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The Molecular Mechanism of Migraine

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ABSTRACT

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Migraine is a common, episodic neurological disorder that includes headache, nausea and hypersensitivity to sensory stimuli. During the headache phase of migraine, migraine patients can be especially hypersensitive to thermal stimuli. The unpredictable and episodic nature of migraine makes it difficult to treat and much of the mechanism of migraine has yet to be elucidated. A T44A substitution in casein kinase 1δ is inherited with migraine with aura. A transgenic mouse model suggests that animals with this mutation exhibit increased sensitivity to thermal stimuli after injection with nitroglycerin (NTG). We performed behavior assays that measure animal responses to thermal stimuli, after injection with NTG, a known migraine-inducer in human migraine patients. Female animals with the CK1δ-T44A mutation are more sensitive than wildtype littermates, suggesting a sex difference emerges in pain sensitivity in animals that express the CK1δ-T44A but not in wildtype siblings. Female CK1δ-T44A animals are more sensitive to the effects of NTG on pain than male CK1δ-T44A mice. This indicates a potential sex hormone related pain response. Since estrogen is implicated in both migraine and pain response, we test the thermal sensitivity of heterozygous $ER\beta^{KO}/+$ and CK1δ-T44A; $ER\beta^{KO}/+$ mice compared to wildtype and CK1δ-T44A mice. Overall thermal sensitivity is decreased before stress of injection in both male and female $ER\beta^{KO}/+$ and CK1δ-T44A: $ER\beta^{KO}/+$ mice. This demonstrates that ERβ is necessary for thermal nociception in untreated mice. However, after injection with saline or NTG, animals of all genotypes responded to thermal stimuli similarly. This suggests that estrogen signaling through ERβ is likely not part of the pathway of NTG-induced thermal sensitivity or that one copy of ERβ is sufficient for NTG-induced thermal sensitivity. Since ERβ is fully functional in CK1δ-T44A mice and CK1δ-T44A mice have wildtype thermal sensitivity at baseline, we can conclude that CK1δ-T44A does not modulate ERβ to affect thermal sensitivity in untreated animals.

Keywords: [migraine, casein kinase 1δ, estrogen, estrogen receptor β, pain, sex difference]

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LIST OF ABBREVIATIONS

5 HT	5-hydroxytryptamine (serotonin) receptors
AEG-1	Astrocyte elevated gene 1
ATP1A2	Sodium/potassium ATPase
CACNA1A	Calcium channel
CDS	Cortical spreading depression
CGRP	Calcitonin gene-related peptide
CK1δ	Casein kinase 1δ
CK1δ-T44A	Casein kinase 1δ T44A substitution
CK1δ-T44A; $ER\beta^{ko}/+$	Casein kinase 1δ/ heterozygous estrogen receptor β knockout
Cx43	Connexin-43
E2	Estrogen/estradiol
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
$ER\beta^{ko}/+$	Heterozygous estrogen receptor β knockout
FASPS	Familial advanced phase syndrome
FHM	Familial hemiplegic migraine
fMRI	Functional magnetic resonance imaging
GnRH	gonadotrophin-releasing hormone
IP	Intraperitoneally
LEP	Laser-evoked potentials
LH	Lutenizing hormone
NMDA	N-methyl D-aspartate receptor
NO	Nitric oxide
NR	Nuclear receptor
NTG	Nitroglycerin
MA	Migraine with aura
PCR	Polymerase chain reaction
PGCP	Plasma glutamate carboxypeptidase
POA	Pre-optic area of hypothalamus
SCN1A	Sodium channel
SLC1A2	Solute carrier family 1 member 2
SK	Small conductance calcium-activated potassium channels
TPH-1	Tryptophan hydroxylase
TRESK	TWIK-related spinal cord potassium channel
USP	United States Pharmacopeia

CHAPTER 1: INTRODUCTION

General Information

Migraine is a common neurological disorder that can be completely disabling. Migraines are characterized by recurrent episodes that may include headache localized to one cerebral hemisphere, moderate to severe pain intensity, pulsing, nausea, and sensitivity to light, sound, touch, heat or smell[3].

Another symptom of migraine is aura, a disturbance characterized by a scatoma that moves across the visual field. In addition to the visual disturbance, aura can also affect speech and motor function[3a, 4]. This type of migraine is called migraine with aura (MA). A subtype of MA is familial hemiplegic migraine (FHM), which is dominantly inherited and paralysis of half of the body as the aura occurs. Other variants of MA include migraine with prolonged aura and migrainous stroke[3a]. Migraine is also associated with several other disorders including hypomania, depression, anxiety, phobia, panic disorder, stroke, familial dyslipoproteinenmias, essential tremor, paroxysmal dyskinesias, and epilepsy[3a].

Another characteristic of migraine is that it is episodic and therefore difficult to accurately predict. However, some migraine patients can identify an environmental trigger. These include bright lights, caffeine, alcohol, strong odors, cheese, chocolate, stress, and changes in sleeping patterns or hormone levels[5]. While these triggers increase risk to a pre-disposed person, they do not always elicit a migraine response, are not universal, and can change throughout the course of a lifetime within the same person.

Models of Migraine

The unpredictability of migraine makes it difficult to study. To overcome this obstacle, chemicals that elicit some of the traits of migraine have been used to create models to study migraine in both animals and human migraine patients. These models include nitroglycerin-induced hypersensitivity, cortical spreading depression, and meningeal inflammation. For human patients, some migraine attacks have been recorded using functional magnetic resonance imaging.

Nitroglycerin

The hypertension and angina medication nitroglycerin (NTG) can induce migraine in patients pre-disposed to migraine[6]. NTG-induced migraine can induce aura in patients with histories of aura, and it is likely the NTG headache is the result of both vasodilatation in cranial blood vessels and stimulation of dural afferents[6b,7]. NTG can also induce hypersensitivity to sensory stimuli including touch and heat in migraine patients[8]. Thus, NTG provides a way to induce a migraine-like attack in pre-disposed persons in a controlled manner, which is useful for studying migraine.

NTG has also been used as a model to trigger migraine in animal studies. In rats, NTG, a NO donor, has been shown to increase spinal responses in afferent neurons, sensitize the trigeminal afferents that innervate the dura, and increase response to pain[9]. NTG also induces FOS expression in the trigeminal nucleus caudalis[10], which is suppressed by the anti-migraine drug Eletripan[11]. In mice, NTG induced a dose-dependent and prolonged allodynic response to both thermal and mechanical stimuli. It also increased FOS expression in the trigeminal nucleus caudalis and cervical spinal cord dorsal horn[6b]. A recent study showed that NTG induces thermal

hypersensitivity in mice at both 5 and 10mg/kg doses, which developed within 30 minutes of injection, peaked at 60 minutes and abated after 4 hours[6b]. NTG also induced mechanical hypersensitivity with the von Frey mechanical-sensitivity assay at 10mg/kg 60 minutes after injection, which subsided after 4 hours[6b]. The anti-migraine drug Sumatriptan provided relief of induced allyodynia in both cases. Another study showed pain behavior in rats after NTG injection. A significant reduction in the latency of the tail-flick assay and increase in formalin-induced pain-related behavior at 2 and 4 hours after NTG administration was observed[9e]. NTG was not shown however, to lower the threshold for cortical spreading depression (CSD), a measure of cortical excitability, suggesting that NTG acts downstream or acts independently of CSD[6b]. The pain-induced behaviors exhibited by both rats and mice in previous studies show that NTG provides a useful method to model migraine in animals.

Cortical Spreading Depression

Cortical Spreading Depression (CSD) is a wave of spreading depolarization followed by a wave of inactivity in the cortex that can be caused by chemical, physical, or electrical disturbances[12]. In response to a stimulus, a self-propagating wave of neuron depolarization spreads across the cortex[12a]. This spreading depression of neural activity parallels the path of aura[12b]. These changes in neuronal impulses measured in the cortex of animals maybe the equivalent to the human aura[13]. Arterial dilation precedes the CSD wavefront. Mice expressing alleles associated with familial hemiplegic migraine type 1 show a reduced threshold for induction of CSD[14] as well as faster propagation of CSD[15]. The threshold stimuli required to induce CSD, the frequency of occurrence, and the rate of neuronal recovery are parameters of

CSD that can be compared between animal models of migraine. Medications that have been effective at preventing migraine affect threshold and frequency of CSD induction[14-16].

Meningeal Inflammation

Meningeal neurogenic inflammation results from the activation of the trigeminal neurons that are also activated during migraine[17]. Chemical stimulation via inflammatory factors, low-osmotic buffer, or ionic salts causes sensitization of the dural afferents in the trigeminal ganglion[12b]. This sensitization can be exacerbated by mechanical stimuli. The release of neuropeptides from the dural afferents results in plasma protein extravasations, vasodilatation, degranulation of mast cells and subsequent release of serotonin, histamine, and cytokines[12b, 17]. These substances are pro-inflammatory and produce dural sensitization, causing normally painless motions such as sudden head moving, coughing, or breath-holding to significantly worsen already existing head pain[12b]. One study showed that chemical stimulation of rat dural receptive fields directly excited the neurons and increased the mechanical sensitivity of the rat to previously innocuous stimuli[12b]. The chemical sensitivity and sensitization characteristics of afferents may contribute to intracranial mechanical hypersensitivity, which is common in certain forms of headache and could contribute to the throbbing pain of migraine[12b].

Functional Magnetic Resonance Imaging

Functional magnetic resonance imaging (fMRI) allows the visualization of the soft tissues and the flow of blood in the brain[18]. When a neuron is activated it requires more oxygen and blood flow to the region of activity. This increase in blood flow occurs 1-5 seconds after

neuron excitability and also alters local blood volume levels[18a, b]. Both blood flow changes and blood volume changes can be measured with fMRI. Arterial dilation precedes the CSD wavefront but abates during the spreading depolarization stage. One study showed cerebral blood flow and volume decreased during migraine aura and move at the same rate as CSD[19]. These decreases also abated after the aura was finished, indicating that non-severe ischemia was spreading over the cerebral cortex[19].

Genetic Factors

Many genetic factors contribute to the migraine phenotype. One type of inherited migraine is familial hemiplegic migraine (FHM), a rare form of migraine affecting about 1% of migraine patients. FHM can be caused by mutations in the CACNA1A (calcium channel), ATP1A2 (sodium/potassium ATPase), and SCN1A (sodium channel) ion channels[20]. Other single nucleotide polymorphisms, rs1835740 and KCNK18, have been linked to the more common migraine with aura subtype but are only correlative and do not have a definitive causative connection or molecular mechanism established.

Familial Hemiplegic Migraine (FHM)

Mutations in CACNA1A (calcium channel), ATP1A2 (sodium/potassium ATPase), and SCN1A (sodium channel) have been found to be causative for FHM[20]. CACNA1A is a voltage-gated calcium channel[23, 15, 21]. Calcium channels are composed of multiple-subunits: $\alpha_{1,}$ β, $\alpha_2\delta$[22]. Subunit α_1 is a transmembrane protein, which acts as a voltage sensor and forms the ion-conducting pore in the membrane[23]. FHM is caused by missense mutations in the α_{1a} subunit of

the calcium channel gene (CACNA1A)[23, 15, 21]. In cultured neurons, the mutations in CACNA1A cause a negative shift in voltage dependence and delay in channel inactivation [15]. This causes the channels to respond to smaller depolarizations and stay open longer, enabling more calcium into presynaptic terminals[15, 24]. The increase in calcium leads to increased excitatory neurotransmission[15] as shown in one study with mice having the CACNA1A mutation[24]. Studies have shown that mice with CACNA1A mutations exhibit a phenotype of increased susceptibility, frequency, and propagation speed of CSD, a physiological correlate of aura[15-16]. Female mutant mice have a greater propensity than male mice to CSD[14.] One study showed this susceptibility diminished after ovariectomy, but could be partially restored with estrogen supplements[15-16]. Electrical stimulation thresholds for wildtype and mutant female mice are 50% less than that of males[16]. Eikermann-Haerter also showed that CSD propensity could be increased in mutant males via orchiectomy, indicating a possible role for androgens in the suppression of migraine[15].

Missense mutations in the ATP1A2 gene, which encodes the NA,K-ATPase α2 subunit, have also been identified in FHM . These mutation result in reduced activity[25] or decreased affinity for K^{+}[26] of the Na^{+} /K^{+} pump and impair uptake of K^{+} from the extracellular space[27]. One specific missense mutation in the NA,K-ATPase channel is the T345A substitution, which was identified in a Finnish family with FHM[26]. In rats, this mutation did not alter cell growth or catalytic turnover but it did cause a significant decrease in apparent K^{+} affinity. This affinity is the basis for a reduced rate of K^{+} uptake, which delays the recovery phase of nerve impulses important in FHM[26].

Another FHM-linked mutation was discovered when sequencing chromosome 2q24. The sequencing revealed a heterozygous missense mutation in the neuronal voltage-gated Na^{+} channel gene SCN1A. The GLN1489Lys mutation was present in three different families with

FHM and resulted in a charge-altering amino acid exchange in the hinged-lid domain[28]. This affects the fast inactivation of the channel which is located in cortical neurons[29] and is essential for the generation and propagation of action potentials in the brain. Whole cell recordings of Na^+ currents in cells expressing the highly homologous SCN5A sodium channel showed the mutation induced a 2-4 fold accelerated recovery from fast inactivation. This means it allows higher firing rates and thus, enhance excitability in the neuronal network, which contributes to migraine[28].

Migraine Genes

A genome-wide association study of migraine found that in 95 % of 2731 migraine cases a single nucleotide polymorphism in the minor allele of rs1835740 on chromosome 8q22.1 was associated with migraine[30]. This single nucleotide polymorphism is located between the genes encoding astrocyte elevated gene 1 (AEG-1) and plasma glutamate carboxypeptidase (PGCP), indicating a connection to glutamate signaling. This connecting region could regulate one or both of these genes. AEG-1 downregulates SLCA2, a gene that encodes the major glutamate transporter in the brain, while increased glutamate in the synaptic cleft or downregulation of SLC1A2 increases PGCP activity. Since glutamate is a major neurotransmitter in the brain, this increased amount of glutamate could lead to neuronal hyperexcitability, and is likely to increase the occurrence of migraine because of enhanced sensitivity to CSD and central sensitization[30].

In a family with dominantly-inherited migraine with aura, a frame shift mutation in the gene, KCNK18, was found to segregate with the migraine trait. KCNK18 encodes TRESK, a TWIK-related spinal cord potassium channel previously implicated in pain pathways and anesthesia[31]. TRESK is expressed in the trigeminal ganglion where neurons are activated during

migraine. The mutation results in a complete loss of function of the ion channel, which also dominantly-negatively suppresses wild-type channel function, leading to migraine pathology[31]. However, studies have only shown these two genes to be correlative with migraine and have yet to establish causative evidence or possible mechanism for migraine incident.

Casein kinase 1δ (CK1δ)

Casein kinase 1δ (CK1δ) is the first gene to have been shown to have a causative link to migraine with aura. A recent study identified a novel mutation in a family with dominantly-inherited migraine with aura. The family was also diagnosed with familial advanced phase syndrome (FASPS), a circadian disorder[32]. Upon being screened for genes linked to this disorder, a mutation in CK1δ was identified in those family members affected with migraine with aura. A T44A substitution in the protein encoded by this gene segregates with a circadian phenotype and migraine with aura[20]. CK1δ phosphorylates Per2, another constituent of the circadian clock whose phosphorylated state modulates the rate of its degradation[33]. The T44A substitution is not in the active site or the activation domain of CK1δ, so it is likely the mutations affects the protein's interactions with other proteins[32]. The mutant kinase showed reduced function *in vitro* for phosphorylation of Per1, Per2, Per3 and α-casein. Mouse and fly models expressing the mutant allele exhibit abnormal circadian phenotypes[32]. Transgenic mice with this mutation exhibited behaviors characteristic of migraine with aura. Thresholds to induce aura can be measured through CSD and vascular changes. Sensitivity to mechanical and thermal stimuli is also another method of identifying migraine in mouse models. Together, pain behavior assays and changes in CSD thresholds with associated vascular dynamics demonstrated that CK1δ is causative for migraine with aura[20]. The target of CK1δ that is causative for migraine is unknown

since it is a ubiquitous and promiscuous kinase. CK1δ phosphorylates many targets that could be involved in migraine such as connexins that form gap junctions, NMDA receptors, glutamate signaling proteins, and estrogen receptors[20].

CK1δ Regulates Gap Junctions

In the brain, the wave of CDS that mirrors aura during migraine propagates via the gap junctions that connect cells. Gap junctions are membrane channels that allow direct communication through electrical, biochemical, or metabolic interaction between adjacent eukaryotic cells[61, 62, 63, 34, 65, 35]. Gap junctions are channels made up of two hemi-channels, each composed of six connexin proteins[65,35b]. These channels allow the diffusion of nutrients, wastes, metabolites, second messengers, and ions between cells[65,35b]. CK1δ regulates formation of gap junctions. Phosphorylation of connexin-43 (Cx43) is necessary for gap junction assembly and CK1δ phosphorylates Cx43 serines 325, 328, or 330[36]. A general CK1 inhibitor decreased phosphorylation of Cx43 at these sites[36]. Upon the addition of a CK1δ-specific inhibitor, a decrease in both gap junction formation and Cx43 phosphorylation occurred in cells. CK1δ was inhibited and connexin-43 was localized at plasma membranes and not cell junctions[36]. The mechanism of this interaction has not yet been completely determined, but these data indicate that CK1δ is responsible for phosphorylation of connexin-43 and subsequent gap junction formation[36], which are important in the progress of migraine.

CK1 Regulates Synaptic Transmission

Migraines propagate through the brain via excitation of neurons, or fast synaptic transmission, which occur when neurons are stimulated by neurotransmitters such as glutamate. CK1 has been shown to regulate fast synaptic transmission. The glutamate receptor mGluR1 uses CK1 to inhibit NMDA-mediated synaptic currents. A recent study showed that CK1 decreased NMDA receptor activity in the striatum by increasing phosphatase 1 and/or 2A activity, which subsequently dephosphorylated NMDA receptors. This is the first evidence of CK1 regulation of synaptic regulation in the brain[37] and is potentially important in migraine since aura is thought to mirror the neuronal depolarization wave of CSD. Migraine patients experiencing aura have delayed synaptic recovery, which could potentially be related to CK1-regulated NMDA inhibition.

Estrogen

CK1δ has been shown to phosphorylate estrogen receptor α (ERα) directly and via the AIB1 protein[38], indicating a link between CK1δ and estrogen receptors.

Estrogen is part of a group of steroid compounds that diffuse readily across the cell membrane into the cytoplasm. Estrogen has many functions in the central nervous system and many of these are modulated by intracellular receptor/transcription factors that interact

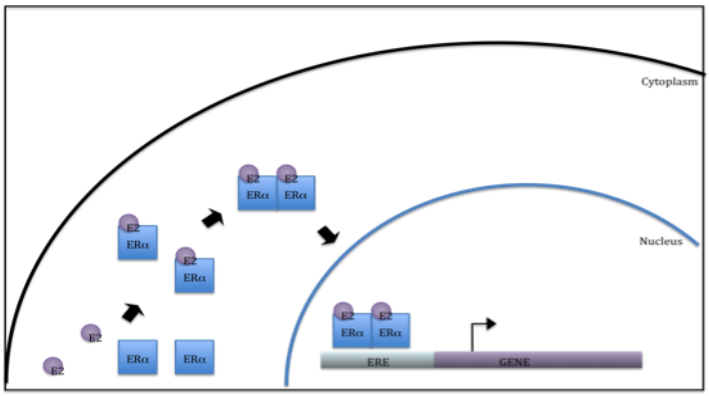

Figure 1: Free estrogen binds to estrogen receptors (α), which then dimerize and translocate to the nucleus to regulate gene expression.

with steroid response elements on specific genes[39]. As shown in Figure 1, estrogen binds an estrogen receptor (ER), where it translocates to the nucleus. Activated ERs dimerize and bind to DNA to regulate gene expression. ERs are part of the nuclear receptor (NR) superfamily and are transcriptional factors that recruit transcriptional machinery to upregulate gene expression[12]. There are at least two types of ERs, alpha (α) and beta (β). Though homology and mode of action are similar, ERα and ERβ are expressed in a variety of different cells. ERα is expressed predominately in endometrial, ovarian stroma, hypothalamus and breast cancer cells. And ERβ is located in kidney, bone, heart, lungs, intestinal mucosa, endothelial and most relevantly, brain cells[49, 40]. Estrogen has many effects on brain function including gap junction formation, serotonin fluctuation, and trigeminal vasodilatation indicating a potential connection with migraine.

Estrogen and Gap Junctions

Estrogen plays a role in gap junction formation. The expression and regulation of connexin proteins, which make up gap junctions, are controlled by a number of factors including, neurotransmitters, growth factors, protein kinases, and steroid hormones, such as estrogen[61]. Cx43 is an important estrogen-regulated gap junction protein and is most prevalent in hypothalamic and uterine tissue[61, 65, 68]. Estrogen and progesterone are released by hypothalamic stimulation and these hormones then act on the hypothalamus to regulate reproductive behavior[61, 41], such as estrus cycling and sexual behavior in rats and mice[61].

High levels of estrogen and progesterone, like those during pregnancy, increase both connexin expression and gap-junction quantity in the hypothalamus in female rats[42]. This increase in the quantity of gap junctions and Cx43, causes an increase in hypothalamic

stimulation[61]. One study showed female rats treated with estrogen, progesterone, or both, increased Cx43 levels in the preoptic area (POA), while estrogen and progesterone decreased Cx43 in the POA of male rats[61]. Estrogen plays important roles in gap junction and Cx43 formation in the brain and this could have implications in migraine due to a correlation of aura with CSD. The delay of CSD recovery in migraine with aura patients might correlate to the number or efficiency of gap junctions and connexins in the brain. Since estrogen regulates gap junctions in the brain, it is likely the occurrence of certain migraines could be due to changes in estrogen signaling.

Gap junction and estrogen are also linked because gap junctions affect regulation of the estrous cycle, uterine stromal cell communication, and neovascualrization[68]. Central control of reproduction occurs in the hypothalamus, so hormonal regulation of hypothalamic gap junctions is important in the regulation of reproduction. Female Cx43 knockout mice have abnormal estrous cycles[61]. Estrogen and progesterone coordinate the cellular differentiation that enables a blastocyte to implant in the uterus[68, 43]. Neovascularization accompanies this differentiation and is the formation of a vascular network in the stromal bed that supports placenta growth and therefore early embryo survival[68, 44]. The communication of gap junctions has a vital role in neovascularization during pregnancy. One study showed the conditional deletion of Cx43 in stromal cells of pregnant mice caused a disruption in the formation of new blood vessels in these cells, which lead to a termination of fetal growth and subsequent miscarriage[68].The absence of Cx43 in mice models and in vitro human cells resulted in aberrant stromal cell differentiation and a decrease in angiogenic factors, including the vascular endothelial growth factor, which causes neovacularization[68]. This indicates an important link between hormone-regulated cell-cell

communication and the maintenance of the vascular network that supports embryonic growth[68]. Estrogen signaling and uterine gap junction regulation with subsequent neovascularization is important in the study of migraine because it suggests a link to aura. Aura, which is thought to propagate through cells via gap junctional connections, is also affected by vascularization.

Estrogen and Serotonin

Estrogen is linked to anxiety and depression through the serotonin pathway and treatments of serotonin decrease anxious behavior[52,45]. Like migraine, more women than men experience these disorders though prevalence increases with menopause[46]. Anxiety and depression are comorbid with migraine[3a] and estrogen contribution to migraine can result from altered serotonin receptor activity[47].

Serotonin receptor agonists are the drugs that are most effective for treating migraines. Triptans, are selective agonists for the serotonin (5-hydroxytryptamine or 5-HT) 1B, 1D and sometimes 1F receptors[48]. Serotonin levels have been shown to change during the estrous cycle[49]. Tryptophan hydroxylase (TPH-1) is the rate-limiting step in serotonin synthesis. A study of proestrus, trigeminal ganglion TPH-1 mRNA levels were two-fold greater and TPH-1 protein levels 1.4 times higher than at diestrus[60]. However, direct treatment with estrogen did not increase TPH-1 protein levels or 5HT-1B or 1D receptor number[60]. Serotonin was present in trigeminal neurons containing calcitonin gene-related peptide (CGRP), a vasoactive neuropeptide indicative of pain; in neurons binding IB4, a marker of nonpeptidergic nociceptors; and in neurons containing 5HT-1B receptors[60].

Serotonin levels change during migraine[50]. Anti-migraine drugs induce the stimulation of serotonin receptors, which inhibit the release of vasoactive neuropeptides like CGRP, while

neuronal calcium channels increase the release of serotonin[51]. Serotonin acts at 5-HT receptors to increase intracellular calcium by freeing calcium stores and activating a calcium influx pathway[52]. For this reason calcium levels increase when triptan drugs are used to alleviate migraine.

Serotonin release, metabolism, reuptake, and synthesis are regulated by estrogen and estrogen receptor β[52]. Studies using the elevated plus maze paradigm showed that diestrous mice were more anxious than their control counterparts[52] and females more anxious than males[53]. However, another study reported that ovariectomized mice receiving estrogen treatment had increased anxiety compared to ovariectomized mice receiving no supplement[54]. Estrogen receptor beta knockout ($ER\beta^{KO}$/ $ER\beta^{KO}$) mice, which lack a functional β estrogen receptor were more anxious than the wildtype mice[52]. Knockout mice had lower levels of serotonin in stria terminalis, preoptic area and hippocampus and dorsal raphe[52]. Low levels of serotonin are associated with anxiety and depression and migraines frequently occur concurrently or after episodes of transient depression, indicating a correlation.

Estrogen and Vasodilatation

Estrogen affects vascular cells and arterial function. The trigeminal neurons innervate the blood vessels in the intracranial meninges and dural venous sinuses[55], and it is thought that changes in blood vessel diameter underlie the pain in migraine headache. The neuropeptide, CGRP is a vasodilator that increases blood levels during migraine[56]. Estrogen rapidly increases the diameter of arteries in ovariectomized female mice by stimulating the production of nitric oxide and activation of ERK/MAP kinase and phosphatidylinositol 3-kinase. Mice that were injected with a nitric oxide inhibitor did not have an increased arterial diameter or increased

activity of either of these kinases. The same result was found when ERα and ERβ knockout mice were exposed to estrogen[57]. Early studies showed stimulation of meningeal and cerebral blood vessels produced a throbbing pain that was absent when parts of the brain not associated with blood vessels were stimulated[58], indicating a mechanism for pain in migraine.

Migraine and Estrogen

Hormonal changes are one of the many triggers of migraines and estrogen in particular has been linked to migraine[3b, 59]. Migraines affect three times more women than men, increase in frequency during puberty and pregnancy, and occur less frequently after menopause[47a, 49].

Estrogen cycles and levels are some of the key differences between men and women and both human migraine patients and animal models have shown sex differences in migraine occurrence and behavior. Pre-pubertal girls and boys have a 4% occurrence of migraines[60]. However, the lifetime prevalence for women is 18% and only 6% for men. For 80% of women, onset of migraine occurs between ages 10-39 (Figure 2) and fluctuates greatly due to reproductive status and hormonal supplements[61].

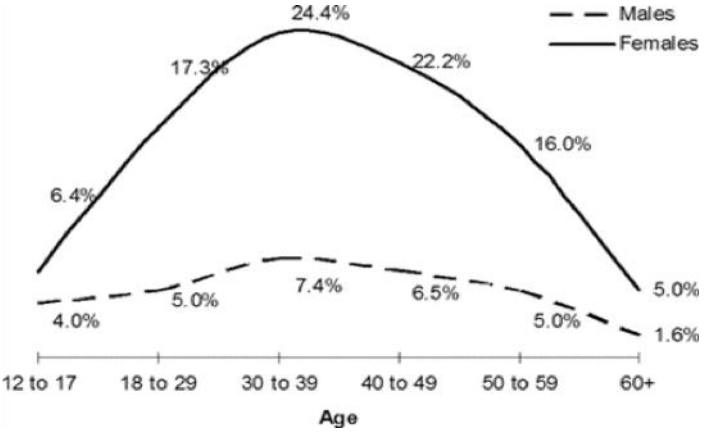

Figure 2: The lifetime prevalence of migraine in women is three times greater than in men [1].

One animal study showed sex differences exist between male and female wild type mice in mechanical stimulation and carrageenan-induced inflammation of hind paw, indications of migraine-like pain. Female mice exhibited a lower paw-withdrawal threshold than male wildtype

mice. In ERα and ERβ knockout mice, there was no significant difference in the male mice paw-withdrawal thresholds, but female knockouts had higher response thresholds, eliminating the sex difference seen previously in wildtype mice. Eliminating ERα or ERβ eliminates sex differences between male and female mice[62], suggesting an important role of estrogen in migraine.

Migraine attacks have also been correlated to changes in estrogen levels and specifically the decline in estradiol level[3b, 47a] that occurs most dramatically at the beginning of the menstrual cycle. This is when the onset of migraines is most frequent in women[47a].

Menstrual Migraines

Migraines associated with estrogen level fluctuations are termed menstrual migraines. Two classifications of menstrual migraines have been defined. A pure menstrual migraine is defined as one that occurs within 2 days of the start of the menstrual cycle and no other time during the cycle[63]. This is when the level of estrogen drops the most dramatically (Figure 3). A menstrual-related migraine is one that occurs within in 2 days of the start of the cycle but occasional attacks occur on other days of the cycle, when estrogen levels fluctuate[63].

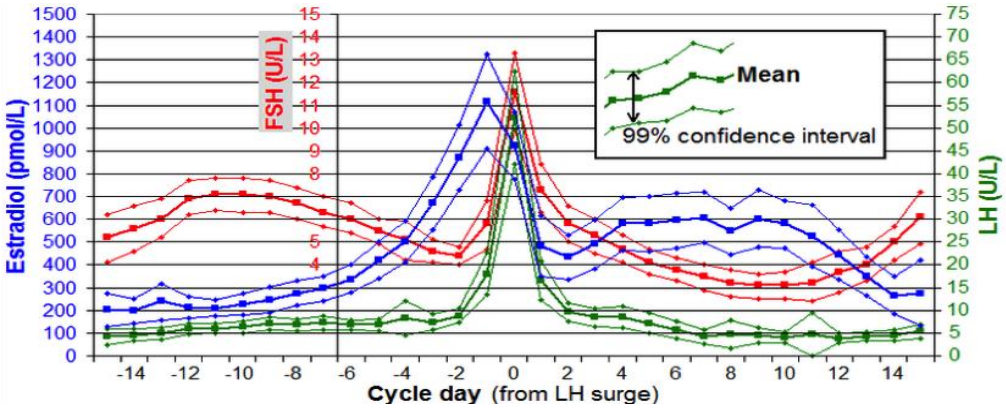

Figure 3: Hormone changes through the estrous cycle. The blue line shows the estrogen levels. The red line is follicle stimulating hormone (FSH) and the green line is Luteinizing hormone (LH)[2].

Correlations between migraine occurrence and estrogen levels during pregnancy and menopause also provide evidence that estrogen fluctuation may be involved in migraine. Estrogen levels increase during each trimester of pregnancy and dropped off sharply after delivery[61d, 64]. Nearly 80% of migraine patients studied did not have migraines in the third trimester but 94% of these reported the occurrence of migraines after giving birth[61d, 64]. Breast feeding suppresses estrogen level fluctuation and women experience fewer migraines during lactation[65]. At menopause, estrogen levels decrease permanently and the occurrence of migraines also decreases. In a review of 556 menopausal women, 82% reported migraines before menopause and only 14% reported headache symptoms after menopause[66]. Thus, the fluctuations of estrogen levels have a strong correlation to the incidence of migraine.

Estrogen Signaling and Pain

Nociceptors are sensory receptors that respond to potentially damaging stimuli. They are expressed in the initial cells that signal pain and depend on ion channels in order to facilitate the transduction of pain[67]. The pain pathway functions when a noxious stimulus causes depolarizations, which initiate action potentials from the peripheral sensory system to the synapse in the central nervous system. Here, the action potentials activate a neurotransmitter release at the presynaptic terminal[67]. Understanding the signal pathway of pain is important in the development of pain therapies. By determining if some ion channels, neuropeptides, growth factor receptors, or signal transduction cascades are different from other neurons and are unique in the type of tissue, it could be possible to target specific nociceptors for pain treatment without targeting other sensory neurons[67].

Estrogen has long been thought to be involved in pain regulation and signaling. Various studies have shown correlations of estrogen involvement in pain. However, the effects of estrogen on pain signaling are complex. Studies have shown that estrogen can increase or decrease pain depending on the type and location of pain[68]. Acute pain responses seem to be attenuated by estrogen. Some studies reported longer latencies to acute stimuli in ovariectomized female animals with hormone regulation as compared to non-hormonal regulated controls[69]. Estrogen treatment also decreased the number or intensity of pain responses in gonadectomized male and female rats[70]. However, other studies reported estrogen had no affect on acute pain response[36, 69b, 71]. Estrogen treatment in women in the early follicular stage, when estrogen is low, has been shown to result in an increased pain threshold[72]. Women who underwent an acute pain assay reported decreased pain perception in the follicular stage, when estrogen is higher[72a]. It seems that location plays a role in the effect estrogen has on pain. When estrogen was located in the peripheral nervous system (PNS), either endogenously or through replacements, pain was alleviated. Yet, male rats who were injected with estrogen directly into the central nervous system had increased pain behaviors[73]. Chronic pain also seems to be intensified by estrogen. In female rats with arthritis, greater thermal sensitivity occurred in the proestrous phase of the menstrual cycle, when the estrogen levels are high[74]. Female animals also had increased mechanical sensitivity during the proestrous and estrous phases[75]. Male rats with spinal cord injury showed an increase in pain response with estrogen modulation[76].

Estrogen withdrawal has been linked to migraine because the onset of most menstrual migraines occurs at the beginning of the estrous cycle, when the estrogen levels sharply decline[47a, 77]. Migraine is based on altered neuronal activity and excitability [78]. Laser stimulation can create event potentials in the brain which mimic migraine. The amplitude of these laser-

evoked potentials (LEPs) is a quantitative index of the neuronal excitability. They decrease during repeated stimuli showing a progressive decrease in neuronal response[77]. The decrease in the amplitude of sensory cortical responses to repeated stimuli to avoid brain overstimulation is defined as habituation[79]. In migraine patients between attacks, the LEP amplitudes are normal in basal conditions, but reduced habituation occurs with repeated stimuli[80]. During migraine, migraine patients have increased LEP amplitudes and increased pain perception[77, 81]. The effects of estrogen during the pre-menstrual and late luteal phases of the menstrual cycle of migraine and non-migraine patients showed migraineurs had increased LEP amplitudes and decreased habituation compared to controls[77], but both groups had increased pain sensitivity, increased amplitude and decreased habituation in the pre-menstrual phase[77]. This indicates that estrogen withdrawal may decrease habituation and increase pain perception.

Sumatriptan

Sumatriptan, a type of triptan, which acts on serotonin receptors, is the most commonly used drug to treat migraine pain[82]. Animal studies suggest that the effectiveness of Sumatriptan on migraine pain coincides with the level of the neuropeptide CGRP[48, 82-83]. In migraines, activated meningeal nociceptors in the trigeminal ganglion release high levels of CGRP resulting in vasodilatation and mast cell degranulation, which leads to trigeminal ganglion sensitization[84]. Sumatriptan is effective because it causes contraction of cerebral arteries and reduces neurological inflammation by blocking the release of nociceptors neuropeptides like CGRP[82]. This blocking of nociceptors in the pain pathway helps stop sensitization of dural afferents and alleviates pain[82]. A study of human migraine patients showed that when Sumatriptan alleviated

nitroglycerin-induced migraine attack, the plasma CGRP levels were significantly decreased. However, if the migraine did not improve, CGRP levels were not affected[82].

Sumatriptan also alleviates migraine by increasing intracellular calcium levels that act in the MAPK phosphorylation pathway, which inhibits CGRP expression. The prolonged calcium increase decreases CGRP promoter activity and subsequently relieves pain [85]. Intercellular calcium fluctuations also control voltage-dependent calcium channels, which regulate neurotransmitter release and excitability and are important in the incidence of FHM [21, 85a].

While the exact mechanism for migraine remains unclear, many factors indicate the important role of estrogen. The prevalence of migraine in woman over men and the onset of migraine sexual dimorphism at puberty, factored with the correlation of both the monthly and lifetime cycling to migraine, suggest that estrogen may be important in the mechanism of migraine. Serotonin is also likely part of the migraine pathway. This is suggested by evidence that serotonin levels change according to estrogen cycling and serotonin mediates estrogen-created anxiety.

Nociceptors rely on ion channels to signal the pain cascade pathway. Specifically, calcium channels seem to have the most relevance in the migraine pathway. FHM migraine is the result of mutations in the calcium channel and cellular calcium released after serotonin receptor stimulation increases serotonin levels that can then free more calcium. This mechanism is why intracellular calcium levels increase after sumatriptan administration.

Vasodilatation also plays a role in the pain mechanism of migraine. Increases in blood flow to the brain during migraine are evident from fMRIs. This change in vascular pressure may be the reason for pain in migraine. Increased levels of CGRP, which is a vasodilator, are also observed during migraines. Sumatriptan success also parallels CGRP levels. However,

sensitization of meninges may also contribute partially or entirely to migraine pain. During migraine, activated meningeal nociceptors release neurotransmitters and signal pain. Estrogen plays a complex role in pain perception, decreasing acute pain and increasing chronic pain. Low estrogen also decreases neuronal habituation and increases pain perception.

Gap junctions can be regulated by estrogen signaling and CKIδ activity. During pregnancy, when high levels of estrogen are present, levels of Cx43 increase. A sexual dimorphism also occurs when estrogen increases Cx43 levels in female rats but decreases it in male rats. Cx43 levels are important for proper neovascularization and fetal growth and normal parturition and may be important in vascularization associated with migraine. The number of gap junctions in the brain may be related to the delay of cortical spreading depression, a parallel of aura. Not only does CK1δ phosphorylate gap junction proteins, it also phosphorylates estrogen receptor α. The research present in the field indicates an intricate pathway for migraine, though much remains to be elucidated.

We want to determine the effect of the CK1δ-T44A substitution on thermal sensitivity to heat and if this sensitivity is affected by the gender of the animal. We want to establish if CK1δ-T44A female mice experience a more pronounced pain difference than the wildtype female and CK1δ-T44A male mice. We use the Hargreaves pain assay to assess any differences in pain reaction post NTG injection. We also want to determine the effect of CK1δ on ERβ and if ERβ heterozygote mice are hypersensitive/hyposensitive compared to wildtype and CK1δ-T44A mutant mice after NTG injection. We also want to determine if this thermal sensitivity changes with CK1δ-T44A; $ER\beta^{ko}$/+ mice.

CHAPTER 2: THE EFFECTS OF GENDER ON THERMAL SENSITIVITY IN CK1δ-T44A AND WILDTYPE MICE

Introduction

A mutant allele of CK1δ-T44A was found to segregate with migraine with aura[20]. This mutation leads to increased susceptibility to nitroglycerin-induced mechanical and thermal hypersensitivity, lower threshold to aura, increased cranial arterial dilation and is causative for migraine with aura[6b].

One study showed CK1δ-T44A male animals responded to lesser mechanical stimuli than did their wildtype littermates after administration of NTG. The NTG dose required to produce hypersensitivity to mechanical stimuli in CK1δ-T44A mice was seven times lower than the NTG dose required to produce a similar effect in controls[6b]. Similar allodynia responses to NTG have been noted in human migraine patients[8b]. Also, a significantly smaller volume of chemical stimuli was required to elicit CSD in CKIδ-T44A mice compared to wildtype animals, and these lower CSD thresholds in CK1δ-T44A animals were reproducible. During constant stimulation over one hour, significantly more CSD events were elicited in CKIδ-T44A mice than in wildtype mice[86].

During CSD, arteries in the cortex change in diameter in both wildtype and CKIδ-T44A mice. Arteries dilate preceding the CSD wavefront and are followed by a large constriction, a second large dilation, and a post-CSD dilation. Baseline diameters and second dilatations were similar for both groups but constriction is less pronounced in CKIδ-T44A than wildtype arteries[6b]. Though CK1δ-T44A and wildtype male animals are different in both induction of CSD and thermal pain thresholds, these studies have not been completed in female mice.

Correlative evidence suggests a link between migraine occurrence and estrogens signaling because migraines affect three times more women than men, increase in frequency during puberty and pregnancy, and occur less frequently after menopause[47a, 49]. Therefore, we explored the sex difference in response to thermal stimuli between CK1δ-T44A male and female mice. We wanted to determine if the same pattern occurs in female CK1δ mutant mice and if these effects are more severe than in the male mice.

Materials and Methods

Materials: The wildtype mice came from Jackson Labs (Sacramento, CA), the CK1δ-T44A mice came from the Louis Ptacek lab (UCSF, San Francisco, CA) and the ERβ mice came from the Jan-Ake Gustafsson lab (Karolinska Institutet, Department of Biosciences and Nutrition, Sweden). The nitroglycerin (NTG injection, USP 5mg/ml) was from American Regent, Inc. The saline (0.9% sodium chloride injection, USP) was from Hospir, Inc. The tail lysis buffer was DirectPCR (Tail) from Viagen. The primers were made in-house on a Mermade 6 (Bioautomation Corporation) DNA synthesizer. The polymerase was GoTaq DNA Polymerase purchased from Promega. The Hargreaves apparatus was purchased from The University of San Diego.

Experimental Animals—All experiments were conducted on C57Bl6 wildtype, CK1δ-T44A, ERβ^{ko}/+, or CK1δ-T44A; ERβ^{ko}/+ mice. All genotypes have a C57Bl6 background and weighed between 18 and 30 grams during experiments. Animals were acclimated to a 12 hour light-dark cycle and experiments were performed between 8 a.m. and 2 p.m. at 22°C.

Nitroglycerin Administration—A stock of 5 mg/mL nitroglycerin dissolved in 30% alcohol, 30% propylene glycol, and water was further diluted fresh each day in 0.9% saline.

Doses of 1, 5, 10 mg/kg were administered Intraperitoneally (IP). Control mice received a dose of 0.9% saline solution.

Hargreaves Assay—Animals were habituated to the testing apparatus for 60 minutes prior to determination of baseline nociceptive thresholds. To determine thermal nociceptive thresholds, the Hargreaves assay focuses radiant heat on the hind paw of each animal and measures the time in seconds until the mouse removes the hind paw from the source of heat. The Hargreaves apparatus has a pressure-sensitive plate that turns off the heat source and stops a timer when the paw is lifted in response to pain. The cut off valve of 20.48 seconds was set to prevent tissue injury. This timer is accurate to one hundredth of a second. Radiant heat intensity was set at 5%. Three determinations were averaged for every time point with at least 1 minute between each trial. Initial measurements were taken as a baseline reference before injection with saline or NTG. The mice were then with saline or NTG and measurements were taken post injection at time 90 minutes. The assay was performed by a single researcher who was blind to both dose and genotype.

Genotyping—Mice were weaned at 25 days, ear marked, and tail clipped for genotyping. A 0.5cm section was clipped from the end of each animal's tail. Each tail fragment was lysed overnight at 55°C in tail lysis buffer and 1 mg/ml of proteinase K solution. The proteinase K was then inhibited by incubation at 85°C for 45 mins. The crude cell lysate was used in PCR to identify either CK1δ T44A mutant or ERβ heterozygote mice. To identify the CK1δ-T44A mutant mice, primers 5'ACACAGTCTGCTGACTGCATCTC and CTCAGGTCTTTCAAATTCTCATT were used. The program used in the Mastercycler ep (Eppendorf) cycler was 94°C for 5', 94°C for 30", 60°C for 1', 72°C for 1:30', 72°C for 7' and 4°C hold with 35 cycles. The products were subjected to electrophoresis on a 1% agarose gel.

Lanes with a band at 800 bp show the CK1δ-T44A genotype and lanes without a band correspond to wildtype animals.

Statistical Analysis—For each paw of each animal, three paw removal measurements were taken for every time point (baseline and 90 minutes after injection). Heat sensitivity thresholds from each group of mice treated with equivalent doses of NTG or saline were averaged. Two-tailed student T-TESTS were performed comparing genotype to genotype, saline to NTG, and baseline to 90 minutes for all genotypes and genders. p values are given in the text. Error bars represent SEM. The number of animals tested for each genotype and each dose of NTG are indicated in the figure legends. Male and female animals are treated as different genotypes and were separated for all data.

Results

To determine the difference between CK1δ-T44A mutant and wildtype mice in response to thermal stimuli after NTG injection, we performed a Hargreaves assay for animals of both genotypes. This experiment measures the animal's response to a thermal stimulus by determining latency to animal response to a calibrated heat source. Initial baseline paw withdrawal measurements of each animal were recorded and withdrawal measurements were again taken 90 minutes after injection with saline and various doses of NTG.

To control for possible stress resulting from injection, both CK1δ-T44A and wildtype mice received saline injections after baseline responses were recorded (Figure 4). For both transgenic CK1δ-T44A and wildtype animals, the latency to response after the saline injection was similarly reduced. At baseline, the transgenic mice responded in an average of 10.01 ± 0.32 (s). Ninety minutes after injection these mice responded at 7.59 ± 0.28 (s). Similarly, the

wildtype mice responded in an average of 9.64 ± 0.19 (s) at baseline, which decreased to 7.66 ± 0.20 (s) at 90 minutes post injection. The decrease in latency after 90 minutes is significant for both groups (CK1δ $p= 1.05E^{-5}$ and wildtype $p= 2.70E^{-12}$). However, there was no difference in the latency to response between the CK1δ-T44A mutant and wildtype mice at either baseline or 90 minutes post saline injection. This indicates that the general stress from injection causes a significant change in sensitivity to heat but this difference is not dependent on CK1δ.

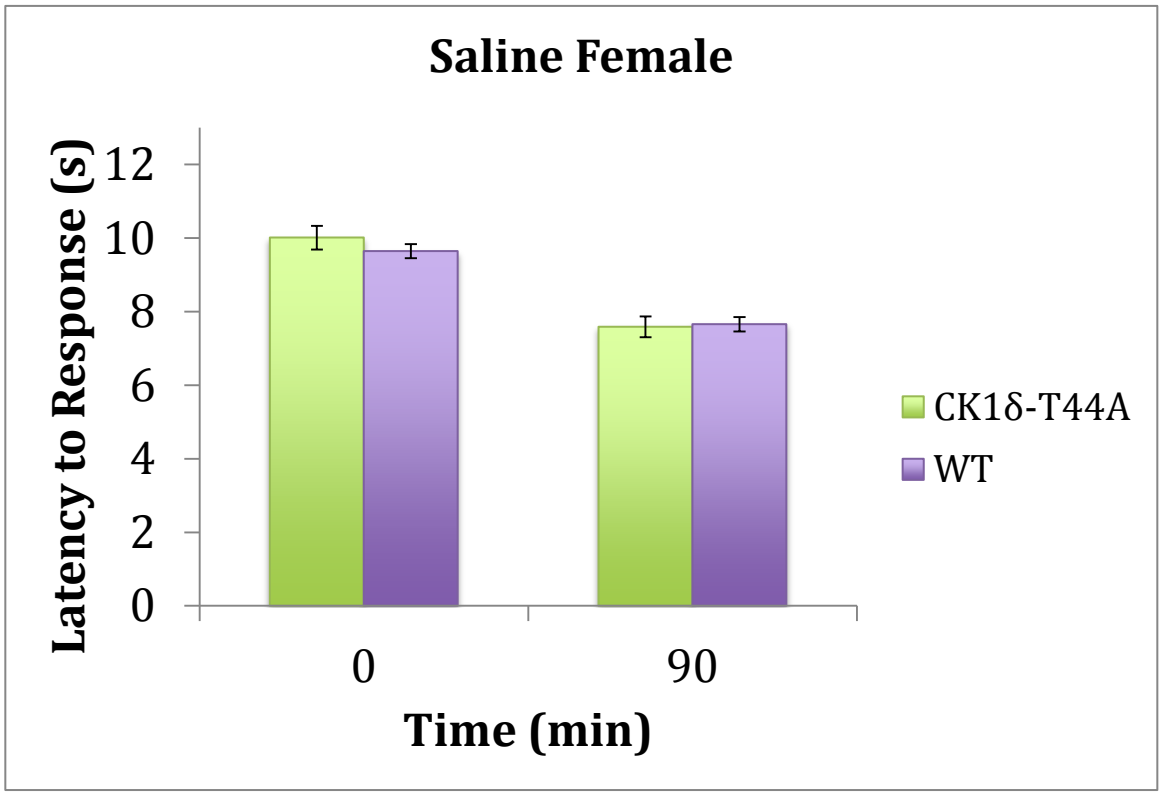

Figure 4: The paw-withdrawal response averages to thermal stimuli of CK1δ-T44A and wildtype mice at baseline and 90 minutes post saline injection. CK1δ-T44A n=13 and wildtype n=26.

To determine if NTG can cause a significant difference in thermal sensitivity between the CK1δ-T44A mutant and wildtype female mice, both groups were injected with 1, 5 and 10 mg/kg NTG. After receiving a 5 mg/kg dose of NTG, transgenic CK1δ-T44A animals responded faster to a calibrated heat source than they did at baseline, while wildtype siblings

remained unaffected by the same dose of NTG (Figure 5). At baseline, before receiving 5 mg/kg NTG, the CK1δ-T44A mutant mice responded with an average of 10.01 ± 0.0.32 (s), which decreased to 6.95 ± 0.43 (s) at 90 minutes after injection. The wildtype mice had a paw withdrawal average of 9.64 ±0.19 (s) at baseline and 9.02 ± 0.37 (s) at 90 minutes post injection.

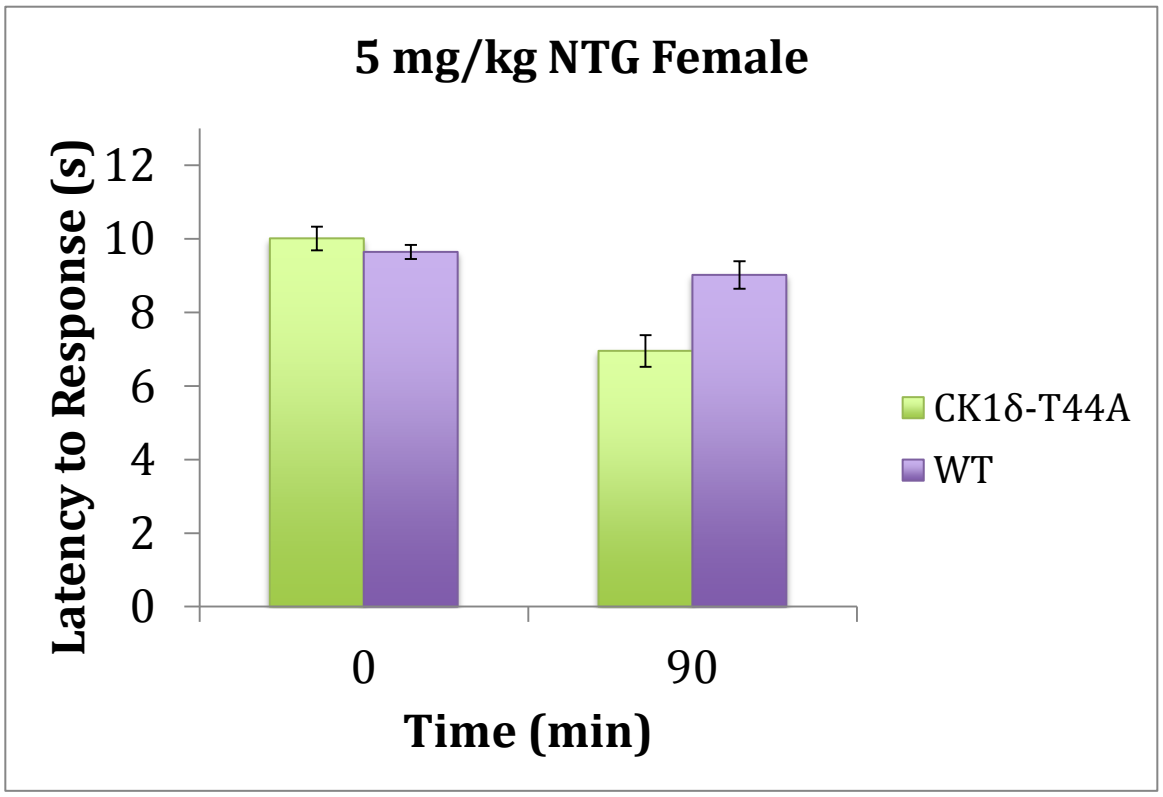

Figure 5: The paw-withdrawal response averages to thermal stimuli of CK1δ-T44A and wildtype mice at baseline and 90 minutes post 5 mg/kg NTG injection. CK1δ-T44A n=14 and wildtype n =26.

While the difference of paw withdrawal response at baseline was not different between the transgenic and wildtype mice, 90 minutes after injection with 5 mg/kg NTG the female CK1δ-T44A mice have a much lower latency to response than their female wildtype siblings, confirmed by a p value of 0.0005. As in human migraine patients, these data show that mice that express CK1δ-T44A have increased sensitivity to thermal stimuli. At 1mg/kg NTG, the difference of paw withdrawal response between female CK1δ-T44A and wildtype mice at 90

minutes post injection was not different (p=0.94). At this dose, CK1δ-T44A mutant mice responded at baseline with an average of 8.42 ± 0.45 (s), this decreased slightly to 7.31 ± 0.55 (s) at 90 minutes after injection. The wildtype females had a baseline latency to response of 9.55 ±0.37 (s), which decreased slightly to 7.27 ± 0.34 (s) at 90 minutes post 1 mg/kg NTG injection. At 10 mg/kg NTG, the difference between the transgenic and wildtype animals 90 minutes post injection was also not significant (p= 0.16). Animals that express CK1δ-T44A responded with an average latency of 8.81 ± 0.52 (s) at baseline, which decreased to 6.75 ±0.45 (s) 90 minutes after injection of 10mg/kg NTG. Wildtype animals responded with an average latency of 8.99 ± 0.32 (s) at baseline, which decreased to 7.61 ± 0.35 at 90 minutes after injection.

To compare the effect of gender on NTG-induce hypersensitivity, latency to response to a heat source was compared in female and male CK1δ-T44A mutant and wildtype mice (Figure 6).

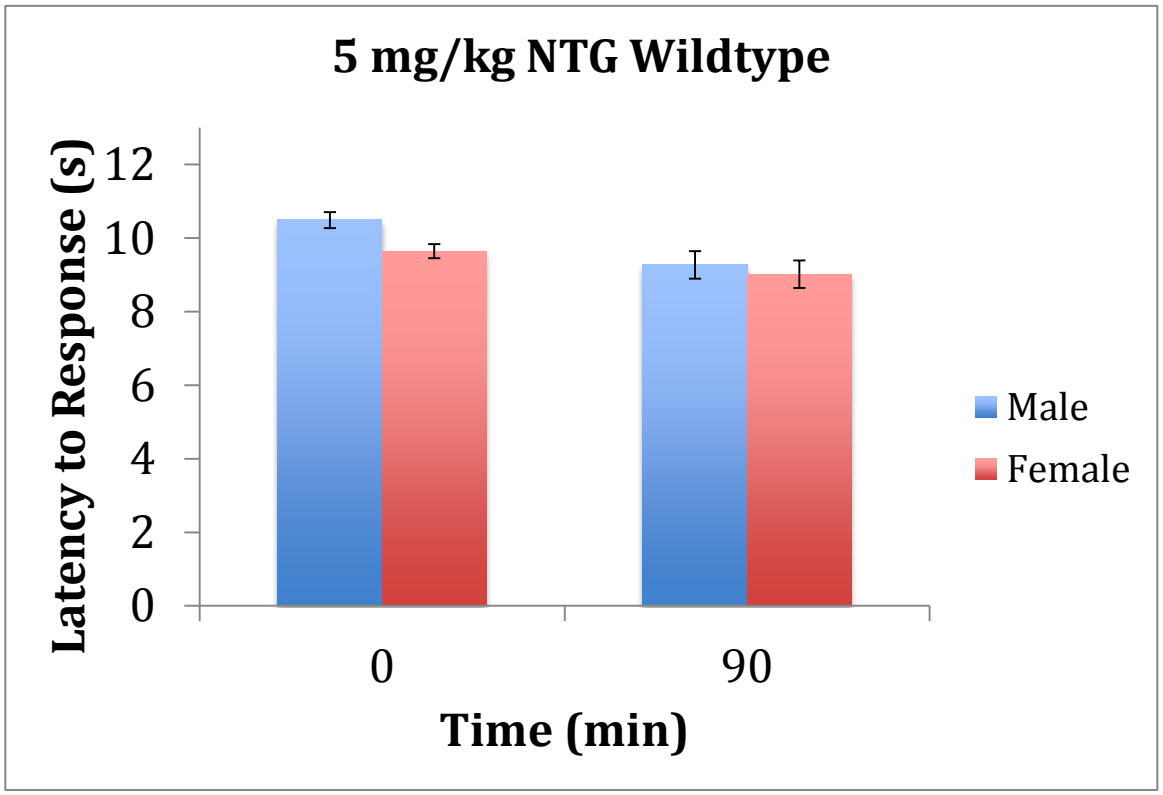

Figure 6: The paw-withdrawal response averages to thermal stimuli of male and female wildtype mice at baseline and 90 minutes post 5 mg/kg NTG injection. Male n=21 and female n=26.

At baseline wildtype males had a longer latency to response than wildtype females (p=0.003). The male wildtype mice had a baseline of 10.49 ± 0.22 (s) and the female mice had a baseline of 9.64 ± 0.19 (s). However, this sex difference disappeared after injection and no difference was found between male and female wildtype mice 90 minutes post injection with saline or 1, 5, or 10 mg/kg NTG.

To determine the effect of CK1δ-T44A on gender, the latency to thermal response was compared between transgenic CK1δ-T44A males and females after injection with 5 mg/kg NTG (Figure 7).

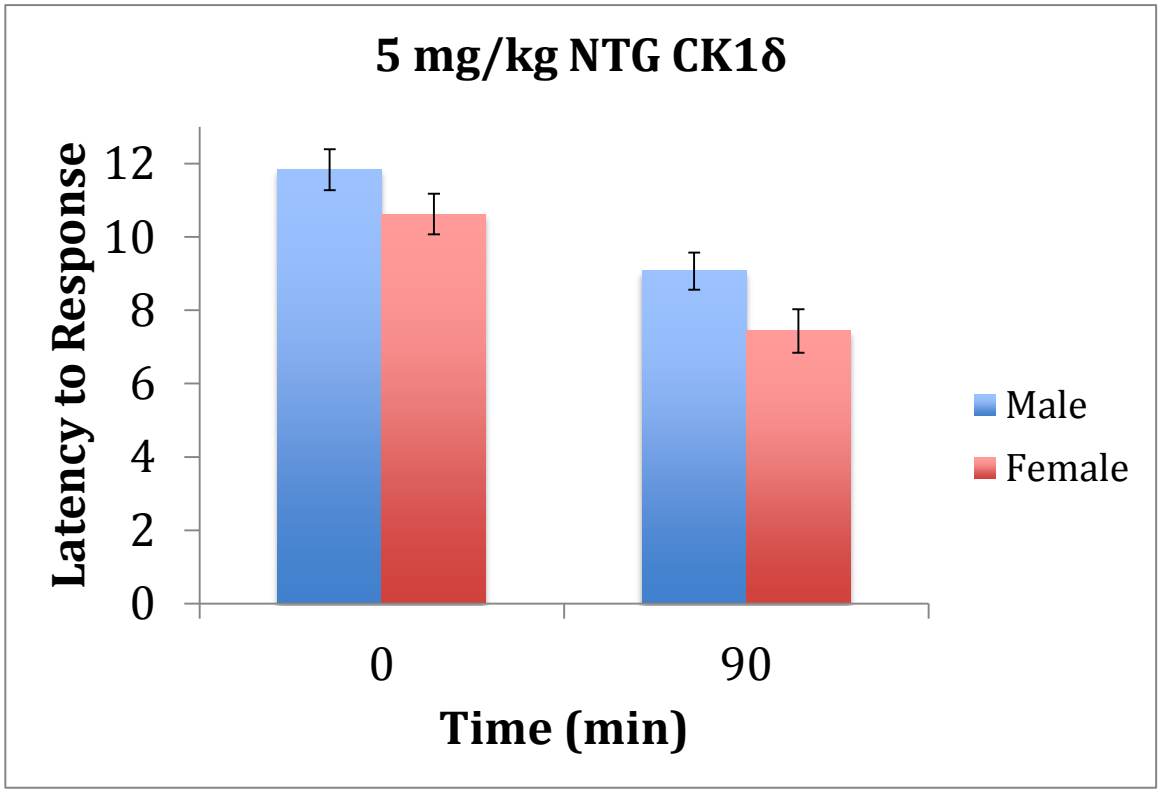

Figure 7: The paw-withdrawal response averages to thermal stimuli of male and female CK1δ-T44A mice at baseline and 90 minutes post 5 mg/kg NTG injection. Male n=21 and female n=14.

At 5 mg/kg, the male CK1δ-T44A mice responded slower than CK1δ-T44A female mice at baseline ($p=6.18E^{-4}$). Male CK1δ-T44A mice responded in 11.28 ± 0.25 (s), while female CK1δ-

T44A mice responded to thermal stimuli in 10.01 ± 0.32 (s). Unlike the wildtype mice, this sex difference is still present after 5 mg/kg NTG injection. Female CK1δ-T44A animals had a significantly shorter latency to response than the mutant males 90 minutes after injection with NTG ($7.39E^{-5}$). The CK1δ-T44A males have a response average of 9.51 ± 0.44 (s), while the CK1δ-T44A females responded with an average of 6.95 ± 0.43 (s), indicating that CK1δ-T44A females are more sensitive to thermal stimuli than CK1δ-T44A males after 5 mg/kg NTG. This sex difference suggests the possible participation of estrogen in NTG-induced sensitivity to thermal stimuli. No difference occurred at saline, 1 or 10 mg/kg doses.

To determine the affect dose had on both male and female mice, the response to heat at 90 minutes was subtracted from baseline response to thermal stimuli to determine the difference

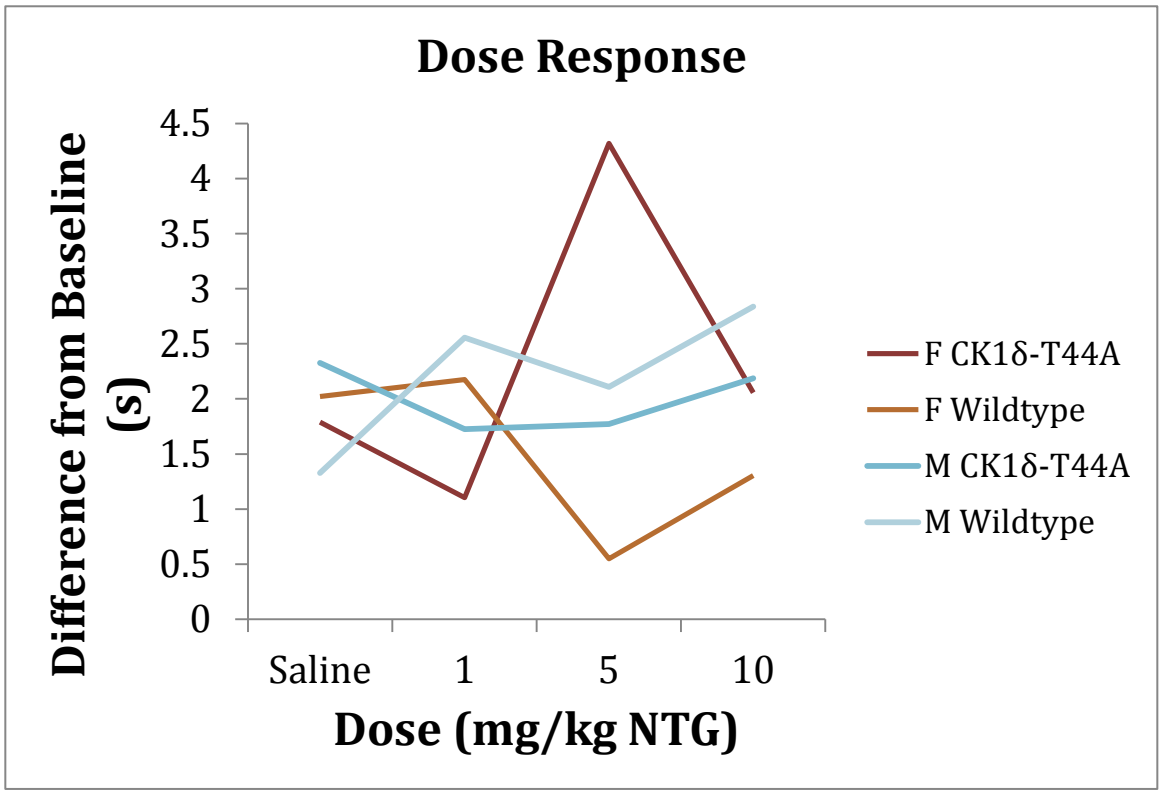

Figure 8: Dose curve for male and female CK1δ-T44A and wildtype animals showing difference from baseline. Female CK1δ-T44A saline n=14, 1 mg/kg n=7, 5 mg/kg n=14, 10 mg/kg n=3. Male CK1δ-T44A saline n=21, 1 mg/kg n=6, 5 mg/kg n=21, 10 mg/kg n=4. Female wildtype saline n=25, 1 mg/kg n=7, 5 mg/kg n=24, 10 mg/kg n=12. Male wildtype saline n=20, 1 mg/kg n=9, 5 mg/kg n=16, 10 mg/kg n=9.

from baseline of wildtype and CK1δ-T44A mice at 1, 5, and 10 mg/kg NTG and saline. The dose curve is shown in Figure 8. Female CK1δ-T44A mice had a significant difference from baseline response compared to wildtype females at 5 mg/kg NTG. Male CK1δ-T44A mice were less sensitive to heat than wildtype mice at 5 mg/kg. The difference from baseline responses at 1 mg/kg and 10 mg/kg vary widely for both male and female wildtype and CK1δ-T44A mice, likely because the number of animals at these doses is small.

Discussion

CK1δ-T44A female mice are more sensitive to thermal stimuli than wildtype females after injection with NTG at a 5 mg/kg dose. This finding is significant since human migraine patients are more sensitive to heat during migraine and the CK1δ mutation in mice is the same mutation that has been identified in a family with inherited migraine with aura. This is consistent with the finding that CK1δ-T44A mice are more sensitive to CSD and greater cranial arterial dilation than wildtype animals. This supports the hypothesis that CK1δ contributes to migraine pathology.

Female CK1δ-T44A animals are more sensitive to thermal stimuli than male CK1δ-T44A mice before and after injection with 5 mg/kg NTG. This sex difference is consistent with observations that women are more susceptible to migraine than men[61c]. Previous studies have suggested that sex hormones are involved with women having lower pain thresholds and higher sensitivity to painful stimulations than men[70a, 87]. This gender difference could be linked to the sex hormone estrogen, which is modulated by CK1δ[38]. This gender difference is important since a significant sex difference is reported in CSD in male and female mice. Female CK1δ and wildtype mice have a lower threshold for inducing CSD than the male mice of their respective

genotypes[86]. A difference in CSD also exists between wildtype and CK1δ-T44A mice, with CK1δ-T44A mice having a lower threshold to induce CDS than wildtype[86]. CK1δ-T44A mice exhibit a gender difference in nociception after NTG injection, which is different from the pain phenotype expressed in wildtype males and females after NTG injection[86]. Since CSD models aura, this could mean that gender might affect occurrence of aura more than nociception. This represents an important link between estrogen signaling and CK1δ in the migraine pain pathway.

CK1δ regulates estrogen and estrogen is a key difference between males and females. Estrogen regulates vasodilatation, which modulates pain response[57-58]. Estrogen acts on estrogen receptors α and β and CK1δ activates these estrogen receptors. The CK1δ-T44A substitution likely decreases the phosphorylation level of the estrogen receptors and the different levels of estrogen present in male and female mice likely have a role in the thermal sensitivity gender difference exhibited by CK1δ-T44A mice that is absent in wildtype siblings.

We conclude that CK1δ-T44A female mice are more sensitive to thermal stimuli than wildtype females because of modified CK1δ function. This is important in migraine because the same CK1δ mutation is identified in a family with inherited migraine with aura. It is also likely that the sex hormone estrogen is involved in the CK1δ migraine pathway because sex hormones have been shown to influence sensitivity to painful stimuli[70a, 87].

CHAPTER 3: THE EFFECTS OF ESTROGEN RECEPTOR BETA ON THERMAL SENSITIVITY

Introduction

Migraine attacks are correlated with changes in estradiol (E2) levels[3b, 47a]. In women, migraine onset is most frequent at the dramatic estrogen decline at the beginning of the menstrual cycle[47a]. Estrogen regulates inflammation and pain[88] and can sensitize pain-sensing neurons[8a]. In addition, estradiol can modulate neuronal activity. Synchronous intracellular Ca^{2+} waves and transients in gonadotrophin-releasing hormone (GnRH), LH releasing, hypocampal, and cortical neurons are amplified by E2 [89]. E2 can affect neuronal excitability by modulating ion channels and receptors including the coupling of the α1-adrenergic receptors to calcium-activated K^+ (SK) channels[90]. These correlations led us to test the hypothesis that estrogen signaling is important for susceptibility to migraine.

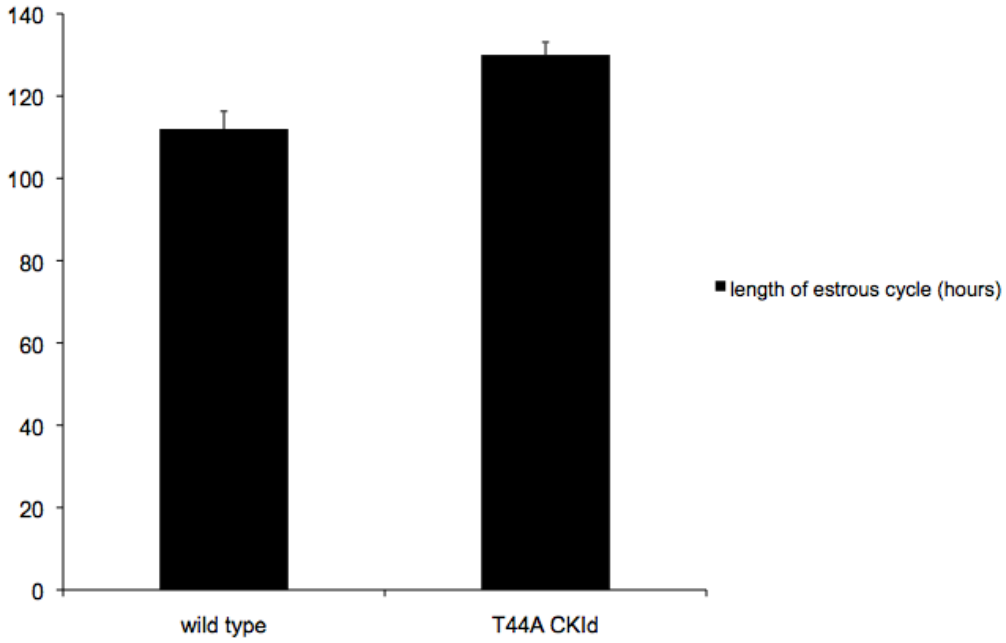

Figure 9: The length of estrous cycles in hours of wildtype and CK1δ-T44A mice.

Transgenic CK1δ-T44A mice have longer estrous cycles (Figure 9), indicating a relationship between the activity of estrogen and CK1δ function. As previously established, CKIδ-T44A animals become hypersensitive to some sensory

stimuli at low doses of NTG and cranial arteries dilate abnormally in CKIδ-T44A mutant animals in response to CSD[86]. Estrogen signals through ERß and ERα to promote arterial dilation in response to nitric oxide—a metabolite of NTG [57, 91]. CKIδ has been shown to phosphorylate ERα *in vitro* and decreased CKIδ function reduces ERα transcriptional activation, but increases the levels of ERα in cells[38]. Thus, it could be that the T44A substitution in CKIδ changes phosphorylation of ERα leading to the increased pain responses after NTG injection and abnormal dilation of cranial arteries with CSD. ERβ is 49% identical and 65% similar to ERα, and ERβ is expressed in the brain. It is unknown if ERβ, like ERα, is regulated by CKIδ. If ERβ is responsible for the pain phenotypes that are downstream of CKIδ in migraine, we would expect that by reducing function of ERβ in animals that express CKIδ-T44A, they will not respond faster to heat after low dose NTG injections.

Materials and Methods

Materials: The wildtype mice came from Jackson Labs (Sacramento, CA), the CK1δ-T44A mice came from the Louis Ptacek lab (UCSF, San Francisco, CA) and the ERβ mice came from the Jan-Ake Gustafsson lab (Karolinska Institutet, Department of Biosciences and Nutrition, Sweden). The nitroglycerin (NTG injection, USP 5mg/ml) was from American Regent, Inc. The saline (0.9% sodium chloride injection, USP) was from Hospir, Inc. The tail lysis buffer was DirectPCR (Tail) from Viagen. The primers were made in-house on a Mermade 6 (Bioautomation Corporation) DNA synthesizer. The polymerase was GoTaq DNA Polymerase purchased from Promega. The Hargreaves apparatus was purchased from The University of San Diego.

Experimental Animals—All experiments were conducted on C57Bl6 wildtype, CK1δ-T44A, ERβ^{ko}/+, or CK1δ-T44A; ERβ^{ko}/+ mice. All genotypes have a C57Bl6 background and weighed between 18 and 30 grams during experiments. Animals were acclimated to a 12 hour light-dark cycle and experiments were performed between 8 a.m. and 2 p.m. at 22°C.

Nitroglycerin Administration—A stock of 5 mg/mL nitroglycerin dissolved in 30% alcohol, 30% propylene glycol, and water was further diluted fresh each day in 0.9% saline. A dose of 5 mg/kg NTG was administered Intraperitoneally (IP). Control mice received a dose of 0.9% saline solution.

Hargreaves Assay—Animals were habituated to the testing apparatus for 60 minutes prior to determination of baseline nociceptive thresholds. To determine thermal nociceptive thresholds, the Hargreaves assay focuses radiant heat on the hind paw of each animal and measures the time in seconds until the mouse removes the hind paw from the source of heat. The Hargreaves apparatus has a pressure-sensitive plate that turns off the heat source and stops a timer when the paw is lifted in response to pain. The cut off valve of 20.48 seconds was set to prevent tissue injury. This timer is accurate to one hundredth of a second. Radiant heat intensity was set at 5%. Three determinations were averaged for every time point with at least 1 minute between each trial. Initial measurements were taken as a baseline reference before injection with saline or NTG. The mice were then with saline or NTG and measurements were taken post injection at time 60 and 90 minutes. The assay was performed by a single researcher who was blind to both dose and genotype.

Genotyping—Mice were weaned at 25 days, ear marked, and tail clipped for genotyping. A 0.5cm section was clipped from the end of each animal's tail. Each tail fragment was lysed overnight at 55°C in tail lysis buffer and 1 mg/ml of proteinase K solution. The proteinase K

was then inhibited by incubation at 85°C for 45 mins. The crude cell lysate was used in PCR to identify either CK1δ T44A mutant or ERβ heterozygote mice. To identify CK1δ-T44A; ERβ^{ko}/+ mice tail lysates were first amplified with CK1δ-T44A identifying primers. Primers 5'ACACAGTCTGCTGACTGCATCTC and CTCAGGTCTTTCAAATTCTCATT were used. The program used in the Mastercycler ep (Eppendorf) cycler was 94°C for 5', 94°C for 30", 60°C for 1', 72°C for 1:30', 72°C for 7' and 4°C hold with 35 cycles. The products were subjected to electrophoresis on a 1% agarose gel. Lanes with a band at 800 bp show the CK1δ-T44A genotype and lanes without a band correspond to wildtype animals.

New PCR reactions containing fresh tail lysate from the same animals were then amplified using ERβ^{ko}/+ primers identify the second genotype. Primers ATCAGCCCATGGGCAGAGTGTG, GTGGATGCCTATGATCACTGTGGA, and CCAGATGCATAATCACTGCAGACG were used. The Mastercycler ep (Eppendorf) cycler was used running the following program: 94°C for 5', 94°C for 30", 56°C for 30", 72°C for 1', 72°C for 7' and 4°C hold with 35 cycles. The products were subjected to electrophoresis on a 1% agarose gel. A lane with only band at 500bp shows wildtype mice, while a lane with a band at 500bp and 300bp or just 300bp indicate one mutant allele for ERβ.

Statistical Analysis—For each paw of each animal, three paw removal measurements were taken for every time point (baseline, 60 and 90 minutes after injection). Heat sensitivity thresholds from each group of mice treated with equivalent doses of NTG or saline were averaged. Two-tailed student T-TESTS were performed comparing genotype to genotype, saline to NTG, and baseline to 60 or 90 minutes for all genotypes and genders. p values are given in the text. Error bars represent SEM. The number of animals tested for each genotype and each dose of NTG are indicated in the figure legends.

Results

To determine the effect of reducing the function of ERβ on the mechanism of migraine, we compared the sensitivity to thermal stimuli as a function of latency to response to a calibrated heat source of heterozygous ERβ knockout ($ERβ^{KO}/+$) mice and wildtype mice. We used saline in order to establish a basis for injection stress (Figure 10). In female mice, $ERβ^{KO}/+$ mice are less sensitive to pain than their wildtype siblings before injection ($p= 3.04E^{-16}$). At baseline, $ERβ^{KO}/+$ mice responded with an average of 12.11 ± 0.22 (s) and wildtype mice responded with an average of 9.64 ± 0.19 (s). Ninety minutes after injection with saline, the latency to response averages for both $ERβ^{KO}/+$ and wildtype mice decreased significantly ($p= 1.60E^{-12}$ and $p= 2.70E^{-12}$, respectively), but were not different from each other (p=0.42). $ERβ^{KO}/+$ mice had an average paw withdrawal of 7.96 ± 0.38 (s) at 90 minutes after saline injection and the wildtype mice responded with an average of 7.66 ± 0.20 (s) at 90 minutes after saline injection.

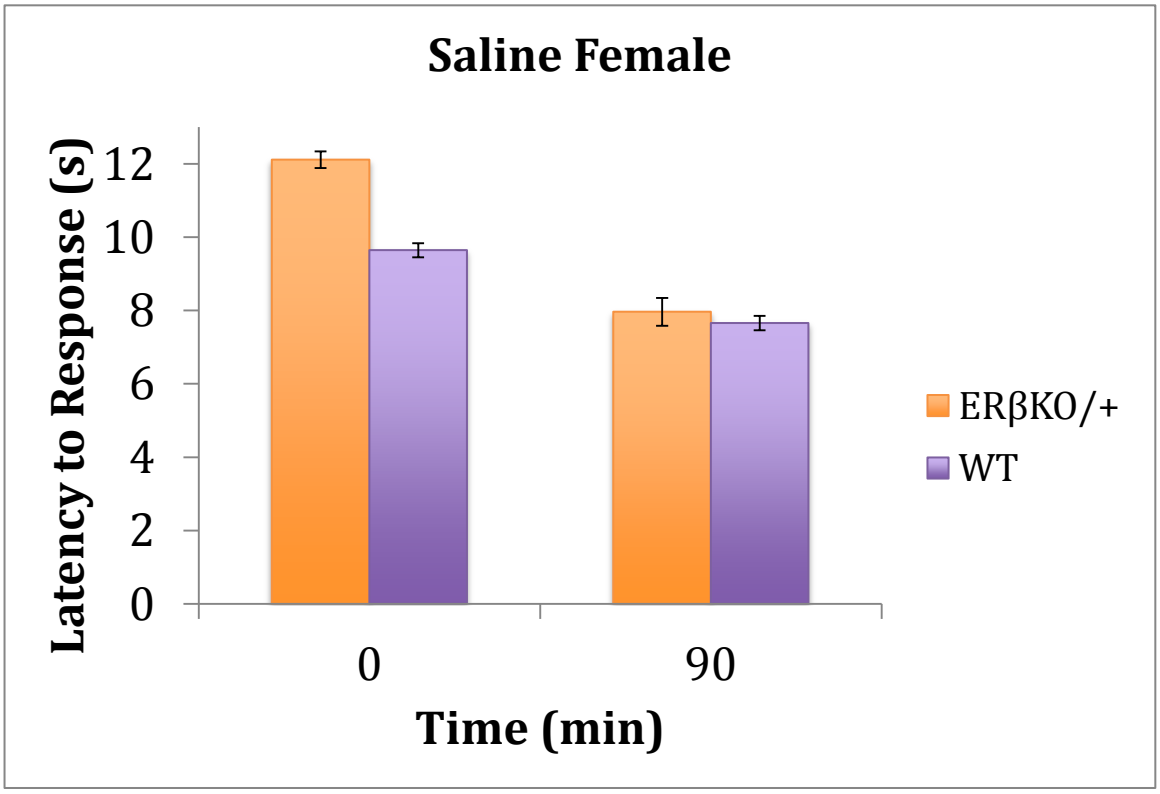

Figure 10: The paw-withdrawal response averages to thermal stimuli of female $ERβ^{KO}/+$ and wildtype mice at baseline and 90 minutes post saline injection. $ERβ^{KO}/+$ n=20 and wildtype n=26.

To determine the effect NTG has on thermal sensitivity of ERβ^{KO}/+ mice, female ERβ^{KO}/+ and wildtype mice were both injected with 5 mg/kg NTG (Figure 11). At baseline, ERβ^{KO}/+ mice responded with an average of 12.11 ± 0.22 (s) and wildtype mice responded with an average of 9.64 ± 0.19 (s). The latency to response 90 minutes after 5 mg/kg NTG injection for ERβ^{KO}/+ mice decreased significantly from baseline response ($p=4.08E^{-29}$), while the response of wildtype mice at 90 minutes post injection was not different from the response at baseline (p=0.13). ERβ^{KO}/+ mice responded faster than the wildtype mice 90 minutes after NTG injection (p=0.04). ERβ^{KO}/+ mice had an average paw withdrawal response of 8.10 ± 0.25 (s), while wildtype mice responded in an average of 9.02 ± 0.37 (s).

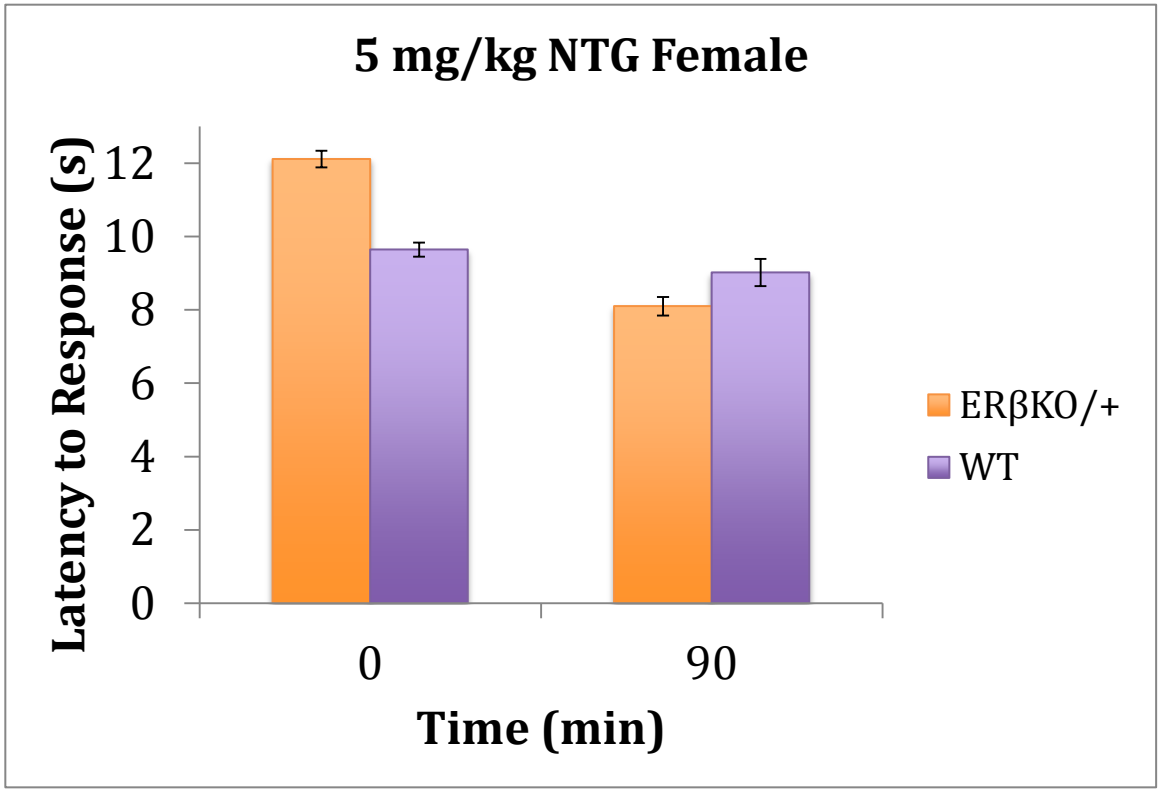

Figure 11: The paw-withdrawal response averages to thermal stimuli of female ERβ^{KO}/+ and wildtype mice at baseline and 90 minutes post 5 mg/kg NTG injection. ERβ^{KO}/+ n= 21 and wildtype n=26.

To determine if only female ERβ^{KO}/+ mice responded differently from wildtype mice to thermal stimuli, male ERβ^{KO}/+ and wildtype mice were injected with saline and 5 mg/kg NTG.

To establish a control for general stress of injection, ERβ^{KO}/+ and wildtype males were injected with saline (Figure 12). At baseline, male ERβ^{KO}/+ responded much slower to thermal stimuli than wildtype male mice ($p=2.41E^{-7}$). Male ERβ^{KO}/+ mice responded with an average latency to response of 12.15 ± 0.22 (s), while wildtype mice responded with an average of 10.49 ± 0.22 (s). However, 90 minutes after saline injection, the previous difference between the ERβ^{KO}/+ and wildtype siblings is no longer present ($p=0.25$). ERβ^{KO}/+ mice had an average paw withdrawal of 9.17 ± 0.25 (s) and wildtype mice had an average paw withdrawal of 8.69 ± 0.27 (s). The initial difference between the female ERβ^{KO}/+ and wildtype animals is again present between the male ERβ^{KO}/+ and wildtype mice, indicating ERβ^{KO}/+ decreases sensitivity to thermal stimuli. This difference is however, nullified by injection stress.

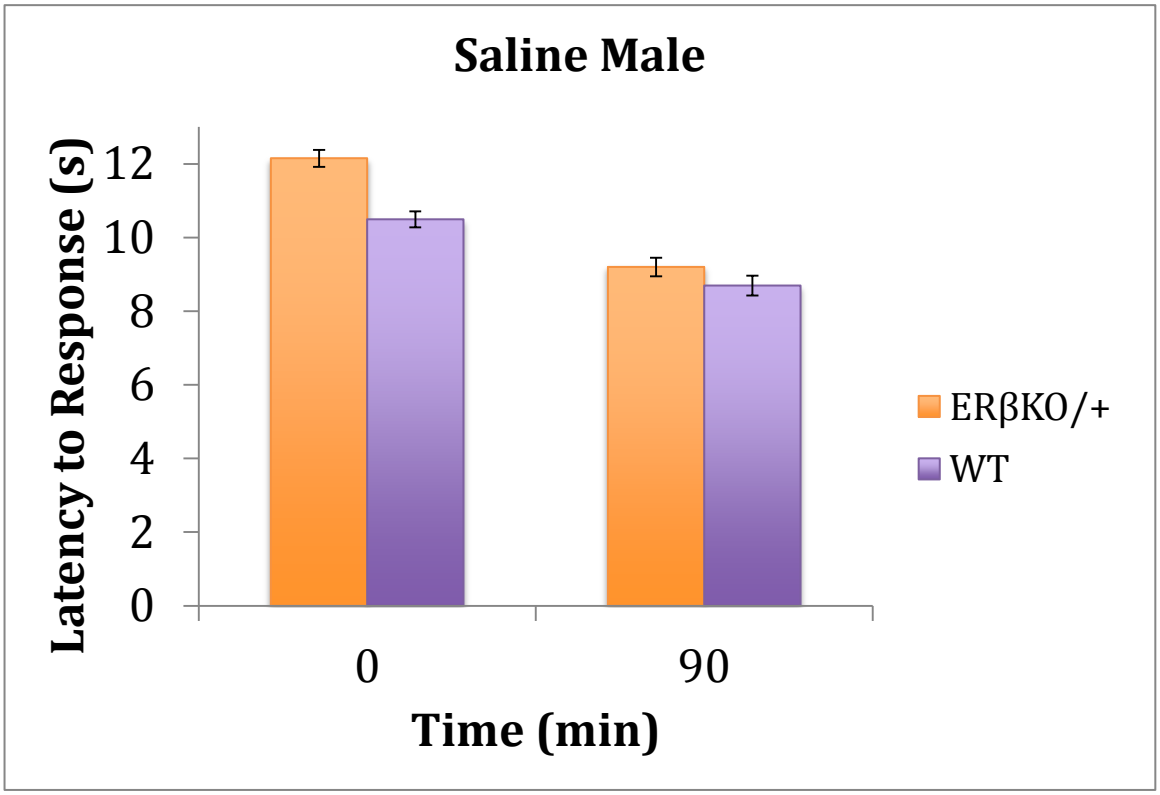

Figure 12: The paw-withdrawal response averages to thermal stimuli of male ERβ^{KO}/+ and wildtype mice at baseline and 90 minutes post saline injection. ERβ^{KO}/+ n=20 and wildtype n=21.

To compare the effect of NTG on thermal sensitivity of ERβ^{KO}/+ mice, ERβ^{KO}/+ and wildtype animals were injected with 5 mg/kg NTG (Figure 13). At baseline, Male ERβ^{KO}/+ mice responded with an average latency to response of 12.15 ± 0.22 (s), while wildtype mice responded with an average of 10.49 ± 0.22 (s). After 90 minutes post injection, the initial difference between ERβ^{KO}/+ mice and their wildtype siblings is no longer present (p= 0.31). ERβ^{KO}/+ mice had a latency to response average of 9.24 ± 0.31 (s) while wildtype mice had a response average of 9.27 ± 0.37 (s).

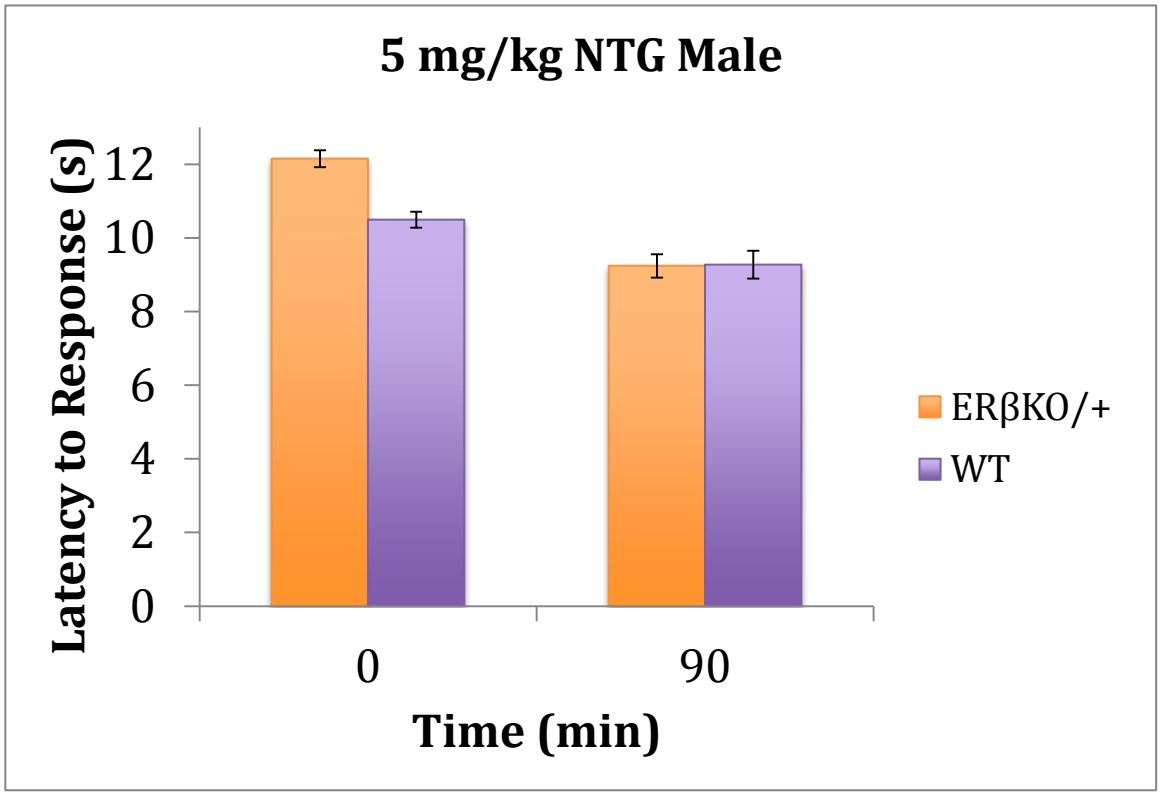

Figure 13: The paw-withdrawal response averages to thermal stimuli of male ERβ^{KO}/+ and wildtype mice at baseline and 90 minutes post 5 mg/kg NTG injection. ERβ^{KO}/+ n= 20 and wildtype n=21.

To determine if ERβ is downstream from CK1δ in the mechanism of migraine, we performed an epistasis experiment. If ERβ is downstream of CK1δ, then reducing ERβ function will suppress the CK1δ-T44A phenotype of shorter latency to response to thermal stimuli. This

means that the CK1δ-T44A; ERβ^{KO}/+ mice will have the same phenotype as ERβ. To determine the effect of CK1δ-T44A on ERβ, the average paw-withdrawal response to thermal stimuli of CK1δ-T44A; ERβ^{KO}/+ animals was compared to the response times of wildtype, CK1δ-T44A, and ERβ^{KO}/+ siblings (Figure 14). At baseline, female CK1δ-T44A: ERβ^{KO}/+ mice had a longer latency to response than wildtype siblings (p= 7.26E^{-9}). CK1δ-T44A; ERβ^{KO}/+ mice responded at baseline with an average of 11.58 ± 0.28 (s), while wildtype mice responded with

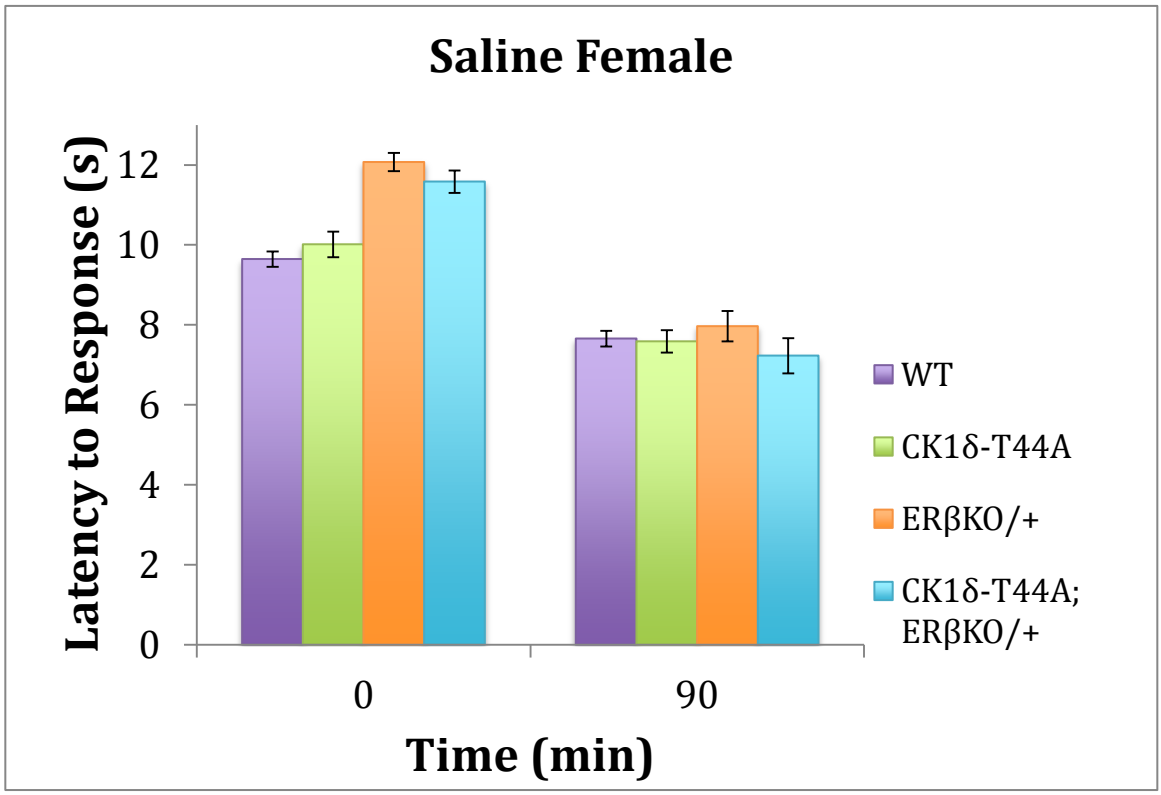

Figure 14: The paw-withdrawal response averages to thermal stimuli of female wildtype, CK1δ-T44A, ERβ^{KO}/+ and CK1δ-T44A; ERβ^{KO}/+ mice at baseline and 90 minutes post saline injection. Wildtype n=26, CK1δ-T44A n=13, ERβ^{KO}/+ n=20 and CK1δ-T44A; ERβ^{KO}/+ n=12.

an average of 9.64 ± 0.19 (s). CK1δ-T44A; ERβ^{KO}/+ mice also responded with a longer latency response than CK1δ-T44A mice at baseline (p= 8.37E^{-5}). CK1δ-T44A; ERβ^{KO}/+ responded to heat in 11.58 ± 0.28 (s) and the CK1δ-T44A mice responded in 10.01 ± 0.32 (s). However, CK1δ-T44A; ERβ^{KO}/+ animals did not respond differently than ERβ^{KO}/+ mice (p= 0.14), which

responded in 12.11 ± 0.22 (s). This result indicates the CK1δ-T44A mutation does not affect the initial decrease in sensitivity to thermal stimuli caused by reducing ERβ function that is present at baseline. To determine the effect of injection stress on thermal sensitivity, all four genotypes of animals were injected with saline (Figure 14). Wildtype mice responded to heat in 7.66 ± 0.20(s), CK1δ-T44A mice responded in 7.59 ± 0.28 (s), ERβ^{KO}/+ mice responded in 7.96 ± 0.38 (s), and CK1δ-T44A; ERβ^{KO}/+ mice responded in 7.23 ± 0.44 (s). After injection with saline, all genotypes experienced a significant increase in sensitivity to thermal stimuli, however there was no difference between any of the genotypes.

To determine if NTG affects the sensitivity to thermal stimuli of ERβ^{KO}/+ and CK1δ-T44A; ERβ^{KO}/+ mice compared to wildtype and CK1δ-T44A mice, all mice were injected

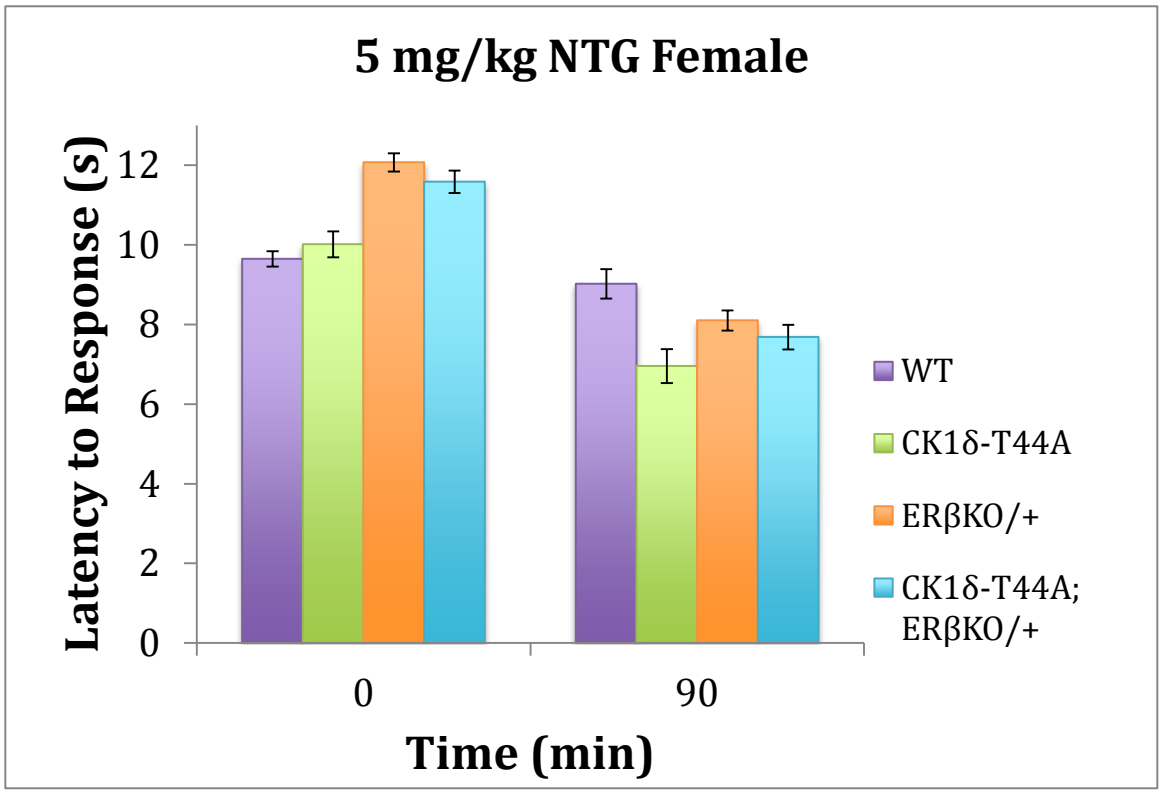

Figure 15: The paw-withdrawal response averages to thermal stimuli of female wildtype, CK1δ-T44A, ERβ^{KO}/+ and CK1δ-T44A; ERβ^{KO}/+ mice at baseline and 90 minutes post 5 mg/kg NTG injection. Wildtype n= 26, CK1δ-T44A n= 14, ERβ^{KO}/+ n= 21 and CK1δ-T44A; ERβ^{KO}/+ n=12.

with 5 mg/kg NTG (Figure 15). At baseline, wildtype mice responded to heat in 9.64 ± 0.19 (s), CK1δ-T44A mice responded in 10.01 ± 0.32 (s), ERβ^{KO}/+ mice responded in 12.11 ± 0.22 (s), and CK1δ-T44A; ERβ^{KO}/+ mice responded in 11.58 ± 0.28 (s). At 90 minutes post NTG injection, CK1δ-T44A mice responded quicker than ERβ^{KO}/+ mice (p= 0.03). Transgenic CK1δ-T44A mice responded with an average of 6.95 ± 0.42 (s) and ERβ^{KO}/+ mice responded with an average of 8.10 ± 0.25 (s). However, at 90 minutes after 5 mg/kg NTG injection, CK1δ-T44A mice which respond in 6.95 ± 0.42 (s), do not respond significantly differently from CK1δ-T44A; ERβ^{KO}/+ which have an average of 7.68± 0.31 (s) (p= 0.17).

Discussion

A recent study shows that female ERβ^{KO}/+ mice have decreased nociception responses compared to wildtype siblings after acute activation afferent neuron nociceptors, but that male ERβ^{KO}/+ mice do not[92]. Our data indicate that both male and female ERβ^{KO}/+ mice are less sensitive to thermal stimuli from a calibrated heat source than wildtype mice at baseline, before injection. This data suggests ERβ is necessary for thermal nociception. Estrogen signaling in pain is complex, but some studies show high levels of estrogen result in increased pain sensitivity[73, 74, 75]. Estrogen alters gene transcription in the trigeminal ganglion, increasing capsaicin-evoked inward currents, calcium influx, and immunoreactive calcitonin gene-related peptides, which increase pain behavior[93]. By reducing ERβ function, the affect of estrogen in pain modulation is decreased, resulting in decreased pain sensitivity.

At baseline, both male and female wildtype and CK1δ-T44A mice respond to thermal stimuli similarly. CK1δ-T44A; ERβ^{KO}/+ mice are less sensitive to thermal stimuli at baseline suggesting that reducing the function of ERβ decreases animal sensitivity to thermal stimuli

regardless of CK1δ function. If CK1δ function is important to modulation of thermal sensitivity through ERβ, then we would expect that reducing function of both would increase the $ER\beta^{KO}/+$ phenotype. Since ERβ is fully functional in CK1δ-T44A mice and CK1δ-T44A mice have wildtype thermal sensitivity at baseline, we can conclude that CK1δ-T44A does not modulate ERβ to affect thermal sensitivity in untreated animals.

Ninety minutes after injection with saline, $ER\beta^{KO}/+$ and wildtype mice respond to thermal stimuli similarly. Saline injection is biologically innocuous, but the injection can cause stress to the animal and stress can increase nociception[94]. Though ERβ modulates thermal nociception in non-stressful conditions, stressful conditions could over-ride the affects of estrogen signaling and could be regulated by a different mechanism. For instance, thermal sensory neurons could be activated by the stressful condition regardless of estrogen signaling. For example, the stress hormone cortisol is implicated in many brain functions such as, the regulation of neuronal cell birth, differentiation and apoptosis, as well as dendritic arborization and synaptic function[95]. Increases in stress raise cortisol levels, and result in increased pain perception[94]. Stress activated the neurons of $ER\beta^{KO}/+$ and wildtype mice and resulted in increased hypersensitivity to thermal stimuli possibly because of a another signaling mechanism like cortisol.

Nitroglycerin (NTG) is broken down into nitric oxide (NO) in the body and activates the trigeminal nucleus caudalis to increase sensitivity to thermal and mechanical stimuli[9a-d]. Migraine patients become sensitive to sensory stimuli at clinical doses of NTG, while people who are unaffected by migraine do not become more sensitive to sensory stimuli at the same dose[8]. When female CK1δ-T44A animals are injected with 5 mg/Kg NTG, they become hypersensitive to thermal stimuli, while wildtype siblings are not affected by the same dose.

Ninety minutes after 5 mg/kg NTG, ERβ^{KO}/+ mice respond to thermal stimuli like wildtype mice. This result means that estrogen signaling through ERβ is likely not part of the pathway to NTG-induced thermal sensitivity. However, these are heterozygous ERβ^{KO}/+ animals, so it could be that one copy of ERβ is sufficient for NTG-induced thermal sensitivity.

While CK1δ-T44A mice respond faster to thermal stimuli than ERβ^{KO}/+, 90 minutes after 5 mg/kg NTG, CK1δ-T44A; ERβ^{KO}/+ mice do not. However, ERβ^{KO}/+ mice are not different from CK1δ-T44A; ERβ^{KO}/+ mice post injection. CK1δ-T44A; ERβ^{KO}/+ show a trend toward less sensitivity to thermal stimuli after 5 mg/kg NTG and CK1δ-T44A mice, though not statistically different. To draw the conclusion that CK1δ is upstream of ERβ and is acting on ERβ to modulate the mechanism of migraine, it is likely necessary to completely eliminate ERβ function in a CK1δ-T44A mutant background (CK1δ-T44A; ERβ^{KO}/ ERβ^{KO}). These mice then need to be tested for hypersensitivity to thermal stimuli after NTG injection. If the effect of the CK1δ-T44A is abolished in these mice, then we can conclude that CK1δ is upstream of ERβ and is acting on ERβ to modulate the mechanism of migraine.

We show that both male and female ERβ^{KO}/+ mice are less sensitive to thermal stimuli than wildtype mice at baseline. This suggests that ERβ plays an important role in thermal nociception. However, since there is no difference between ERβ^{KO}/+ and wildtype mice after saline or NTG injection, thermal sensory neurons could be activated by the stressful conditions, through another pathway, regardless of estrogen signaling. This means that estrogen signaling through ERβ is likely not part of the pathway to NTG induced thermal sensitivity. However, these are heterozygous ERβ^{KO}/+ animals, so it could be that one copy of ERβ is sufficient for NTG-induced thermal sensitivity and it is necessary to generate complete ERβ^{KO}/ ERβ^{KO} mice to fully eliminate the effect of the CK1δ-T44A on NTG-induced hypersensitivity to heat.

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