Sex differences in mitochondrial (dys)function: Implications for neuroprotection

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Abstract

Decades of research have revealed numerous differences in brain structure size, connectivity and metabolism between males and females. Sex differences in neurobehavioral and cognitive function after various forms of central nervous system (CNS) injury are observed in clinical practice and animal research studies. Sources of sex differences include early life exposure to gonadal hormones, chromosome compliment and adult hormonal modulation. It is becoming increasingly apparent that mitochondrial metabolism and cell death signaling are also sexually dimorphic. Mitochondrial metabolic dysfunction is a common feature of CNS injury. Evidence suggests males predominantly utilize proteins while females predominantly use lipids as a fuel source within mitochondria and that these differences may significantly affect cellular survival following injury. These fundamental biochemical differences have a profound impact on energy production and many cellular processes in health and disease. This review will focus on the accumulated evidence revealing sex differences in mitochondrial function and cellular signaling pathways in the context of CNS injury mechanisms and the potential implications for neuroprotective therapy development.

Keywords

Introduction

Mitochondrial dysfunction is a common component of central nervous system (CNS) injury pathophysiology that has received increasing attention as a putative target for neuroprotective therapy development (Fiskum 2000; Robertson et al. 2006; Perez-Pinzon et al. 2012; Hagberg et al. 2014). Animal and human CNS injury studies indicate sexually

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dimorphic behavioral and histopathological outcomes yet animal and *in vitro* studies rarely include females for evaluation of injury and/or treatment responses. The National Institutes of Health of the US mandated the inclusion of women in clinical trials almost a decade ago, but this balanced approach was not adopted by basic science researchers. Recently, the NIH issued a statement that new guidelines will be established requiring the inclusion of both sexes or strong justification for exclusion of one sex in all future federally funded preclinical studies in an effort to increase the translational generalizability and replicability of basic scientific research (Clayton and Collins 2014).

Sex is determined in mammals by chromosome complement and the presence of a single gene on the Y chromosome, SRY, for sex-determining region of the Y chromosome, which codes for tdf, the testis determining factor (Fig. 1). Expression of this gene during a narrow window of early embryonic development directs the bipotential gonad to become a testis. In the absence of SRY the gonad will become an ovary. The testis begins steroidogenesis embryonically, exposing the developing male fetus to high concentrations of testosterone and its aromatized metabolite, estradiol. The fetal brain is rich in estrogen (ER) and androgen receptors (AR), as well as the enzyme aromatase that converts testosterone to estradiol. The ovary remains quiescent at this time, leaving the female relatively free from androgen and estrogen exposure, particularly in the brain. Androgens and estrogens program the developing brain by impacting numerous parameters including cell death and survival, neural and glial genesis, neuronal migration, myelination, synaptogenesis and synaptic pruning and neurochemical phenotype. In adulthood, gonadal and adrenal steroids act on and are influenced by this differentiated neural substrate in some but not all instances. Some adult brain sex differences are a byproduct of differences in gonadal steroids, meaning the sex difference goes away if steroids are either equalized or removed. Thus this is an instance of hormonal modulation as opposed to a primary sex difference *per se*. More recently it has become apparent there is also both a direct and a modulatory role for chromosome complement, with genes on the X or Y chromosome impacting brain and behavior in males and females. To-date there have been no clear connections drawn between a particular sex chromosome gene and a behavioral or phenotypic endpoint, but it is still early days for this research focus (review in (McCarthy 2008; McCarthy and Arnold 2011)).

Sex differences in metabolism have long been observed in humans (reviewed in (Widdowson 1976). Circumstantial evidence for sex differences comes from cases of severe nutrition deprivation observed in Germany following World War II. During the war, all people were given the same rations regardless of age or sex. In order to qualify for additional rations, individuals had to be considered severely malnourished and 60 % of those qualified individuals were male. Further studies of orphan children in 1947 indicated males were more severely malnourished than females. Subsequent studies of nutrition deprivation in pigs and rats revealed a significantly greater mortality in males, coinciding with a greater loss of total body protein, while females lost significantly more fat than males. Accordingly, Widdowson concludes "A large loss of protein from the body is metabolically a far more serious matter than loss of fat, and the greater catabolism of protein in males is probably an important reason why they withstand a deficiency of energy less well than females." (Widdowson 1976). More recently, endurance exercise studies reveal that males rely almost exclusively on carbohydrates and amino acids (97 %) for fuel while females predominantly use fat

(62 %) (Lamont 2005). Furthermore, starvation of mice results in significantly greater survival in females coinciding with increased plasma ketone bodies and associated estrogen mediated increases in mitochondrial uncoupling protein 1 in brown fat of females *vs.* males (Jikumaru et al. 2007). Sex differences in preferred fuel source are also apparent in cardiac and hepatic cells (Djouadi et al. 1998). The primary reliance on proteins and fatty acids for fuel in males and females, respectively, has been confirmed in cultured primary neurons and fibroblasts following nutrient deprivation (Du et al. 2009). Nutrient deprivation activates autophagy; the targeted degradation of cellular components for fuel. Given the evidence for sex differences in human metabolism and preferred cellular biofuels, there may also be sex differences in autophagy. These fundamental sex differences in metabolism under stressful conditions may also be influenced by intrinsic differences in genomic maintenance.

Only 13 of 92 mitochondrial proteins essential for oxidative phosphorylation are encoded by mitochondrial DNA. As a result, mitochondrial-nuclear communication for translation and mitochondrial import of respiratory chain subunit proteins is an essential process for maintaining adequate cellular energy supply. During stressful conditions, the mitochondrial-nuclear DNA coordination is especially critical for cellular survival. Therefore, mechanisms to prevent or repair DNA damage under stressful conditions would be predicted to improve cellular survival. Given the near exclusive maternal inheritance of mitochondrial DNA, there may be fundamental sex differences in mitochondrial metabolic regulation such that female mitochondria are better equipped to cope with stressful conditions and are relatively resilient to DNA damage and mutation to reduce the probability of producing inheritable metabolic disorders. Indeed, microarray analysis of embryonic day 10.5 mouse brains (previous to hormonal influence) identified over 50 differentially expressed transcripts between males and females (Dewing et al. 2003). Of note is the nearly 2 fold increase in expression of DNA replication and repair DNA polymerase delta 1 and GA binding protein subunit alpha (GABP) in female fetal brain *vs.* males (Dewing et al. 2003). Previous studies identify GABP as the nuclear DNA binding motif of the rat and human homologue nuclear respiratory factor-2 (NRF-2) (Virbasius and Virbasius 1993). NRF-2 upregulates the expression of complex II and IV electron transport chain subunits, ATP synthase β, mitochondrial biogenesis protein mitochondrial transcription activator A (TFAM) (Zhang and Wong-Riley 2000; Scarpulla 2002) and is activated by neuronal depolarization (Zhang and Wong-Riley 2000). These findings highlight the possibility that females are hardwired for favorable nuclear to mitochondrial coordination of critical mitochondrial respiratory subunits in response to stress compared to males. The few studies assessing sex differences in mitochondrial function following injury reveal substantial differences in mitochondrial membrane potential, enzyme activity and respiration between male and female rodents after cerebral neonatal hypoxic ischemia (Weis et al. 2012; Demarest et al. 2013). Therefore, the focus of this review is to examine the literature on sexually dimorphic phenomena relevant to metabolic and mitochondrial function in the context of well-established mechanisms of CNS injury.

Excitotoxicity and Calcium

Glutamate excitotoxicity is a widely accepted contributor to neurodegeneration following CNS injury. Under normal physiological conditions, activation of glutamate receptors

increases intracellular sodium, calcium and downstream activation of second messengers, nitric oxide generation, mitochondrial calcium uptake, increased mitochondrial membrane potential (Ψ) and upregulation of rate-limiting TCA cycle dehydrogenases (Duchen 2000a, b). Excitotoxic neurodegeneration is predominantly mediated through extrasynaptic NR2B subunit containing N-Methyl-d-Aspartate receptors (NMDARs), however the exact receptor mediating excitotoxic cell death may differ with age and cellular composition of a given brain region (Chen et al. 1995; Portera-Cailliau et al. 1997; Hilton et al. 2006). Sex differences have been scarcely studied with regard to differential susceptibility to excitotoxic neurodegeneration. However, following severe traumatic brain injury (TBI) in humans, cerebral spinal fluid (CSF) glutamate content is significantly greater in males *vs.* females (Wagner et al. 2005). While female neuroprotection observed following injury is commonly attributed to female hormones, less attention has been paid to the influence of androgens mediating male susceptibility to brain injury (for review see (Quillinan et al. 2014)). For instance, in immature neurons, activation of ionotropic γ-aminobutyric acid receptors (GABA$_A$) is excitatory and contributes to male vulnerability to excitotoxic injury in an androgen dependent manner (Nuñez et al. 2003; Nuñez and McCarthy 2008).

Sex differences in vulnerability to excitotoxic injury can also be influenced by chromosome complement. Sex stratified primary cortical neuronal cultures treated with exogenous glutamate/glycine, NMDA, AMPA or kainate demonstrate a significant decrease in cellular viability in male (XY) *vs.* female (XX) neurons. NMDAR antagonist MK-801 prevents decreases in cellular viability solely in XY neurons (Du et al. 2004) while 17β-estradiol treatment does not improve XY neuronal viability. Other studies exposing hippocampal (Weaver et al. 1997) and cortical neurons (Perrella and Bhavnani 2005) to glutamate demonstrate protection from excitotoxic cell death with exogenous administration of 17β-estradiol, but not in cerebellar granular neurons (Minano et al. 2007). However, these cultures were not sex-specific. Moreover, cultured mesencephalic dopamine neurons exposed to high concentrations of extracellular dopamine display male susceptibility to cell death, but only female neurons are rescued by NMDAR antagonist AP-5 (Lieb et al. 1995). *In vivo* CNS injury studies show some efficacy of NMDAR antagonists in preventing cell death but typically only use male animals or do not report which sex is used (Ford et al. 1989; Gaviria et al. 2000; Rao et al. 2001; Lapchak 2004; Han et al. 2009; Wang et al. 2014). Spinal cord excitotoxicity induced with high and low doses of kainic/quinolinic acid results in 80 % and 40 % mortality of male mice, respectively. Interestingly, all females survive both doses of either drug (Martin 2011). Further complicating the interpretation of sex differences in excitotoxic neurodegeneration is the observation that injection of naïve rats with NMDAR antagonist MK-801 results in significantly more neuronal cell death in females compared to males. However, similar treatment of several strains of mice does not result in the same severity of cell death nor a sex difference (Bender et al. 2010). These findings highlight sex, brain region and species dependent susceptibility to excitotoxic injury which require further investigation.

Regardless of the exact receptor and brain region specificity impacted by excitotoxicity, resulting cell death is calcium dependent (Choi 1985). Mitochondrial calcium buffering is an essential homeostatic process for maintenance of normal cell function. Mitochondrial calcium uptake in the context of excitotoxicity has been intensively studied in isolated

mitochondria and primary neuronal cultures (for review see (Choi 1994; Nicholls 2004)). In general, a situation in which mitochondrial calcium is lower is associated with decreased cellular injury and too much calcium is associated with mitochondrial swelling and the opening of the mitochondrial permeability transition pore (mPTP) (Wang et al. 2001). Opening of the mPTP results in diffusion of molecules (<1,500 kD) from mitochondria to cytoplasm, ATP depletion and acute cell death. To our knowledge there have been no studies assessing putative sex differences in mPTP opening. However, studies of isolated mitochondria reveal rat brain (Kim et al. 2012) and mouse heart (Arieli et al. 2004) mitochondria have a sexually dimorphic capacity for calcium uptake with isolated male mitochondria having greater calcium uptake capacity than female mitochondria. This may be estrogen dependent as 17β-estradiol decreases calcium retention in brain mitochondria of both sexes (Kim et al. 2012) but overiectomy has no effect on calcium uptake in cardiac mitochondria (Arieli et al. 2004). Furthermore, brain mitochondria from cyclophilin D knockout mice have enhanced calcium uptake in both males and females but no sex difference. Cyclophilin D is a key regulator of mPTP opening where genetic knockout or pharmacological inhibition of cyclophilin D (e.g. by cyclosporine A) inhibits mPTP opening and cell death. Interestingly, survival analysis reveals that the increased lifespan normally observed in female *vs.* male wild-type mice is no longer apparent in cyclophiln D knockouts (Kim et al. 2012). These results beg the question - What is the physiological role of enhanced calcium uptake in mitochondria derived from males?

One possible explanation necessitating enhanced mitochondrial calcium uptake capacity by male mitochondria derives from secondary activation of the calcium-permeable transient receptor potential M2 (TRPM2) nonselective cation channels. TRPM2 channels are considered executioners of cell death following oxidative stress. They are activated by hydrogen peroxide and gated by intracellular adenine dinucleotide phosphate ribose (ADPr), (Fonfria et al. 2004) a breakdown product by poly(ADP)-ribose glycohydrolase (PARG) of poly(ADP-ribose) (PAR) polymers formed by poly(ADP-ribose) polymerase 1 (PARP-1). TRPM2 channels are present in both males and females at similar levels in cultured hippocampal neurons. However, electrophysiological evidence (Verma et al. 2012) and reductions in cell death by TRPM2 pharmacological or shRNA inhibition in an *in vivo* model of stroke, (Jia et al. 2011) or shRNA knockdown following *in vitro* oxygen glucose deprivation (OGD), (Verma et al. 2012) indicate that TRPM2 channels are only activated in males following injury. Contrarily, peroxide mediated *in vitro* toxicity shows no sex difference in cell death and TRPM2 inhibition is neuroprotective in both sexes (Verma et al. 2012) suggesting greater oxidative stress and/or PAR/ADPr generation in males following injury contributes to sex differences in TRPM2 mediated cell death.

As mentioned above, calcium propagates numerous cellular signaling cascades. Particularly relevant to CNS injury is the induction of nitric oxide synthase (NOS) and upregulation of TCA cycle enzymes. The TCA cycle enzyme α-ketoglutarate dehydrogenase (α-KGDH) is a potent generator and target of oxidative stress in the brain (Starkov et al. 2004; Starkov 2013) and regulatory mechanisms may limit ROS/RNS generation during times of cellular stress in a sex dependent manner. For example, TCA cycle enzyme regulation in myocardial ischemia suggests that increasing phosphorylation of α-KGDH and aldehyde dehydrogenase-2, reduces oxidative stress and confers cardioprotection in female heart as

compared with male heart (Lagranha et al. 2010). These data suggest that female resilience to injury may be mediated by superior enzyme regulation and decreases in oxidative stress.

Oxidative/Nitrositive stress

Calcium induction of oxidative stress is well documented in brain cells (reviewed in (Duchen 2000a, b; Nicholls 2004; Peng and Jou 2010)). Oxidative and nitrositive stress (ROS/RNS) refers to the balance between the generation of free radicals and their detoxification via resident antioxidant systems. Mitochondria are a major source of cellular ROS/RNS generation. Under pathological conditions, high levels of ROS/RNS can damage proteins, lipids and nucleic acids that must be repaired in order to meet cellular energy demands and ensure cell survival. NOS induction by calcium influx is hypothesized to be a fundamental regulator of cellular energy demand. Nitric oxide (NO) synthesized by NOS is freely diffusible and competes with oxygen at complex IV to reversibly inhibit the rate of oxidative phosphorylation (Brown and Cooper 1994; Brown 1995, 2001, 2007). In this manner, slowing the flow of electrons could serve as a feedback mechanism to regulate the rate of oxidative phosphorylation in response to cellular energy demand under physiological and pathophysiological conditions. NO can also react with superoxide forming the highly reactive and damaging RNS, peroxinitrite (ONOO-) (Brown 2007). Interestingly, neuronal NOS (nNOS) induction is greater in male animals following cerebral ischemic injury (McCullough et al. 2005; Semenas et al. 2010). Pharmacological inhibition or genetic knockout of nNOS is neuroprotective in male mice but actually increases infarct volume in female mice (McCullough et al. 2005). This suggests NO production following injury has a beneficial role in females but whether this is due to vasodilation and restoration of cerebral blood flow or another mechanism is unclear.

Sex differences in NOS signaling *in vitro* have also been investigated. Organotypic hippocampal slices from males are more susceptible to cell death following NMDA exposure or OGD and produce more nitrate/nitrite *vs.* females (Li et al. 2005). nNOS inhibition prior to NMDA exposure or OGD prevents cell death in male slices while having no effect in female slices. Treatment with 17β-estradiol protects both male and female neurons in culture (Li et al. 2005). Treatment of cortical neurons with exogenous ONOO- results in cellular depletion of reduced glutathione (GSH) in XY neurons after 24 h, which is completely rescued by antioxidant N-acetyl-cysteine (NAC) but XX neurons have no detectible depletion of GSH and NAC treatment has no effect (Du et al. 2004). Male specific GSH depletion also occurs in an *in vivo* model of cardiac arrest asphyxia in postnatal day 17 rats (Du et al. 2004). Moreover, NAC treatment within 2 h of rat controlled cortical impact (CCI) TBI prevents GSH depletion, restores mitochondrial respiratory function and calcium buffering capacity; only male rats were used in this study (Xiong et al. 1999). In human infants and children suffering from severe TBI, GSH depletion in cerebrospinal fluid (CSF) is measured 5-7 days post-injury (Bayir et al. 2002) revealing the clinical importance of considering sex as a contributing variable to CNS injury.

The cellular origins of NOS may also be an important contributing variable. A mitochondrial localized NOS (mtNOS) has been proposed (Haynes et al. 2004; Giulivi 2007). While its undeniable existence is complicated by the lack of specific antibodies, mtNOS has been

detected by immune electron microscopy (Bates et al. 1995). As such, it is tempting to speculate it plays an even larger role than nNOS in sexually dimorphic mitochondrial function. Regardless of cytosolic or mitochondrial origin, higher ONOO- generation under conditions of ROS/RNS damage mitochondrial and nuclear DNA thus activating PARP-1 for DNA repair (discussed in detail in cell death section). Additionally, protein nitration of critical antioxidant components may impair anti-oxidant defense systems. Following TBI in mice and humans, nitration of manganese superoxide dismutase (MnSOD/SOD2) impairs detoxification of mitochondrial superoxide (Bayir et al. 2007). Inhibition of neuronal NOS, but not endothelial or inducible NOS, attenuates MnSOD nitration following TBI. Interestingly, while total SOD activity remains unchanged over 72 h following TBI, mitochondrial MnSOD activity is significantly decreased over the same time period (Bayir et al. 2007). Indeed, over-expression of MnSOD in cells is associated with an increase in glutathione peroxidase (GPx) activity, decreases in lipid peroxidation and ONNO- formation ((Keller et al. 1998; Murakami et al. 1998) as cited by (Bayir et al. 2007)).

The antioxidant enzyme GPx is arguably the most important of this category of enzymes in both neurons and glia - providing the main detoxification pathway for hydrogen peroxide (H_2O_2) ((Dringen et al. 1999) as cited by (Nicholls 2004)). H_2O_2 is formed when MnSOD dismutates superoxide radicals formed by electron 'leakage' from the mitochondrial electron transport chain or by other mitochondrial activities. When catabolism of H_2O_2 by GPx (or catalase) is impaired, increasing lipid peroxidation can ensue, compromising cellular and mitochondrial membrane integrity and potentiating bioenergetic failure. Increased mitochondrial GPx activity in females *vs.* males was first observed in liver back in the 1960's (Pinto and Bartley 1969). More recently, Borras et al (2003) demonstrated increases in GPx and MnSOD activity in female derived liver mitochondria. Female derived mitochondria also produce about half the amount of H_2O_2 in liver, and perhaps more importantly, in synaptic and non-synaptic brain mitochondria *vs.* males. Mitochondria from overiectomized females have increased H_2O_2 production and decreased GSH *vs.* males, and these levels can be rescued by estrogen administration. These changes were most dramatic in synaptic mitochondria. Furthermore, female derived brain mitochondria have higher GSH content and four-fold less oxidative damage to mitochondrial DNA *vs.* males (Borras et al. 2003).

Mitochondrial DNA is oxidatively damaged with aging (Mecocci et al. 1993) and theorized as a main determining factor for lifespan (Harman 1956, 1972). In mammalian species, females generally live longer than males. Studies conducted as a part of The Biomarker of Aging Program from the NIH characterized longevity in several rodent strains and identified just two strains of mice where males live longer than females. Specifically, female C57BL6 mice have a 20 % shorter lifespan than males which is associated with decreases in antioxidant proteins and increases in oxidative stress in the hippocampus of 5 month-old mice. In older 24 month-old female C57BL6 mice, hippocampus, cortex and substantia nigra all have higher levels of oxidative stress *vs.* males. Chronic administration of a superoxide dismutase mimetic (SOD; from 12–24 months in drinking water) significantly increases longevity in female mice (p<0.05) compared to male mice (p=0.073) (Ali et al. 2006). Similar differences in longevity and associated increases in oxidative stress have been demonstrated in *Drosophila* (Ballard et al. 2007). Therefore, decreased ROS/RNS generation

and enhanced detoxification by antioxidant systems in females *vs.* males may play an intimate part in sex differences observed across the lifespan.

The production and antioxidant detoxification of ROS/RNS plays a key role in normal cellular physiology and pathophysiology following CNS injury. Emerging evidence suggests ROS/RNS generation and antioxidant defense systems are sexually dimorphic. In humans, ROS damage measured by lipid peroxidation product F_2-isoprostane is higher in male cord blood from preterm human twins (Minghetti et al. 2013) and following TBI in CSF of male adults *vs.* females (Bayir et al. 2004; Wagner et al. 2005). Another study finds higher F_2-isoprostane in CSF of children and infants following severe TBI independent of sex (Varma et al. 2003). Further supporting the notion of sex-dependent oxidative damage are animal studies showing mitochondrial antioxidant enzymes paraoxynase-2 (PON2) (Costa et al. 2013; Giordano et al. 2013), thioredoxin (Trx) (Saeed et al. 2009; Chen et al. 2010), peroxiredoxin 6 (Prdx6) (Di et al. 2012), GPx (Borras et al. 2003) and glutaredoxin (GRx) (Diwakar et al. 2007; Saeed et al. 2009) at higher levels/activity in the female *vs.* male brain. Increased antioxidant enzymes in the female brain may be a result of the generally recognized neuroprotective effects conferred by female hormones. Accordingly, a rat model of global cerebral ischemia-reperfusion demonstrates metestrous (when estrogens are low) female susceptibility to lipid peroxidation, GSH depletion and decreased antioxidant enzyme activity of SOD and catalase *vs.* males (Mohagheghi et al. 2013b). This evidence suggests females may be relatively resilient to ROS/RNS mediated injury *vs.* males due to higher antioxidant enzyme defense systems and that males may particularly benefit from antioxidant treatments following CNS injury.

As mentioned previously, specific brain regions appear to be particularly susceptible to injury. In particular, dopaminergic areas are very sensitive to ROS/RNS mediated damage. Antioxidant enzyme PON2 is highly enriched in astrocytes compared to neurons and highest in dopaminergic brain regions (e.g. striatum) (Giordano et al. 2013), possibly playing a role in the aforementioned female resilience of mesencephalic cultures to excitotoxicity (Lieb et al. 1995; Alano et al. 2010). In the *in vitro* and *in vivo* 6-hydroxydopamine model of Parkinson's disease (PD), male mice have more lactate dehydrogenase release, apoptotic and necrotic cell death *vs.* females. XY neurons also display decreases in mRNA and protein levels of mitochondrial respiratory chain subunits coinciding with decreases in ATP and higher ROS generation *vs.* XX neurons (Misiak and Beyer 2010). Similar ROS and mitochondrial changes are observed in sex-dependent vulnerability of cultured astrocytes in the 1-methyl-4-phenylpyridinium (MPTP) model of PD (Sundar et al. 2011). These molecular mechanisms of ROS/RNS production and antioxidant detoxification may underlie the higher incidence of early onset (age 50–59) PD in human males (Pringsheim et al. 2014) and contribute to their poorer quality of life due to increased burden of symptoms *vs.* females (Lubomski et al. 2014). Dopaminergic regions of the brain (i.e. midbrain & striatum) are also susceptible to injury following TBI (Yan et al. 2007; Hutson et al. 2011; Shin et al. 2012; van Bregt et al. 2012) but sex dependent vulnerability of these regions following TBI is unknown.

Antioxidant defense systems are a necessary vital component for proper mitochondrial function. For example, the antioxidant enzyme GRx is essential to maintenance of

mitochondrial complex I activity where reductions in GRx result in loss of complex I activity. Following ROS/RNS induction by β-N-oxalyl amino-l-alanine lumbosacral spinal cord injection or MPTP exposure of motor neurons, complex I activity in males is severely inhibited while activity is unaffected in females (Kenchappa et al. 2002, 2004; Kenchappa and Ravindranath 2003; Diwakar et al. 2007). In overiectomized and estrogen receptor antagonized (ICI 182,780) females, complex I is also inhibited. In the context of CNS injury, the higher antioxidant capacity of females may provide neuroprotection but evaluation of putative sex differences in mitochondrial function following TBI have yet to be assessed. Therefore, novel approaches to neuroprotection utilizing antioxidant treatments are most likely to benefit males *vs.* females (reviewed in (Fernandez-Gajardo et al. 2014)) and should be evaluated in a sex-specific manner.

Oxidative stress promotes loss of mitochondrial Ψ and mitochondrial fission. This is thought to 'select' mitochondria for degradation by mitophagy. Indeed, oxidative stress is sufficient to induce autophagy/mitophagy ((Scherz-Shouval et al. 2007) as cited by (Au et al. 2010)) and is necessary for the normal progression of autophagic recycling ((Zhu et al. 2007) as cited by (Au et al. 2010)). The following section reviews autophagy in the context of brain injury and sex differences.

Autophagy/Mitophagy

Autophagy/mitophagy generally refers to the cellular process of targeted lysosomal degradation of macromolecules for metabolic recycling into amino acid and fatty acid constituents (Smith et al. 2011). This process is necessary for normal cellular protein and lipid turnover and augmented following excitotoxic, ischemic and traumatic CNS injury (Diskin et al. 2005; Guo et al. 2014; Lin et al. 2014; Ginet et al. 2014; Zheng et al. 2014; Zhou et al. 2014). Recent studies reveal that during starvation induced autophagy, mitochondria supply membranes for autophagasome formation via association with autophagy mediating protein Atg5 and subsequent association with autophagasome protein microtubule-associated-protein-1 light chain 3 (LC3) (Hailey et al. 2010) thus suggesting a critical role for mitochondria in the induction of autophagy. While mitochondrial degradation via mitophagy is a focus of this section, the term autophagy by definition includes mitophagy and will be used interchangeably hereafter as it is the most commonly used terminology.

There is a significant signaling role for the mitochondrial specific diphosphatylglycerol lipid cardiolipin in coordination of mitophagy progression (Kirkland et al. 2002; Chu et al. 2013, 2014). Cardiolipin peroxidation causes a conformational flip from the normal position on the inner mitochondrial membrane to the outer mitochondrial membrane in a phospholipid scrambalase-3 dependent manner. This promotes oxidized cardiolipin association with the lapidated form of autophagasome protein microtubule-associated-protein-1 light chain 3 (LC3-II) (Chu et al. 2013). LC3-II is considered a reliable biochemical marker for preautophagasomal membrane formation and is widely used to detect changes in autophagy.

Sex differences in autophagy are observed using *in vitro* and *in vivo* models of cardiac ischemia, (Chen et al. 2013) cerebral neonatal hypoxia-ischemia, (Weis et al. 2014) and

iron-induced brain injury (Chen et al. 2012). In one of the most informative studies pertaining to sex differences in autophagy, Du et al. (2009) demonstrates fundamental differences following nutrient deprivation of neuronal cultures. They observe a decrease in XY cellular viability and associated increases in LC3-II protein levels compared to XX cells, an observation supported by time-lapsed microscopy confirmation of lysosomal fusion. Pharmacological or siRNA mediated inhibition of autophagy initiating protein Atg7 attenuates loss of cell viability and cell death of XY neurons to levels of XX neurons. Furthermore, XX neurons display phospholipase A2 mediated increases in lipid droplet formation following nutrient deprivation which is not apparent in XY neurons. Treatment of cultures with L-carnitine, a necessary co-factor for import of free fatty acids into the mitochondrial matrix for β-oxidation, improves XY neuronal viability and attenuates cell death after nutrient deprivation but has no effect in XX neurons (Du et al. 2009). Thus, autophagy may play a detrimental role in XY cells under stressful conditions and the relative resistance of XX cells to nutrient deprivation may be attributed to an enhanced capacity to synthesize and utilize free fatty acids as alternative biofuels. Related to these results, we observe a male susceptibility to brain mitochondrial respiratory impairment following cerebral neonatal hypoxic-ischemia and *in vivo* administration of acetyl-L-carnitine post-injury partially prevents respiratory impairment in male mitochondria while having no effect on female mitochondrial respiration (Demarest et al. 2013). These data support the aforementioned hypothesis that human females preferentially utilize lipids while males utilize proteins as primary biofuels at the subcellular level and imply that under stressful conditions, males may need to cannibalize cellular components via autophagy to obtain the requisite protein fuel.

In support of the notion that too much autophagy is detrimental following stressful conditions, Atg7 deficient mice subjected to the Rice-Vanucci model of cerebral neonatal hypoxia ischemia have less hippocampal pyramidal neuron death *vs.* wild-type (Koike et al. 2008). On the contrary, neuroprotection is not observed in older Atg7 deficient mice (Koike et al. 2008). Notably, sex was not specified in this study and may be a contributing factor to the discrepancies between ages. In another study using male animals subject to closed head TBI, rapamycin, an activator of mTOR mediated autophagy induction, is neuroprotective both histologically and behaviorally *vs.* vehicle treated controls (Erlich et al. 2007). Further clouding our sex-specific understanding of the involvement of autophagy following injury, female mice subjected to cerebral neonatal hypoxia ischemia have increased cortical LC3B-II levels compared to males (Weis et al. 2014). The authors interpret this increase as a marker of failed autophagy progression. Autophagy is undoubtedly involved in response to various CNS injuries and evidence suggests it may be sex-dependent but the exact beneficial/detrimental role is incompletely understood. Similarly unresolved in functional significance in response to a CNS injury is the observed synthesis of new mitochondria or mitochondrial biogenesis.

Mitochondrial Quality Control

Mitochondrial quality control (MQC) is essential for maintaining adequate cellular energy supply. Main processes contributing to MQC maintenance include mitochondrial biogenesis, dynamics (fission and fusion balance) and the aforementioned recycling of damaged/aged

organelles via autophagy (reviewed in (Michel et al. 2012)). Targeting MQC processes in CNS injury where mitochondrial dysfunction plays a role in pathogenesis has been advocated (for review see (Anne et al. 2013)).

In animal models of neonatal (Yin et al. 2008) and adult ischemic stroke, mitochondrial biogenesis is increased in the ischemic hemisphere (Xie et al. 2014). It is hypothesized that this is an endogenous compensatory response attempting to restore ATP levels by increasing mitochondrial number. *In vitro* OGD of sex specific cerebral granule neurons (CGNs) shows sex-dependent responses with XY cells maintaining cellular ATP, mitochondrial Ψ and less cell death *vs.* XX CGNs. These results are attributed to mitochondrial biogenesis shown by increases in mtDNA, mitochondrial biogenesis proteins peroxisome proliferator-activated receptor-γ coactivator 1alpha (PGC1α), TFAM, nuclear respiratory factor 1 (NRF-1), mitochondrial heath shock protein 60 (HSP60) and cytochrome c oxidase (COXIV) in XY while XX CGNs lack or have suppressed responses (Sharma et al. 2014).

The functional consequences of enhanced mitochondrial biogenesis following brain injury are unknown. Nonetheless, promoters of mitochondrial biogenesis as therapeutics have been investigated. Gemfibrozil (an activator of mitochondrial biogenesis) treatment of rats following global cerebral ischemia induces TFAM and NRF-1 in pre-treated metestrous females but suppresses levels in males (Mohagheghi et al. 2013a). Correspondingly, treatment with gemfibrozil inhibits caspase-dependent apoptosis in females by upregulating antioxidant defenses but promotes caspase-dependent and caspase-independent cell death in male hippocampus (Mohagheghi et al. 2013b) (cell death signaling discussed in the next section). Selenium pretreatment of murine hippocampal neurons or mice is neuroprotective in models of ischemic stroke by reducing oxidative damage and promoting mitochondrial biogenesis via PGC-1α and NRF-1 induction but was not tested in female cells/animals (Mehta et al. 2012). On the opposing side of mitochondrial biogenesis, enhanced rates of mitochondrial fragmentation or fission have been reported to contribute to cell damage following CNS injury (Cao et al. 2013; Slupe et al. 2013; Kashani et al. 2014).

Mitochondrial fission and fusion processes are generally thought to segregate functional and dysfunctional mitochondria in order to maintain a healthy population of efficient energy producing organelles within a given cell (for review see (van der Bliek et al. 2013)). The overall concept is that dysfunctional mitochondria tend toward fission while healthy functional mitochondria undergo fusion and maintain an elongated tubular morphology thereby protecting mitochondria from autophagic degradation (Rambold et al. 2011). During injury, upregulation of fission proteins (e.g. FIS1, DRP-1) is associated with damage while fusion proteins (Mitofusins 1 and 2, OPA1) are considered beneficial (Jahani-Asl et al. 2011). DRP-1 is upregulated in disease processes and inhibition by siRNA preserves mitochondrial function and attenuates cell death in a hippocampal cell model of excitotoxicity (Zhang et al. 2014). Moreover, DRP-1 levels are decreased or cleared by induction of autophagy in primary rat striatal neurons (Purnell and Fox 2013). Very few studies have investigated the role of mitochondrial dynamics in a sex-specific manner. One study of cultured astrocytes indicates sex differences in mitochondrial dynamics and cell survival in response to estradiol or progesterone treatment. Specifically, treatment with progesterone or estradiol increases cell number, fusion and fission protein expression in

female astrocytes and decreases cell number by inducing apoptotic cell death in male astrocytes (Andrabi et al. 2008). Expression of apoptotic proteins BCL-2 and BAX are upregulated after progesterone treatment in female astrocytes but decreased in male astrocytes indicating opposing effects of progesterone on critical apoptosis mediating proteins (Arnold et al. 2008). The detrimental effect of progesterone on male derived astrocytes may be a contributing factor to the recent discontinuation of the progesterone phase III clinical trial for treatment of TBI based on lack of evidence for neuroprotection in 875 enrolled participants (www.ninds.nih.gov/research/tbi). Indeed, at least one preclinical TBI study reported a reduction of anti-apoptotic proteins (BCL-2, AKT) and the astrocyte marker GFAP in male rats administered progesterone (Djebaili et al. 2005). Given the putative clinical implications of furthering our understanding of sex-specific regulation and functional consequences of mitochondrial biogenesis and dynamics, enhancing future research efforts in this area is warranted.

Cell Death Pathways

We now know cell death is not simply apoptotic or necrotic as once proposed, but rather a continuum encompassing apoptosis, necrosis, programmed necrosis, necroptosis and parthanatos. Each of these modes of cell death has unique defining features but one thing in common, mitochondrial involvement (reviewed in (Fatokun et al. 2014)). Prevention of cell death and resulting cognitive behavioral deficits is the essence of neuroprotection. The general dogma is that males are more susceptible to CNS injury primarily due to the lack of the inherently neuroprotective effects of female hormones which can converge on mitochondria (reviewed in (Nilsen and Brinton 2004; Simpkins et al. 2005; Simpkins and Dykens 2008; Arnold and Beyer 2009)). While hormones clearly play a role in sex differences, other intrinsic hormone-independent cell signaling mechanisms are likely involved since sex differences can be modeled in cell culture absent of circulating hormones (Du et al. 2004; Li et al. 2005) and are present during embryonic development prior to hormone involvement (Dewing et al. 2003).

Sex differences in cell death pathways is one of the most heavily studied areas of sexually dimorphic neurotrauma and neuroprotection. Since TBI research rarely includes females, the majority of our knowledge comes from animal stroke models. Primary cultured neurons also show sex differences in cell death pathways (Du et al. 2004; Li et al. 2005). It has been proposed that male cell death is a product of caspase-independent cell death while female cell death is caspase dependent with bioenergetic failure (decreased ATP) and neuronal NOS induction being the common preceding features observed in both sexes (reviewed in (Turtzo and McCullough 2010; Hill and Fitch 2012; Zuo et al. 2013)). Briefly, caspase independent cell death is mediated by PARP-1 activation and mitochondrial to nuclear translocation apoptosis inducing factor (AIF) while caspase-dependent cell death (reviewed in (Hyman and Yuan 2012)) is mediated by cytochrome c mitochondrial to cytosol translocation, apoptosome formation, caspase cleavage and activation of complement. Both pathways result in DNA fragmentation and cell death. In the caspase-independent pathway mitochondrial to nuclear translocation of AIF occurs in combination with endonuclease G (EndoG) mediated DNA fragmentation as shown by AIF/Endo G nuclear colocalization following transient focal ischemia in mice (Lee et al. 2005). In caspase dependent cell death

DNA fragmentation is initiated by caspase-3 cleavage and release of caspase activated DNase (CAD) from inhibitor of caspase activated DNase (ICAD) (Enari et al. 1998). The different endonucleases contributing to DNA fragmentation downstream of cell death signaling pathways offer unique neuroprotective targets for sex specific treatments. However, sex dependent specificity of endonucleases needs to be confirmed.

It is important to appreciate that these pathways are not exclusive to either sex but overlap considerably (Fig. 2). For example, cytochrome c release and active caspase 3 are observed in males as well as females following injury (Clark et al. 2000; Djebaili et al. 2005). The sex 'specific' distinction comes primarily from neuroprotection studies demonstrating that caspase inhibitors are solely neuroprotective in females, while having little to no effect in males. Females also have an endogenous caspase inhibitor, X-linked inhibitor of apoptosis (XIAP) expressed at higher levels than males independent of estradiol or overiectomy in adult mice. However, protein levels in cytosolic and mitochondrial fractions are lower in females *vs.* males. This may be due to miR-23a regulation of XIAP mRNA (Siegel et al. 2011). Similarly, mRNA expression of Smac/DIABLO, an endogenous inhibitor of XIAP, is higher in females *vs.* males but protein levels are lower. Following stroke Smac/DIABLO mRNA and protein is decreased more in females than males. This suggests females have a genetic advantage in inhibition of caspase dependent cell death signaling. Accordingly inhibition of XIAP with embelin in adult male and female mice after stroke exacerbates lesion volume in females but has no effect in males (Siegel et al. 2011). Furthermore, treatment of female rat pups with embelin following rat cerebral neonatal hypoxic ischemia injury exacerbates anatomical damage and behavioral deficits *vs.* vehicle treated controls (Hill et al. 2011). These results imply that under conditions eliciting the same degree of caspase activation in both sexes, females may be more resilient to caspase dependent cell death *vs.* males.

Female resilience to injury may also be afforded by PARP-1. Genetic studies have demonstrated that, following experimental stroke, male PARP-1 homozygous knockout mice have a substantially diminished infarct size while female PARP-1 knockout mice have increased lesion volume (McCullough et al. 2005; Yuan et al. 2009). Instead of sex-specificity, it is helpful to think of a cell death continuum where each sex has a predominant or 'preferred' cell death pathway but there is still overlap between male and female cell death signaling (Fig. 2). Intriguingly, following TBI in human infants and children, poly(ADP-ribose) (PAR), the product of PARP-1, is detected at higher levels in the CSF of males *vs.* females and positively correlated with age (Fink et al. 2008). Excitotoxicity, oxidative/nitrositive stress, autophagy and cell death all converge on PARP-1 (Huang and Shen 2009; Fatokun et al. 2014). PARP-1 is activated by ROS/RNS mediated DNA damage and consumes nicotinamide adenine dinucleotide (NAD+) to form cytotoxic PAR. Cell death studies evaluating the involvement of PAR have coined the term *parthanatos*, PAR for poly(ADP-ribose) and the Greek word for death, *thanatos* (for review see (Andrabi et al. 2008; Fatokun et al. 2014)). Accumulation of PAR polymers serves as a cell death signal by stimulating mitochondria to release AIF, (Wang et al. 2009, 2011) but not cytochrome c, possibly via the mPTP (Baek et al. 2013). PAR can be catabolized by PARG into ADPr, suppressing cytotoxic levels of PAR, however, ADPr can activate the aforementioned TRPM2 calcium channel in males. TBI studies report mixed results on behavioral

improvement with PARP-1 inhibition. For instance, Clark et al. (2007) reports improvement on the spatial learning task, the Morris water maze (MWM) but no histopathological improvement following CCI in male mice with PARP-1 inhibitor INO-1001 while Stoica et al. (2014) report inhibition of microglial activation and limited neuronal preservation without MWM improvement after PARP-1 inhibitor, PJ34, treatment. PARP-1 inhibition presumably inhibits NAD+depletion following injury.

NAD+depletion is necessary and sufficient for neuronal cell death (parthanatos) (Alano et al. 2010) and contributes to astrocyte cell death (Alano and Ying 2004). NAD+metabolism is altered during the pathophysiology of acute brain injury (reviewed in (Owens et al. 2013)). Male mice have twice as much baseline NAD+ *vs.* females. Following middle cerebral artery occlusion, NAD+depletion occurs in males and overiectomized females but not in intact females (Siegel and McCullough 2013). In PARP-1 knockout mice, stroke induced NAD+depletion is prevented in males but aggravated in females. Treatment with nicotinamide, an NAD+precursor, reduces infarct volume in both sexes of PARP-1 knockout mice but has no effect in wild type females. So why do males have twice as much NAD+compared to females? Since males have higher baseline ROS/RNS production and lower antioxidant defense systems compared to females, excess NAD+may be necessary for PARP-1 to routinely repair oxidatively damaged DNA.

Recent studies suggest a mitochondrial localization of PARP-1 and that NAD+consumption specifically by intramitochondrial PARP-1 contributes to oxidative stress induced cell death (Du et al. 2003). PAR polymerized proteins are detected in mitochondrial subcellular fractions from liver (Masmoudi and Mandel 1987) and brain (Masmoudi et al. 1988; Du et al. 2003). PAR coimmunoprecipitates with AIF in HeLa cells and increases following NMDA exposure (Wang et al. 2011). Whether the intramitochondrial PAR observed is a product of nuclear or mitochondrial localized PARP-1 and whether cytosolic or mitochondrial NAD+depletion is the cause of cell death is controversial. Pankotai et al. (2009) observed that a component of α-KGDH complex (DLDH) also consumes NAD+and addition of DLDH to isolated mitochondria results in PAR formation demonstrating PARP-like activity of DLDH. Thus, intramitochondrial PARP-like proteins may contribute to the observed PAR formation within mitochondria. On the other hand, PARP-1 itself coimmunoprecipitates with inner mitochondrial transmembrane protein, mitofilin, suggesting intramitochondrial localization. Moreover, PARP-1 coimmunoprecipitates with mitochondrial DNA and α-DNA ligase III in HeLa cells (Rossi et al. 2009). These results suggest that if PARP-1 is indeed present in the mitochondrial matrix, it may be involved in mitochondrial DNA repair. As such, it is tempting to speculate that increased male production of ROS/RNS and consequential DNA damage induces PARP-1 activity more in males *vs.* females, thus necessitating higher basal NAD +levels for homeostatic maintenance.

Summary and future directions

There exists substantial evidence for fundamental sex differences in brain metabolism. A model adapted from the collective evidence presented in this manuscript portraying sex differences in pathophysiological cell signaling is depicted in Fig. 3 and highlights of each

section are summarized in Table 1. Thus far these differences have been largely ignored and necessitate full consideration when designing future experiments. Appropriate inclusion of both sexes in future studies will help fill significant knowledge gaps. Some unanswered questions for future experimental evaluation include the role of mitochondrial quality control, the mPTP, and AIF and caspase dependent endonucleases in sexually dimorphic cell death signaling. Evaluation of putative sex specific brain regional vulnerability following TBI may also provide insight into the cognitive and behavioral consequences of CNS injury observed in humans. Furthermore, segregating cell culture studies by genotype and including a female sex comparison in CNS injury studies will evoke generalizability of experimental findings and appropriate translational relevance. While it may be unrealistic that all hormonal states (e.g. estrus *vs.* proestrus) are accounted for in every experiment, there are experimental strategies that can be followed to distinguish between hormonal, environmental and chromosomal influences on sex differences (McCarthy et al. 2012). Future appreciation of sexually dimorphic mitochondrial metabolism, cell signaling and pathophysiology will ultimately aid in the generation of novel efficacious neuroprotective treatment strategies for both men and women suffering from CNS injury.

Acknowledgments

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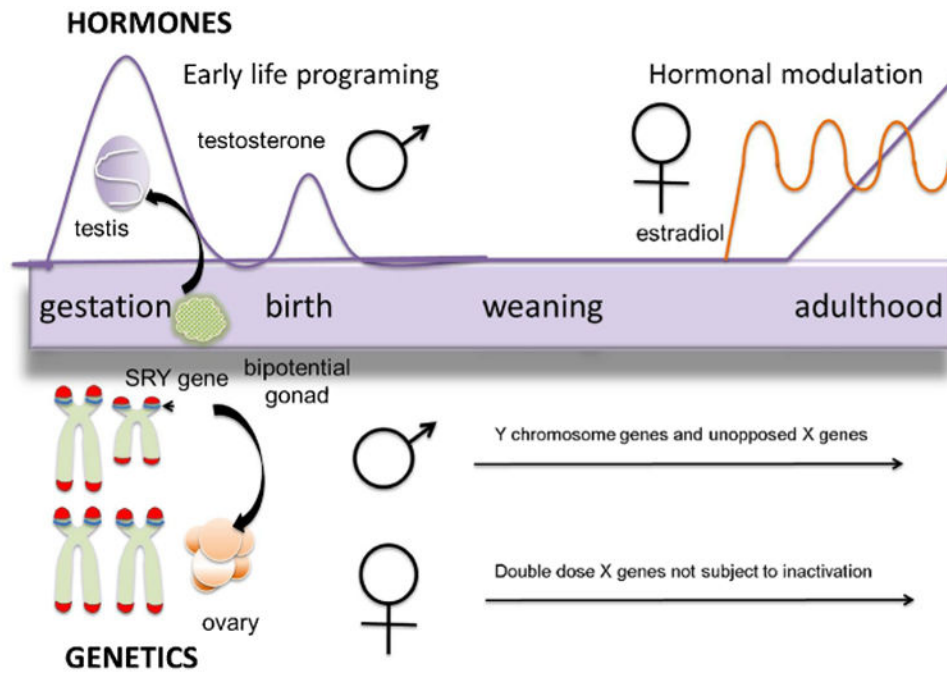
Fig. 1.

Sources of Sex Differences in the Brain. The bipotential gonad is directed towards testis development by the SRY gene on the Y chromosome. Testosterone production begins during fetal life and surges again at birth and then remains low until puberty. Steroids impact numerous endpoints in the developing brain, many of which will endure into adulthood. In females the ovary develops due to the lack of SRY and remains quiescent until puberty at which time estradiol production is cyclical. Genes on the X and Y chromosome are capable of influencing brain in behavior throughout life in manners still not well understood
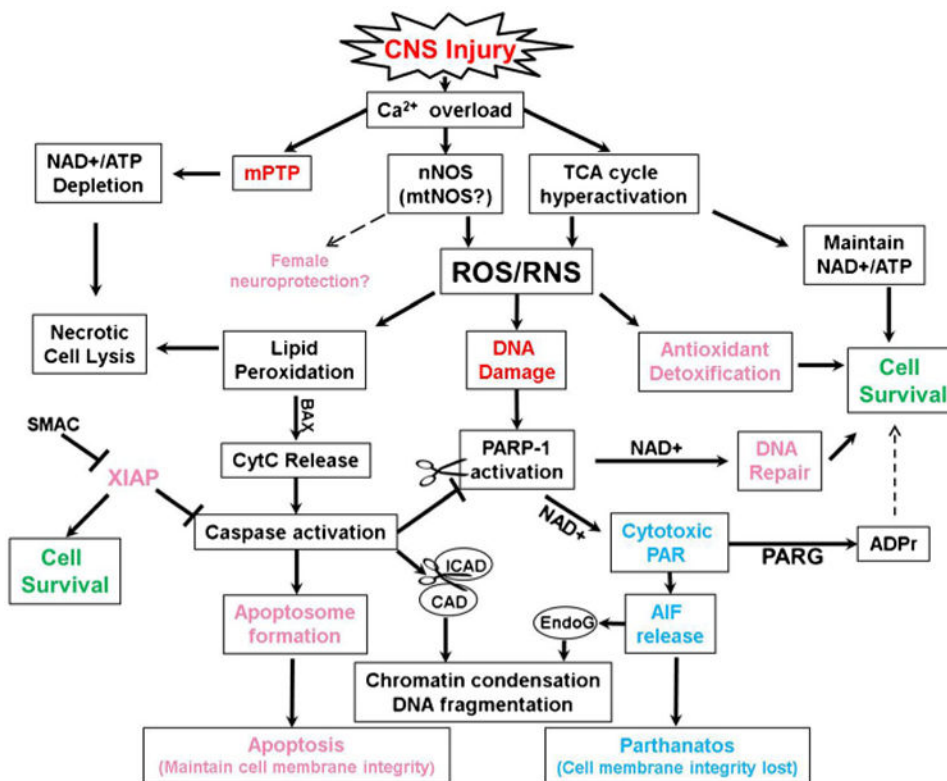
Fig. 2.

Sex Dependent Cell Death Proclivity and Interactions. Male predominant pathways are in *blue*, females in *pink*, and interacting pathways in *black*. Abbreviations: mitochondrial permeability transition pore (mPTP); neuronal/mitochondrial nitric oxide synthase (nNOS/mtNOS); tricarboxylic acid cycle (TCA cycle); nicotinamide adenine dinucleotide (NAD+); Reactive oxygen species (ROS); reactive nitrogen species (RNS); adenosine triphosphate (ATP);cytochrome c (CytC); poly(ADP-ribose) polymerase 1 (PARP-1); poly(ADP-ribose) (PAR); poly(ADP-ribose) glycohydrolase (PARG); ADP-ribose (ADPr); apoptosis inducing factor (AIF); X-linking inhibition of apoptosis (XIAP); Second mitochondria-derived activator of caspases (SMAC). Inhibitor of caspase activated DNase (ICAD); Caspase activated DNase (CAD); Endonuclease G (EndoG)
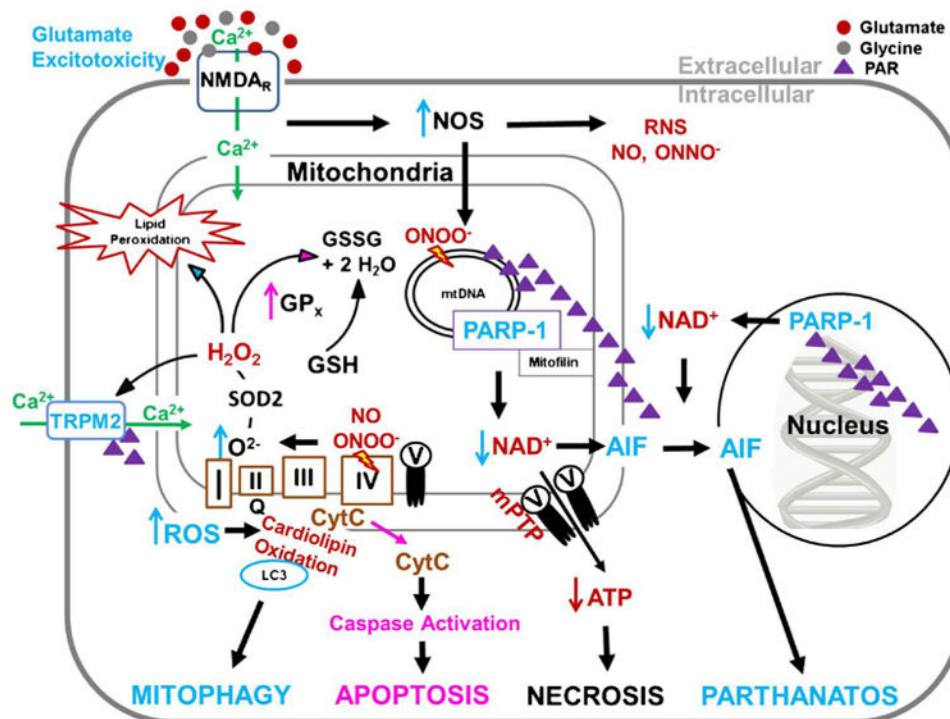
Fig. 3.
Summary of Sexually Dimorphic Cell signaling following CNS Injury. *Male predominant pathways are represented in blue and female predominant pathways in pink*. Following CNS injury, extracellular glutamate activates extrasynaptic $NMDA_R$s triggering Ca^{2+} influx. Intracellular Ca^{2+} activates NOS, to a higher degree in male cells. Mitochondrial Ca^{2+} uptake increases oxidative phosphorylation, ROS generation, subsequent DNA damage and mitochondrial swelling. ROS mediated damage is greater in male cells likely due to poorer antioxidant defense systems *vs.* females, (i.e. glutathioned peroxidase; GPx), which activates PARP-1 consumption of NAD+to generate PAR and ADPr. In surviving cells, ROS oxidation of cardiolipin, likely more common in males, causes a conformational flip to the outer membrane where oxidized cardiolipin binds LC3 to promote mitophagy, removing damaged organelles. In male cells, the combination of peroxide and ADPr activates TRPM2 cation channels further exacerbating Ca^{2+} influx and oxidative damage. In severe injury, Ca^{2+} overload can lead to activation of the mitochondrial permeability transition pore (mPTP), acute loss of ATP and necrosis or mitochondrial release of cytochrome c and AIF. Cytochrome c release results in caspase-dependent apoptosis in female cells while the combination of NAD depletion and AIF release leads to caspase-independent parthanatos in male cells

Table 1

Highlights: Sex differences in mitochondrial (dys)function

Excitotoxicity and Calcium

- *In vitro* and *in vivo* CNS injury models demonstrate male susceptibility to glutamate excitotoxicity compared to females.

- Following injury, intracellular calcium and mitochondrial calcium uptake is greater in males than females.

Oxidative/Nitrositive Stress

- In isolated brain mitochondria and following *in vivo* stroke models, males produce more ROS/RNS than females.

- The female brain is equipped with higher levels of endogenous antioxidant defense systems than the male brain.

Autophagy/Mitophagy

- Under metabolic stress, female cells including neurons predominantly use fats while males utilize proteins as biofuels.

- Activation of autophagy occurs in a sex dependent manner following *in vitro* and *in vivo* CNS injury models.

Mitochondrial Quality Control

- Mitochondrial biogenesis is activated in response to *in vivo* and *in vitro* CNS injury and may be sexually dimorphic.

- Following CNS injury, mitochondrial dynamics favor fission over fusion and may differ between males and females.

Cell Death Pathways

- Male and female brain cell death proclivity is caspase independent and caspase dependent, respectively.

- Following CNS injury, nNOS and PARP-1 are detrimental in male brain but paradoxically neuroprotective in female brain.