiments outlined above, all culture mediums (at 15 DIV) contained PI for quantification of cell death.

Fluorescent microscopy and data analysis

Fluorescent microscopy was employed to measure cell death as indicated by cellular uptake of PI. Images were taken using SPOT Advanced software (version 4.0.2) for Windows with a 5× objective on an inverted Leica DMIRB microscope (W. Nuhsbaum Inc., McHenry, IL, USA) fitted for fluorescence detection (mercury-arc lamp) and connected to a personal computer via a SPOT 7.2 color mosaic camera (W. Nuhsbaum Inc.). Propidium iodide has a maximum excitation wavelength of 536 nm and was excited using a band-pass filter that excites wavelengths between 515 and 560 nm. The emission wavelength of PI in the visual range is 620 nm. Densitometry was conducted using Image J software (National Institutes of Health, Bethesda, MD) to quantify cell death in the primary neuronal layers of the dentate gyrus (granule cell layer) and the CA3 and CA1 regions (pyramidal cell layers) of the hippocampus.

Non-specific background fluorescence was subtracted from each hippocampal slice prior to statistical analysis and conversion to percent control values. A two-way analysis of variance (ANOVA) was conducted (treatment × sex) within each hippocampal region (CA1, CA3, DG). Separate analyses were conducted using data derived from each hippocampal region given the a priori hypothesis that ethanol-withdrawal-related neurotoxicity would be greatest in the CA1 region (Prendergast et al., 2004). When appropriate, Fisher’s LSD post hoc analyses were interpreted. The significance level was set at $P < 0.05$.

## RESULTS

### Caffeine exposure in ethanol-naive and ethanol-withdrawn hippocampal cultures

A two-way treatment × sex ANOVA comparing propidium iodide uptake in the primary neuronal layers of the dentate gyrus and the CA3 and CA1 regions of the hippocampus in ethanol-naive and ethanol-withdrawn cultures exposed to 0, 5, 20 or 100 μM caffeine was conducted. Withdrawal from an initially calculated concentration of 50 mM ethanol did not result in greater PI uptake compared to control cultures in either female or male cultures, in any hippocampal region. Similarly, 24 h exposure to caffeine in ethanol-naive tissue did not produce toxicity in pyramidal cells of the CA1 region or in granule cells of the dentate gyrus. In contrast, modest but significant neurotoxicity was observed in the pyramidal cell layer of the CA3 region $[F(7, 676) = 0.862, P < 0.001; \text{Fig. 1}]$ and was not dependent on the sex of the tissue. Prior 10-day exposure to ethanol did not alter caffeine-induced neurotoxicity during ethanol withdrawal in the CA3 region (Fisher’s LSD post hoc, $P = 0.698$) but did alter effects of caffeine on PI uptake in the CA1 and DG regions, as described below. Representative images of PI uptake in ethanol-naive tissue are displayed in Fig. 2.

In contrast to the CA3 region, a significant treatment × sex interaction was present in the granule cell layer of the DG $[F(7, 663) = 3.299, P < 0.01]$. Female cultures exposed to either 5 μM or 20 μM caffeine during EWD sustained significantly greater toxicity compared to male cultures (Fisher’s LSD post hoc, $P < 0.05$) and female EWD cultures (Fisher’s LSD post hoc, $P < 0.05$). Ethanol-withdrawn female cultures exposed to 5 μM or 20 μM caffeine had ~13–28% greater PI uptake compared to EWD female cultures, whereas male cultures had only ~5–10% greater PI uptake when exposed to caffeine during EWD. Interestingly, exposure to 100 μM caffeine, a concentration nearly five times the $K_d$ value of caffeine for both the A₁ and A₂A receptor subtypes in rat, did not produce toxicity in ethanol-naive or ethanol-withdrawn cultures (Fig. 3). The toxicity produced by caffeine in EWD female cultures was clearly dependent on prior ethanol exposure, as significant toxicity was not produced by caffeine exposure at any concentration in ethanol-naive female cultures in the DG.
Fig. 1. Effects of 24-h caffeine exposure (5, 20 or 100 μM) at 15 days in vitro on neurotoxicity in ethanol-naïve male and female hippocampal slice cultures. Propidium iodide uptake was significantly increased in the CA3 pyramidal cell layer with exposure to 20 μM caffeine. *P < 0.05 versus control. No sex differences were observed.

Similar to the DG, a significant treatment × sex interaction was present in the pyramidal cell layer of the CA1 region [F(7, 677) = 4.666, P < 0.001]. The CA1 region of female cultures was more susceptible to EWD-induced injury in the presence of 20 μM caffeine, demonstrated by a significant sex difference in PI uptake (Fig. 3). PI uptake was increased by ~12% in female hippocampi, as compared to ethanol-withdrawn hippocampi not exposed to caffeine. Further, in female cultures, exposure to 20 μM caffeine during EWD produced significantly greater toxicity than 20 μM caffeine in ethanol-naïve cultures.

DISCUSSION

The present studies were designed to study the effect of caffeine exposure on neurotoxicity in ethanol-naïve and ethanol-withdrawn hippocampal slice cultures derived from male and female rats, independent of the acute hormonal milieu. Pyramidal cells of the CA1 region and granule cells of the DG in female hippocampi were significantly more vulnerable to toxicity produced by caffeine exposure during ethanol withdrawal than were pyramidal and granule cells of male hippocampi. Importantly, neither withdrawal from 10-day exposure to 50 mM ethanol nor caffeine exposure in ethanol-naïve cultures produced toxicity in the CA1 region or DG. Previous studies reporting caffeine-induced neurotoxicity have required much higher concentrations to produce toxicity (>250 μM). At these high concentrations, caffeine’s mechanism of action is difficult to discern. Non-selective effects of caffeine include inhibition of phosphodiesterase, intracellular calcium release via ryanodine receptor activation and blockade of GABA_A receptors (Fredholm et al., 1999). Failure to observe toxicity during withdrawal from 50 mM ethanol has been noted previously using this model (Self et al., 2005; Butler et al., 2008). However, this concentration was preferred for its relevance to the study of binge drinking, as it approximates a plasma ethanol concentration of 230 mg/dL. Despite lack of overt toxicity from ethanol withdrawal alone, this ethanol exposure and withdrawal regimen has been shown to produce NMDA-dependent toxicity upon exposure to a variety of substances, including corticosterone; the HIV-1 protein Tat and an A1 receptor antagonist (Self et al., 2004; Mulholland et al., 2005; Butler et al., 2008). Additionally, the concentrations of caffeine used in the current studies (5, 20 and 100 μM) approximate plasma levels found in humans after moderate caffeine consumption (Fredholm et al., 1999).
The importance of the A1 receptor subtype in mediating caffeine’s effects is reflected in studies showing that neurotoxicity observed in female cultures is due to effects downstream of the A1 receptor at NMDA receptors. A1 receptors are G_{i/o} protein-linked receptors that confer inhibition in the CNS by presynaptic inhibition of neurotransmitter release (Fredholm and Dunwiddie, 1988) and postsynaptic hyperpolarization (Segal, 1982; Li and Henry 1992). Specifically in regard to NMDA receptors, A1 receptor activation has been shown to decrease glutamate release (Clark and Dar, 1989; Di Iorio et al., 1996; O’Kane and Stone, 1998; Lopes et al., 2002) and reduce NMDA-induced cell death (Finn et al., 1991). Conversely, A1 receptor antagonism with DPCPX has been shown to amplify the release of excitatory amino acids in rat’s hippocampus (Di Iorio et al., 1996).

Toxicity produced by caffeine in the hippocampus is likely mediated by antagonism of A1 receptors and subsequent relief of inhibition on NMDA receptors. A similar conclusion for the importance of the A1 receptor has been drawn with regard to caffeine’s effects on motor behavior. It is well accepted that acute caffeine produces dose-dependent biphasic effects in terms of motor stimulation in rodents, such that lower doses (up to ∼30 mg/kg) produce motor-activating effects and higher doses produce motor-depressing effects (Daly and Fredholm, 1998; El Yacoubi et al., 2000; Gasior et al., 2000; Halldner et al., 2004). Using selective agonists and antagonists, investigations into the relative contributions of A1 and A2A receptor blockade in the effects of caffeine on motor behaviors have found mixed results (e.g. El Yacoubi et al., 2000; Halldner et al., 2004). However, it seems clear that caffeine’s effects are dose dependent and the motor behaviors observed depend on whether administration is acute and chronic and whether the rodent is acclimated to the environment (El Yacoubi et al., 2000; Karcz-Kubicha et al., 2003). Antoniou et al. (2005) conducted a factor analysis over a wide spectrum of motor behaviors and concluded that the A1 receptor subtype was more critical in mediating the motor effects of caffeine as compared to the A2A receptor subtype. That is, the motor-activating effects of caffeine are more closely mirrored by an A1 antagonist (CPT) than by an A2A selective antagonist (MSX-3). Also, CPA, a selective A1 agonist, was more effective than CGS 21680, a selective A2A agonist, in attenuating the motor-activating effects of caffeine (Antoniou et al., 2005). Likewise, acute caffeine effects on motor behavior are found to correlate with in vivo A1 receptor density (Kaplan et al., 1992, 1993). It has also been suggested that A1–A2A heteromers, which to date have only been observed in experimental transfected cells and rat striatum, mediate caffeine’s effects (Ferre et al., 2008).

Behavioral effects of caffeine administration during ethanol withdrawal have been little studied, though some work does suggest that caffeine may influence the nature of ethanol withdrawal-related behaviors (e.g. seizure). Malec et al. (1996) reported that caffeine itself did not worsen audiogenic seizures in rats, but it reduced depressing effects of adenosine analogs when co-administered during ethanol withdrawal. However, this study did not examine neuronal injury and it is not clear if seizures and neuronal injury are highly correlated in ethanol-withdrawn rodents. Several studies have noted caffeine’s mediation of ethanol intoxication behaviors. In mice, IP or ICV administration of low doses of caffeine antagonizes, whereas high doses accentuate, motor incoordination (Dar, 1988). Chronic

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**Fig. 3.** Effects of caffeine exposure (5, 20 or 100 μM) during the 24 h withdrawal period after 10-day ethanol exposure on neurotoxicity in male and female hippocampal slice cultures. Toxicity produced by caffeine exposure in the DG and CA1 hippocampal regions was sex dependent, whereas sex differences were not detectable in the CA3 region. *P < 0.001 versus control cultures (CA3); **P < 0.05 versus male cultures, EWD female cultures and female ethanol-naive cultures exposed to caffeine.

Taken together, the current data suggest sex-dependent alterations produced by prolonged ethanol exposure in the adenosinergic receptor system and/or effectors downstream of adenosine receptors that make the female hippocampus more susceptible to injurious effects of A1 receptor manipulation during ethanol withdrawal.

Caffeine exerts its primary effects via antagonism of adenosine A1 and A2A receptors in the brain (Fredholm et al., 1999). Both receptor subtypes are antagonized by low concentrations of caffeine and are co-localized widely in the hippocampus (Rebola et al., 2005). The current data support the primary importance of the A1 receptor subtype in mediating caffeine’s effect on neurotoxicity during ethanol withdrawal, as these data parallel sex differences in response to specific A1 receptor antagonism with DPCPX during ethanol withdrawal using the same model (Butler et al., 2008). These previous studies showed no sex difference in A1 receptor density in ethanol-naive or ethanol-exposed cultures, suggesting that neurotoxicity observed in female cultures is due to effects downstream of the A1 receptor at NMDA receptors. A1 receptors are G_{i/o} protein-linked receptors that confer inhibition in the CNS by presynaptic inhibition of neurotransmitter release (Fredholm and Dunwiddie, 1988) and postsynaptic hyperpolarization (Segal, 1982; Li and Henry 1992). Specifically in regard to NMDA receptors, A1 receptor activation has been shown to decrease glutamate release (Clark and Dar, 1989; Di Iorio et al., 1996; O’Kane and Stone, 1998; Lopes et al., 2002) and reduce NMDA-induced cell death (Finn et al., 1991). Conversely, A1 receptor antagonism with DPCPX has been shown to amplify the release of excitatory amino acids in rat’s hippocampus (Di Iorio et al., 1996).
caffeine also affects the response to acute ethanol, such that chronically caffeinated mice show greater motor incoordination compared to controls (Dar and Wooles, 1986). Accordingly, humans report feeling less intoxicated/impaired when caffeine and alcohol are co-administered (Ferreira et al., 2006; Marczinski and Fillmore, 2006), caffeine antagonizes alcohol’s impairment in a laboratory task of inhibitory control (Marczinski and Fillmore, 2003) and caffeine and alcohol co-administration results in greater tolerance to ethanol compared to either drug alone (Fillmore, 2003). Taken collectively, these data support a central nervous system interaction between ethanol and the adenosinergic system, though the potential neurotoxic effects of caffeine use during ethanol withdrawal warrant further investigation.

In sum, these data suggest that female brain adapts differently than the male brain to prolonged ethanol exposure in a manner that influences neuronal excitability and, potentially, neuronal injury. This difference in adaptation to ethanol exposure is clearly independent of the hormonal milieu, possibly reflecting innate differences in responses to ethanol. Notably, these data are not in kind with data demonstrating that female rodents recover from ethanol withdrawal much more rapidly than do male rats (Devaud and Chadda, 2005), suggesting a discordance between withdrawal seizure and neuronal injury. This difference in adaptation to ethanol exposure warrants further investigation.

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Sex Differences in Caffeine Neurotoxicity 573


