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Isoflurane preconditioning protects neurons from male and female mice against oxygen and glucose deprivation and is modulated by estradiol only in neurons from female mice

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Abstract

The volatile anesthetic, isoflurane, can protect the brain if administered before an insult such as an ischemic stroke. However, this protective “preconditioning” response to isoflurane is specific to males, with females showing an increase in brain damage following isoflurane preconditioning and subsequent focal cerebral ischemia. Innate cell sex is emerging as an important player in neuronal cell death but its role in the sexually dimorphic response to isoflurane preconditioning has not been investigated. We used an in vitro model of isoflurane preconditioning and ischemia (oxygen and glucose deprivation, OGD) to test the hypotheses that innate cell sex dictates the response to isoflurane preconditioning and that 17 β -estradiol attenuates any protective effect from isoflurane preconditioning in neurons via nuclear estrogen receptors. Sex-segregated neuron cultures derived from postnatal day 0 to 1 mice were exposed to either 0% or 3% isoflurane preconditioning for 1 hour. In separate experiments, 17 β -estradiol and the non-selective estrogen receptor antagonist ICI 182,780 were added 24 hours before preconditioning and then removed at the end of the preconditioning period. Twenty-three hours after preconditioning, all cultures underwent 2 hours of OGD. Twenty-four hours following OGD, cell viability was quantified using calcein-AM fluorescence. We observed that isoflurane preconditioning increased cell survival following subsequent OGD regardless of innate cell sex, but that the presence of 17 β -estradiol before and during isoflurane preconditioning attenuated this protection only in female neurons independent of nuclear estrogen receptors. We also found that independent of preconditioning treatment, female neurons were less sensitive to OGD compared to male neurons and that transient treatment with 17 β -estradiol protected both male and female neurons from subsequent OGD. More studies are needed to determine how cell type, cell sex and sex steroids like 17 β -estradiol may impact on anesthetic preconditioning and subsequent ischemic outcomes in the brain.

Keywords

Estradiol; isoflurane; neurons; preconditioning; sex differences

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1. Introduction

Isoflurane has been shown to protect brain tissue (Kitano et al., 2007a) and isolated neurons if administered before insults that model ischemic stroke (Wise-Faberowski et al., 2001; Kapinya et al., 2002; Li et al., 2008; McMurtrey and Zuo, 2010). However, this “preconditioning” protection is specific to male brains, with female brains showing increased brain damage following isoflurane preconditioning and subsequent ischemia (Kitano et al., 2007b). The specific neuronal component of this sexually dimorphic response has not been investigated, as current literature assessing neuronal responses to isoflurane preconditioning has relied on mixed-sex cultures. Indeed, innate cell sex is emerging as an important factor in neuronal cell death following a myriad of insults associated with neurodegeneration (Du et al., 2004; Heyer et al., 2005; Li et al., 2005; Du et al., 2008; Lang and McCullough, 2008), but its role in the neuronal response to isoflurane preconditioning has not been examined.

The major female sex steroid in women and rodents, 17 β -estradiol (E2), has primarily been shown to be neuroprotective if administered before or during an ischemic insult (Cimarosti et al., 2005; Raval et al., 2006; Lang and McCullough, 2008; Liu et al., 2009; Raval et al., 2009). E2 has also been shown to protect cortical neurons from oxygen and glucose deprivation (OGD), an in vitro model of ischemia. However, current in vitro reports have been limited to neuronal cultures that were not stratified by animal sex (Xu et al., 2006) or hippocampal slice cultures that could not resolve specific neuronal responses (Cimarosti et al., 2005; Li et al., 2005). The nuclear estrogen receptors (ERs), subtype α and β , can mediate E2 neuroprotection depending on innate cell sex (Bryant and Dorsa, 2010), the specific cellular insult (Mize et al., 2003; Singh et al., 2006; Wang et al., 2006; Zhao and Brinton, 2007; Bryant and Dorsa, 2010), or a combination of these and other factors. We have previously shown that E2 attenuates the brain’s protective response to isoflurane preconditioning via the nuclear ERs (Wang et al., 2008a), yet whether E2 exerts this effect on isoflurane preconditioning protection through an ER-dependent mechanism at the neuronal level remains unknown.

We used isolated male and female cortical neurons from mice and an in vitro model of isoflurane preconditioning and ischemia (OGD) to determine (1) if innate cell sex dictates the response to isoflurane preconditioning and (2) whether E2 attenuates any protective response to isoflurane preconditioning in neurons via nuclear ERs.

2. Experimental Procedures

2.1 Establishing Sex-Segregated Cortical Neuron Cultures

Animal procedures were in compliance with the National Institutes of Health guidelines for the care and use of animals in research. All experimental protocols were approved by the Institutional Animal Care and Use Committee. Male and female C57BL/6 (Charles River, Wilmington, MA) mouse pups (postnatal day 0 to 1) were segregated by sex based upon a larger genital papilla and ano-genital distance in males versus females. Our laboratory has previously validated this sex-segregation technique using PCR analysis for the male-specific gene, *Sry*, and the sex-independent autosomal gene, *Myogenin* (Liu et al., 2008; Johnsen and Murphy, 2011).

Male and female mice within each litter were segregated and the brains from each sex were pooled to establish separate male and female cortical neuron cultures. Cortices were dissected in an ice-cold Hanks Balanced Salt Solution buffer followed by dissociation in papain for 20 min at 37°C. The digestion reaction was stopped with the addition of 0.04 mg/ml of soybean trypsin inhibitor and 0.04 mg/ml of DNase was added to prevent clumping.

The cells were washed twice in growth media consisting of Neurobasal A without phenol red to prevent phenol red-induced estrogen signaling (Berthois et al., 1986), 2% B27, 2 mM Glutamax, and 100 units of penicillin and streptomycin. Male and female neuronal cultures were each plated at a density of 1.5×10^5 cells/cm² onto plastic wells (Costar, Sigma-Aldrich, St. Louis, MO). The wells were pre-treated with 50 µg/ml poly-D-lysine for 3 hours at 37°C and later rinsed in sterile water and air-dried. The cells were allowed to settle for 2 hours before being gently swirled and rinsed with fresh media to remove non-adherent cells. Cells from each sex of a given litter were plated across 3 different culture plates designated as isoflurane preconditioning without OGD, sham preconditioning + OGD, and isoflurane preconditioning + OGD. Each 24-well plate (6 wells across 4 rows) had 3–4 well replicates per row plated with cells, with each row designated for a different drug treatment depending on the experiment (e.g. untreated; vehicle; E2; the ER antagonist, ICI 182,780; or E2 + ICI 182,780). The neurons were maintained in growth media in a humidified incubator at 37°C and 5% CO₂ for the duration of the experimental period, except during preconditioning and OGD.

Cell culture reagents including Neurobasal A, B27, soybean trypsin inhibitor, Glutamax, streptomycin and penicillin, and poly-D-lysine were from Life Technologies (Invitrogen), Carlsbad, CA. The papain was from Worthington Biochemical Corporation, Lakewood, NJ.

2.2 Preconditioning Neurons With Isoflurane

Neurons were preconditioned with isoflurane using a gas exchange system. A gas exchange system was chosen as opposed to adding liquid isoflurane to the culture media in order to better maintain the gas:liquid anesthetic equilibrium (Kudo et al., 2001; McDougall et al., 2008) and thereby better model in vivo anesthetic preconditioning. Similar designs have been reported for exposing neurons and other cell types to volatile anesthetics (Miyazaki et al., 1997; Kapinya et al., 2002; Li et al., 2008). Two air-tight, plastic chambers (Billups-Rothenberg, Del Mar, CA) were filled with medical air and 5% CO₂, with or without 3% isoflurane. For preconditioning, neurons were exposed to either 3% isoflurane in one chamber or sham preconditioned with 0% isoflurane in the other chamber. An anesthetic monitor (POET II, Criticare Systems, Waukesha, WI) was attached to the outflow of the isoflurane preconditioning chamber to determine when the isoflurane concentration had reached 3% in the chamber. The inflow and outflow tubing was then clamped and each chamber was placed in a 37°C incubator during the 1 hour preconditioning period. Following the preconditioning period, the culture plates were removed from the chambers and returned to normal growth conditions for 23 hours before OGD. The treatment paradigm is illustrated in Figure 1. The 3% isoflurane concentration, 1 hour preconditioning period, and 23 hour period following preconditioning were chosen based upon reports using similar models of volatile anesthetic preconditioning in isolated brain cells (Johnsen and Murphy, 2011; Miyazaki et al., 1997; Kapinya et al., 2002; Li et al., 2008).

2.3 17β-Estradiol And ICI 182,780 Pretreatments

The effects of E2 (Sigma-Aldrich) and the non-selective nuclear ER α and β antagonist (Harms et al., 2001), ICI 182,780 (ICI, Tocris Bioscience, Bristol, UK), were examined by dissolving these compounds in either 100% ethanol (for E2) or 100% sterile DMSO (for ICI) to generate 1 mM stock concentrations of each compound. Serial dilutions were then made to achieve the working concentrations. For E2, 1 µl of 1 mM E2 solution was added per 10 ml of growth media (0.01% volume) to achieve a 100 nM E2 solution, which was further diluted separately 1:10 to achieve a 10 nM E2 solution. For ICI, a 1 µM solution was achieved by diluting 10 µl of 1 mM stock solution in DMSO into 10 ml of growth media (0.1% volume). Each “vehicle” control received the same volume dilutions of either ethanol (E2), DMSO (ICI), or both (E2 + ICI). On day in vitro 8, E2 and/or ICI were added 24 hours

before preconditioning and then removed with two washes in growth media immediately following preconditioning on day in vitro 9 (Figure 1).

2.4 OGD And Cell Survival Assessment

OGD was conducted when neurons reached day in vitro 10. The cells were gently rinsed twice in Neurobasal A without glucose or other nutrients and placed in a chamber deprived of oxygen (< 0 ppm, Coy Laboratory Products, Grass Lake, MI) at 37°C for 2 hours. Control cells (isoflurane preconditioned without OGD, “100% Cell Survival”) were rinsed alongside the experimental isoflurane preconditioned + OGD and sham preconditioned + OGD groups but then returned to normal growth media and conditions. Following the 2 hour OGD period, the treated cells were returned to normal growth media and conditions.

Cell survival was assessed 24 hours following the end of OGD using calcein-AM (Life Technologies) with an automated plate reading protocol as previously reported (Forte et al., 2007). Calcein-AM is a membrane-permeable non-fluorescent molecule that, upon entering an intact living cell, is cleaved by endogenous esterases to produce the highly-fluorescent, membrane impermeable molecule, calcein. This fluorescence can be quantified to represent “cell survival”. Calcein was excited by 490 nm light and the emitted fluorescence was filtered at 535 nm for quantification. Each well was scanned at 9 different points to account for intra-well density differences, with 3–4 replicate wells per row. The average fluorescent value from all these points in a row (27 to 36 points total) was used to generate the final fluorescent value for that particular treatment. In each experimental round, the final fluorescent average of the isoflurane preconditioned without OGD plate was designated as “100% Cell Survival” by which the other corresponding fluorescent averages from the sham preconditioned + OGD and isoflurane preconditioned + OGD plates were divided to generate “% Cell Survival”. This design has the advantage of being an unbiased, high-throughput, internally-controlled method of quantifying cell survival while compensating for cell density and plating efficiency differences within wells, among different wells, and throughout different cultures.

2.6 Statistics

Two-way analysis of variance (ANOVA) of the difference of paired means (i.e. “repeated measures”) was used to allow for a paired littermate analysis, where compared factors were “cell sex” and “preconditioning” (*Results 3.1*) or “drug treatment” and “preconditioning” (*Results 3.2*). This analysis controlled for inherent variability in absolute cell survival values between different cultures. Every ANOVA was followed by the Student Newman-Keuls post-hoc test to analyze differences between and within factors of cell sex, preconditioning, and/or drug treatment (vehicle, E2, ICI, or E2 + ICI). Each culture from a given litter represented an $n = 1$. There were 11 paired male and female cultures for the sham preconditioned vs. isoflurane preconditioned experiments (Figure 2), 9 paired male and female cultures for the E2-treated experiments (Figure 3), and 7 paired female cultures for the 100 nM ICI ± E2 experiments. Data is given as the mean ± standard error of the mean and $p < 0.05$ was considered significant. Unless otherwise indicated in the text as the ANOVA output, significance in the figures and text represents the post-hoc Student Newman-Keuls output. SigmaStat [v2.0] statistical software (SPSS Inc., Chicago, IL) was used for all statistical analyses.

3. Results

3.1 The Effect Of Innate Cell Sex Following Isoflurane Preconditioning And OGD

Using a model of *in vitro* isoflurane preconditioning with sex-segregated neuron cultures, we found that isoflurane preconditioning protected both male (Figure 2A, sham

preconditioned, $47.7 \pm 4.3\%$ vs. isoflurane preconditioned, $54.8 \pm 5.9\%$ cell survival, $p < 0.05$) and female neurons (Figure 2A, sham preconditioned $61.7 \pm 3.0\%$ vs. isoflurane preconditioned 71.6 ± 4.0 , $*p < 0.01$) from subsequent OGD. However, there was no interaction between cell sex and preconditioning treatment ($p = 0.25$ for statistical interaction). The sex-independent ANOVA (pooled male and female cell survival outcomes) revealed that isoflurane preconditioning increased cell survival $8.5 \pm 3.4\%$ compared to sham preconditioning regardless of innate cell sex (Figure 2B: sham preconditioned, $54.7 \pm 3.3\%$ vs. isoflurane preconditioned, $63.2 \pm 4.8\%$; $p < 0.01$). Independent of preconditioning, we found that female neurons showed a $15.4 \pm 3.4\%$ increase in cell survival following OGD compared to male neurons (Figure 2C: male, $51.2 \pm 5.4\%$ vs. female, $66.6 \pm 3.6\%$; $p < 0.001$).

3.2 The Effect Of E2 On The Response Of Male And Female Neurons Following Isoflurane Preconditioning And OGD

In male neurons, the effect of different preconditioning treatments (sham or isoflurane preconditioning) is not altered by the level of E2 (interaction between preconditioning and E2, $p = 0.74$). The difference in the mean values between the sham and isoflurane preconditioning groups is greater than would be expected by chance after allowing for effects of differences in E2 ($p < 0.05$). This indicates that male neurons continued to demonstrate isoflurane preconditioning-induced protection from subsequent OGD regardless of E2 treatment ($p < 0.05$). Conversely, the difference in the mean values among the different levels of E2 is greater than would be expected by chance after allowing for effects of differences in preconditioning treatments ($p < 0.05$). This indicates that independent of preconditioning, E2 protected male neurons from subsequent OGD compared to vehicle ($p < 0.05$). Individual preconditioning and E2 means with post-hoc analyses are represented in Figure 3A. There were differences in cell survival between 10 nM E2 treated sham vs. isoflurane preconditioning groups (Figure 3A, $*p < 0.05$), 100 nM E2 treated sham vs. isoflurane preconditioning groups, (Figure 3A, $*p < 0.05$) and 100 nM E2 vs. vehicle treated isoflurane preconditioning groups (Figure 3A, $++p < 0.05$).

In contrast to male neurons, the effect of different preconditioning treatments in female neurons does depend on the level of E2 is present (interaction between preconditioning and E2, $p < 0.001$), where E2 attenuated isoflurane preconditioning protection from OGD. This is evident in that the difference in the mean values between sham preconditioning and isoflurane preconditioning was not great enough to exclude the possibility that this difference is simply due to random sampling variability after allowing for the effects of differences in E2 ($p = 0.452$). Conversely, the difference in the mean values among the different levels of E2 is greater than would be expected by chance after allowing for effects of differences in preconditioning treatments ($p < 0.05$). This indicates that independent of preconditioning, E2 protected female neurons from subsequent OGD compared to vehicle ($p < 0.05$). Individual preconditioning and E2 means with post-hoc analyses are represented in Figure 3B. There were differences in cell survival between vehicle treated sham vs. isoflurane preconditioning groups (Figure 3B, $*p < 0.05$), 10 nM E2 vs. vehicle treated sham preconditioning groups (Figure 3B, $+p < 0.05$) and 100 nM E2 vs. vehicle treated sham preconditioning groups (Figure 3B, $+p < 0.05$).

We used the non-selective nuclear ER antagonist, ICI, to determine if E2's attenuation of the protective response to isoflurane preconditioning in female neurons is mediated through ER α and ER β . Interestingly, 100 nM E2 continued to attenuate isoflurane-induced protection in female neurons in the presence of ICI (sham preconditioning + 100 nM E2 + ICI, $76.7 \pm 4.8\%$ vs. isoflurane preconditioning + 100 nM E2 + ICI, $76.3 \pm 3.2\%$) as did ICI alone (sham preconditioning + ICI, $76.7 \pm 3.3\%$ vs. isoflurane preconditioning + ICI, $77.5 \pm 4.9\%$). After allowing for the effects of differences in drug treatment, there were no

significant differences in the mean values among the different preconditioning treatments ($p=0.339$). There were also no significant differences in the mean values among the different drug treatments after accounting for the effects of differences in preconditioning treatments ($p = 0.920$). The effect of either sham or isoflurane preconditioning does not depend on what drug treatment (ICI or ICI + E2) is present (interaction between preconditioning and drug treatment, $p = 0.719$).

4.0 Discussion

Using sex-stratified isolated cortical neurons derived from mice, we have shown that isoflurane preconditioning protects male and female cortical neurons from subsequent OGD and that the sex steroid, E2, attenuates the protective isoflurane preconditioning response only in female neurons independent of the nuclear estrogen receptors. Ancillary to our primary hypotheses, we also found that regardless of preconditioning, female neurons are less sensitive to OGD compared to male neurons and that transient E2 exposure protects both male and female neurons from subsequent OGD.

Although isoflurane exposure itself has been shown to be neurotoxic in some experimental paradigms, the cytoprotective properties of isoflurane preconditioning in vivo and in vitro have typically been associated with shorter exposure times similar to what was used in the current study as well as with lower isoflurane doses (Kapinya et al., 2002; Kitano et al., 2007b; Li et al., 2008; Miyazaki et al., 1997; Wei et al., 2007). For example, in vitro preconditioning in rat primary cortical neuronal cultures with 1.2% or 2.4% isoflurane for 60 minutes was observed to be protective against the toxic consequences of continuous exposure to 2.4% isoflurane for 24 hours (Wei et al., 2007). We have also shown that a 2 hour exposure of sex-stratified mouse cortical astrocyte cultures to 3% isoflurane induced less than 0.5% cell death 48 hours later when compared to male and female Triton X-100-treated controls (Johnsen and Murphy, 2011). Based on these observations, it seems unlikely that our findings were confounded by any intrinsic toxic effect of isoflurane exposure with the 3% concentration and 1 hour exposure time used in this study.

One of the potential limits of our study is that we may have missed delayed neuronal death due to apoptosis as we only analyzed cell death 24 hours following OGD. OGD is an in vitro model of ischemia that induces multiple modes of cell death including apoptosis that ultimately result in cell death. Rather than focusing on different types of cell death mechanisms like apoptosis, our objective in this study was to simply determine if isoflurane preconditioning can protect neurons from cell death induced by ischemia regardless of cell death mechanism initiated, and if so, whether the level or amount of cell death rather than mechanisms leading to cell death are different between male and female cells in the presence or absence of E2. However, we recognize that our conclusions presented below are limited to cell death mechanisms operating at 24 hours after OGD since we did not examine cell death at more delayed time points or induced by other injury paradigms.

4.1 Isoflurane Preconditioning Protects Neurons From Subsequent OGD Independent Of Innate Cell Sex

We have demonstrated that isoflurane preconditioning similarly protected isolated male and female cortical neurons from OGD. Considering recent findings from our lab demonstrating that isoflurane preconditioning protects both male and female astrocytes from subsequent OGD (Johnsen and Murphy, 2011), our data suggest that the cellular default in the brain in the absence of E2 is a protective response to isoflurane preconditioning regardless of innate cell sex and that sexually dimorphic responses to isoflurane preconditioning may result from other factors in the intact brain (Wang et al., 2008a). This notion is supported by the general consensus of current in vitro isoflurane preconditioning studies demonstrating a protective

response in mixed-sex neuronal cultures that are removed from a sex steroid milieu (Wise-Faberowski et al., 2001; Kapinya et al., 2002; Li et al., 2008; McMurtrey and Zuo, 2010). Further support that the cellular default in the brain may be a protective response to isoflurane preconditioning is provided by in vivo experiments demonstrating a protective response to isoflurane preconditioning following subsequent brain ischemia in ovariectomized females, which have reduced circulating female sex steroids, compared to a detrimental isoflurane preconditioning response in young intact females (Wang et al., 2008a).

4.2 E2 Attenuates The Protective Isoflurane Preconditioning Response Only In Female Neurons Independent Of Nuclear Estrogen Receptors

Since the presence of E2, the primary estrogen in women and female rodents, has been linked to a deleterious response of female mouse brain to isoflurane preconditioning (Wang et al., 2008a), we hypothesized that E2 would attenuate the apparent default protective response to isoflurane preconditioning in male and female neurons. Interestingly, we found that the presence of E2 before and during isoflurane preconditioning attenuated the protective response only in female neurons, while male neurons exposed to E2 continued to show a protective response to isoflurane preconditioning compared to sham preconditioning. These findings are consistent with previous in vivo reports showing that E2 attenuates isoflurane preconditioning protection in ovariectomized female mice (Wang et al., 2008a) and that male mice, which normally have low circulating E2, demonstrate a protective response to isoflurane preconditioning.

One interpretation of these findings is that while the neuronal default is a protective response to isoflurane preconditioning regardless of innate cell sex, the mechanisms of this protective response may be different between male and female neurons, of which E2 alters only the mechanism in female neurons. There have been no investigations directly linking sex-specific isoflurane preconditioning mechanisms with E2, although one report has demonstrated differences in Akt phosphorylation between brains from gonadally-intact male and female mice following isoflurane preconditioning (Kitano et al., 2007b). Here it was demonstrated that isoflurane preconditioning increased Akt phosphorylation, a protective response, in male mouse brain compared to female brain, but the role of E2 in this sexually dimorphic Akt phosphorylation was not assessed. Moreover, the volatile anesthetic, xenon, has been shown to protect rodent brain independent of animal sex via Akt and HIF-1 α pathways (Limatola et al., 2010), though many of the mechanisms of xenon preconditioning in the brain appear to be substantially different from those of isoflurane (Wang et al., 2008b; Derwall et al., 2009). However, xenon's high cost and limited approval continues to limit its clinical application.

In addition to isoflurane preconditioning inducing different protective mechanisms between male and female neurons, it is equally possible that E2 induces different mechanisms between male and female neurons, of which the mechanism in female neurons alters the default protective isoflurane preconditioning response. While reports investigating differences in E2 signaling between male and female neurons are scarce, it has been shown that neuronal cell death-inducible putative kinase, a negative Akt modulator (Du et al., 2003) and E2-responsive gene (Ise et al., 2005) is increased in female cortex compared to males (Kitano et al., 2007b). Expression of this kinase, however, was not modulated by isoflurane preconditioning (Kitano et al., 2007b).

The nuclear estrogen receptors, ER α and β , on the other hand, have been linked to the E2-mediated attenuation of isoflurane preconditioning in female animals (Wang et al., 2008a) and to differential responses following glutamate exposure to isolated male and female neurons (Bryant and Dorsa, 2010), but whether E2 acts through nuclear ERs in female

neurons to attenuate isoflurane preconditioning has not been directly tested. By using the ER antagonist ICI (Harms et al., 2001), which binds strongly to both nuclear ERs and prevents the dimerization required for most genomic and non-genomic ER action (Bjornstrom, 2005), we found that nuclear ERs do not exclusively mediate E2's attenuation of isoflurane preconditioning in female neurons. This is not likely due to differential ER α and β expression as both receptor subtypes are expressed in female neurons (Bryant and Dorsa, 2010) and are functionally antagonized by ICI (Bjornstrom, 2005). It may be that E2 attenuates isoflurane preconditioning in female neurons via non-nuclear ERs such as GPR30 (Maggiolini and Picard, 2010), although GPR30 and other non-nuclear ER mediated mechanisms have not yet been investigated for a potential role in anesthetic or non-anesthetic preconditioning.

4.3 Female Neurons Are Less Sensitive To OGD Compared To Male Neurons Independent Of Preconditioning Treatment

It has been appreciated for some time that female rodent brain is less sensitive to ischemia compared to males, but most investigations have focused on the role of post-pubertal sex steroids as primary mediators in this sexually dimorphic outcome (Singh et al., 2006; Vagnerova et al., 2008; Liu et al., 2009). The role of innate cell sex in ischemic injury remains under-investigated despite increasing evidence that sex-specific neuronal sensitivities have been reported following oxidative and excitotoxic damage (Du et al., 2004), hypoxia (Heyer et al., 2005), autophagy (Du et al., 2008), and OGD in brain slices (Li et al., 2005). This report is the first to demonstrate that isolated female cortical neurons are less sensitive to OGD compared to male cells independent of preconditioning (Figure 2C). One reason why other laboratories may not have seen sex differences in neuronal cell death outcomes following OGD might be the use of neuronal cultures derived from prenatal rather than postnatal brains as was done in these studies. Masculinization of the mouse brain occurs when the fetal testes in males produce an adult-level spike of testosterone at embryonic days 16 to 17 and a second surge on the day of birth (postnatal day 0) while feminization occurs in the absence of these testosterone surges (Gondos, 1980; Pontis et al., 1979, 1980; Motelica-Heino et al., 1988). Hence neurons from prenatal brain are less sexually differentiated compared to neurons from postnatal or adult brains. The results presented here from postnatal neurons suggest that innate neuronal cell sex may have an important role in male versus female ischemic brain injury outcomes.

4.4 Transient Pretreatment With E2 Protects Neurons from Subsequent OGD Independent Of Preconditioning In Male And Female Neurons

Independent of isoflurane preconditioning, transient pretreatment with E2 protected both male and female neurons (pooled sham preconditioned and isoflurane preconditioned cell survival outcomes, $p < 0.05$). E2 pretreatment is known to protect brain tissue and isolated brain cells from subsequent OGD (Harms et al., 2001) and other insults (Du et al., 2004; Heyer et al., 2005). However, the results presented here are the first to show that E2-mediated protection from ischemic insult in isolated cortical neurons can persist for up to 24 hours following E2 removal from the cellular environment in isolated male and female neurons. While these findings were ancillary to our investigation of E2's effect on isoflurane preconditioning, they do have important implications towards expanding our current understanding of cellular responses to E2 in the brain. Similar results have been reported showing that transient E2 exposure protects female hippocampal slices (Raval et al., 2006) and brain tissue (Raval et al., 2009) from ischemic insult via intra- and extracellular Ca^{2+} , calcium/calmodulin-dependent kinase II, cAMP response element-binding protein. Our findings extend such E2 protection in models of ischemic injury to isolated male and female cells.

5. Conclusions

There is considerable clinical interest in applying the neuroprotective potential of isoflurane preconditioning during the perioperative period so as to reduce the risk of brain damage following surgeries at risk for ischemic stroke, such as coronary artery bypass grafting and carotid endarterectomy (Kitano et al., 2007a; Wang et al., 2008b). However, a better understanding of the sexually dimorphic response to isoflurane preconditioning is crucial before such neuroprotective strategies can be implemented in men and women. Our results have important implications towards increasing our understanding of the sex differences in the response to isoflurane preconditioning as well as potential implications towards ischemic stroke outcomes in men versus women.

Highlights

- Isoflurane preconditioning protects neurons from ischemia independent of cell sex
- Estradiol attenuates isoflurane preconditioning protection only in female neurons
- Estradiol's effect on isoflurane preconditioning is receptor-independent
- Female neurons are less sensitive to ischemia than male neurons
- Transient estradiol exposure protects both male and female neurons from ischemia

List of Abbreviations

ANOVA	analysis of variance
DMSO	dimethylsulfoxide
E2	17 β -estradiol
ER	estrogen receptor
ICI	ICI 182,780
IsoPC	isoflurane preconditioning
OGD	oxygen and glucose deprivation
PCR	polymerase chain reaction
ShamPC	sham preconditioning

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Figure 1.

Schematic of the preconditioning and drug treatment paradigm in sex-segregated cortical neuron cultures from postnatal day 0 – 1 day mice. E2, 17 β -estradiol; ICI, ICI 182,780; IsoPC, isoflurane preconditioning; OGD, oxygen and glucose deprivation; ShamPC, sham preconditioning.

Figure 2.

Isoflurane preconditioning (IsoPC) increased cell survival in male and female cortical neurons, while female neurons were protected from OGD regardless of preconditioning. **(A)** Mean cell survival of sham preconditioning (ShamPC) and IsoPC-treated male and female cortical neurons following OGD. * $p < 0.05$ ShamPC vs. IsoPC in male cells, * $p < 0.01$ ShamPC vs. IsoPC in female cells; $n = 11$. **(B)** Sex-independent paired difference analysis of variance (ANOVA) of cell survival outcomes between ShamPC and IsoPC groups. Each line connects cell survival outcomes from the same litter ($n = 1$); * $p < 0.05$; $n = 11$. **(C)** Preconditioning-independent paired difference ANOVA of cell survival outcomes between

male and female cortical neurons. Each line connects the cell survival outcome from the same litter ($n=1$); $*p < 0.001$; $n = 11$.

Figure 3.

17 β -estradiol (E2) attenuates isoflurane preconditioning (IsoPC)-induced protection from oxygen and glucose deprivation (OGD) in female but not male neurons. **(A)** IsoPC protects male neurons from subsequent OGD independent of E2. * $p < 0.05$, ShamPC vs. IsoPC; ** $p < 0.05$, E2 IsoPC vs. vehicle IsoPC; $n = 9$. **(B)** E2 attenuates IsoPC protection following subsequent OGD in female neurons. * $p < 0.01$, ShamPC vs. IsoPC; + $p < 0.05$, E2 ShamPC vs. vehicle ShamPC; $n = 9$.