Gender, age, and stress plasticity in BK channel expression in the mouse adrenal medulla

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Abstract

Catecholamine release from chromaffin cells in the adrenal medulla is a vital element of the stress response. The activity of large conductance Ca^{2+}-and-voltage-gated potassium channels (BK channels) is an important regulator of chromaffin cell excitability. The subunits β2, β4, and the stress-regulated Slo-α splice variant, STREX, modulate the kinetic properties of BK channels and their sensitivity to chemicals. Previous research has found gender dimorphisms in the expression of Slo-α, β2, and STREX mRNA in adrenal medullae from adult SJL mice, with males showing significantly higher expression than females. Recent literature has also identified chronic-stress-dependent increases in the expression of Slo and STREX mRNA in the adult male SJL adrenal medulla. The SJL mouse strain is of interest, as adult SJL males differ from females in size, behavior, and adrenal morphology. This investigation used quantitative real-time RT-PCR to examine changes in the expression of Slo, STREX, β2, β3, and β4 mRNA in the SJL mouse adrenal medulla with respect to gender, stress, and age. Among adult mice tested for Slo, STREX, and β2, males showed significantly higher mRNA expression in all subunits compared to females. Male adolescent mice showed higher mRNA expression of Slo, STREX, β2, β3, and β4 compared to females, significantly for β2. Stress increased the expression of all BK subunits in adolescent mice, significantly for β2, β3 and STREX. In age comparisons, adolescent mice showed significantly higher expression of β2 mRNA and significantly lower expression of Slo and STREX mRNA compared to adults. These results suggest male mice have more excitable chromaffin cells than do females and provide evidence for a role of chronic stress in shaping excitability and, in turn, levels of catecholamines in the blood.

Introduction

The stress response is orchestrated by the HPA (Hypothalamus-Pituitary-Adrenal) Stress Axis, revealing a complex, nuanced, and elegant system of interconnection and regulation. Evidence of stress in an animal is most clearly manifest in the presence of chemicals in the bloodstream, most notably glucocorticoids and catecholamines. The adrenal gland thus plays a vital role in the stress response, with the adrenal cortex releasing glucocorticoids, such as cortisol and corticosterone, and the adrenal medulla releasing the catecholamines epinephrine and norepinephrine. Catecholamine release is orchestrated by chromaffin cells, the primary constituents of the adrenal medulla, which are themselves subject to close regulation.

A major regulator of chromaffin cell excitability is the action of large conductance potassium channels (BK channels), which are, in turn, regulated by glucocorticoids and pituitary hormones (Lovell et. al 2004, Lovell & McCobb 2001). BK channels are calcium- and voltage-gated channels found within various physiological systems, most interestingly in cells involved in the HPA axis. These channels are composed of four alpha subunits encoded by *Slo*, as well as up to four accessory β subunits, β1-4 (Salkoff et. al 2006, Lu et al 2006). BK channels provide the basis for rapid firing in adrenal chromaffin cells by allowing for faster action potential repolarization (Solaro et. al 2005). Enhanced repetitive firing ability can, in turn, result in greater amounts of catecholamine release by chromaffin cells, constituting a critical element of the sympathetic nervous system's "fight or flight" response. Facilitating the channel's role in the stress response, inclusion of an alternative splicing product known as STREX (STress Regulated EXon) increases the channel's activity and ability to

promote repetitive firing (Xie and McCobb 1998). In adrenal chromaffin cells, a direct consequence of reduced STREX expression is reduced excitability (Lovell and McCobb 2001, Xie and McCobb 1998). The β subunits and STREX splicing expand the repertoire of regulatory mechanisms by allowing channel activity to be modulated by stress steroids. The β subunits confer sensitivity of BK gating to modulatory effects of steroid hormones that take effect immediately. The β subunits are differentially sensitive to steroids. For example, β2, which includes an inactivation domain, is particularly sensitive to dehydroepiandrosterone (DHEA), while β4 is more sensitive to corticosterone (CORT) (King et. al 2006). Steroid hormones also regulate BK channel activity by regulating the phosphorylation state of BK-α (Slo) subunits, and the presence of the STREX exon is a critical player in determining how a BK channel responds (Shipston et al., 1996, Tian et al., 2001, Tian et al., 2004). On a much slower timescale, removal of the pituitary in rats results in a decrease in the abundance of STREX mRNA over a period of days, unless adrenocorticotropin hormone (ACTH) is provided through injection (Lovell and McCobb 2001, Xie and McCobb 1998).

The idea of gender differences represents an interesting paradigm in exploring the stress response. Evidence exists for gender dimorphisms in both disease susceptibility and HPA axis function, as estradiol, for example, potentiates stress response in females (Kudielka and Kirschbaum 2005). In males, testosterone has also been found to modulate alternative splicing of *Slo* (Mahmoud et. al 2004). Evidence for gender dimorphism also exists specifically in the adrenal medulla. In unstressed tree shrews, for instance, males show a higher abundance of STREX as compared with females (McCobb et. al 2003). In adult control SJL mouse adrenal medullae, Ahmed (2007) observed

gender dimorphisms in the expression of Slo, STREX, β2, and β4, with males demonstrating significantly greater expression of these genes compared to females. Among adolescent SJL mice, Dias (2008) found a significant difference between male and female adrenal medullary expression of Slo and β2 but not of β4 or STREX, again with males showing greater abundances of mRNA than females where there were differences. In the context of this evidence, corroborating the gender-dimorphic results in the SJL mouse adrenal medulla in adult mice in Ahmed (2007) and expanding upon the results in young mice in Dias (2008) are paramount goals of this investigation. In addition to demonstrating sexual dimorphisms in channel subunit expression, SJL mice are of interest as SJL males have smaller adrenals than SJL females (Dias 2008) and are behaviorally aggressive (Chatterjee et al 2009).

In addition to gender differences in stress axis function, another key perspective is identifying how the function of the stress axis is itself plastic to stress experience. In tree shrew adrenals, for instance, chronic stress influences the splicing of *Slo*, reducing the abundance of *Slo* transcripts including STREX (McCobb et al 2003). In chromaffin cells from the rat adrenal, removal of the source of ACTH through hypophysectomy decreased the abundance of STREX, but injection of ACTH maintained STREX levels (Xie and McCobb 1998). In a study of chronically stressed SJL adult male mice, Chatterjee et. al (2009) found stress increased the abundance of Slo and STREX mRNA in the adrenal medulla and produced a qualitative decrease in β2 and β4 subunit mRNA abundance. Relative to Slo, the abundances of the β2 and β4 subunits were found to decrease significantly in response to stress (Chatterjee et al 2009). Combining the variables of gender and stress, Dias (2008) found a significant difference between stressed males and

stressed females in β2, but no such difference in Slo, β2, β4, or STREX in young SJL mouse adrenal medullae.

A third perspective on the context of the stress response is that of age, which, in turn, raises the question of how and when aspects of the stress response (including gender and/or stress plasticity) arise in mouse development. Among recently deceased human subjects, Elhamdani et al (2002) found adrenal chromaffin cells from older individuals to demonstrate less efficacious excitation-secretion coupling than those from younger individuals, which may in part account for the observation of a decline in the levels of epinephrine with age. In Chatterjee et. al (2009), the finding of a bigger effect of stress on BK subunit expression levels among 6-week-old mice versus 9-week-old mice suggested the potential of age as a variable of interest in the study of adrenal chromaffin cell excitability. While the studies in Dias (2008) were conducted on young SJL mouse adrenal medullae, a better understanding of excitability in young mice can come from considering the BK-associated gene expression of young mice in the context of direct comparisons with adult mice.

The primary goal of this investigation is to ascertain whether mRNA expression patterns of BK-associated genes provide additional information about the time course of the development of gender differences. An absence of evidence for gender dimorphisms in mRNA production in prepubescent mice would suggest the gender difference arises during puberty, while a similar finding in young mice would suggest the difference arises much earlier in development. Another goal of the investigation is to identify effects of stress on the expression of these genes. Furthermore, as gene expression is analyzed using quantitative real-time PCR, the nature of the experiment constrains the number of

samples and genes that can be tested at once. Thus, with sample sizes between 10 and 20 mice per experiment, the only way to make appropriate conclusions about gene expression patterns involves assembling a larger sample size from multiple experiments with requisite considerations for inter-experimental variability. In order to address these questions, quantitative real-time RT-PCR experiments were performed to assess the mRNA expression of Slo, β2, β3, β4, and STREX genes relative to the housekeeping gene succinate dehydrogenase-A (SDHA) in the adrenal medullae of stressed and non-stressed groups of male and female prepubescent SJL mice, as well as on non-stressed adult mice.

Materials and Methods

Animals

The animals used in the q-RT-PCR experiment on prepubescent mice comprised a sample of 33 3-4 week old SJL mice, divided into four groups based on gender and stress treatment. Specifically, the sample included 10 control males, 8 control females, 8 stressed males, and 7 stressed females. Mice were housed in cages with mice of the same gender and stress conditions. Stress was administered via 5-minute forced swim tests every 7 hours from 7 AM to 9 PM (Dias 2008). For mice in control and stressed groups, a forced swim test was performed immediately before sacrifice. Mice were euthanized by cervical dislocation.

The animals used in the q-RT-PCR experiment on adult mice consisted of 36 mice over 8 weeks in age, including 19 males and 17 females. These mice were kept in similar living conditions as the control adolescent mice and were not subject to any

stress. Among these mice, 24 were euthanized with CO, while the remainder of the mice were euthanized by cervical dislocation.

Experimental information for all animals is summarized in the Supplementary Information, Tables 5 and 6.

RNA Extraction from Tissue and Reverse Transcription

RNA Extraction and Reverse Transcription protocols were followed as per Lai and McCobb (2002). Adrenal medullae were isolated from whole adrenals in Ringer's solution under a dissection microscope. Combined adrenal medullae from each mouse were disrupted using a plastic mortar and pestle (Fisher Scientific) and homogenized with a 20-gauge syringe (Becton Dickinson). Total RNA was harvested from the tissue with Qiagen's RNeasy Mini Kit and quantified with a UV NanoDrop spectrophotometer. To obtain cDNA, a maximum of 1.7 μg RNA was used in 20 μL reverse transcription reactions containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3mM $MgCl_2$, 10 mM DTT, 1 mM dNTP, 200 units of Superscript II reverse transcriptase (Invitrogen), 20 units of RNase-Out (Invitrogen), and 10 μM oligo(dT).

Primer Design

For the experiment on adolescent mice and the qPCR run for 12 of the adult mice, the same primers were used as in Dias (2008) for experimental assays and for constructing calibration series. Primers for *Slo, PanBK, β2, β3, β4, STREX,* and *SDHA* were ordered from Integrated DNA Technologies' 25 nmol custom oligo synthesis service. The primers for β2, β3, β4, STREX, and SDHA were designed using the

PrimerSelect application in the DNAStar Lasergene software package. Sequences for these primers were as follows: β2 [F: 5'-GACCCAGAAGGAAACCAGAAGAGT-3', R: 5'-ACACCCCCAGCCATCATACAAGTT-3'], β3 [F: 5'-TCCCCATGCCTGCAGGTGTTCGTA-3', R: 5'-GGTCCTGATGGCCTCGTCGTCGTA-3'], β4 [F: 5'-CTACACAGCGACCAGCACCAG-3', R: 5'-ACAGGGCGGGATATAGGAGCACTT-3'], STREX [F: 5'-CAGGCCGTGTGCGTGGTAAC-3', R: 5'-ACGGAAACTGGTGGAGCAATCAT-3'], SDHA [F: 5'-TGGGGAGTGCCGTGGTGTCATTG-3', R: 5'-CTGTGCCGTCCCCTGTGCTGGTAT-3']. The primer for *Slo* came from Chen et. al (2005) and had the following sequence: [F: 5'-CTCCAATGAAATGTACACAGAATATCTC-3', R: 5'-CTATCATCAGGAGCTTAAGCTTCACA-3'].

Experimental assays for 24 of the adult mice used the primers in Ahmed (2007). For β2, the sequences were as follows: [F: 5'-GACGAGAAAAGAAATATCTACCAGAAAAT-3', R: 5'-CAGCCTTCAGAGCTGTCACAGT-3']. For the calibration series for β2, the following sequences were used: [F: 5'-CAGTGGCCGGACCTCTTCATCT-3', R: 5'-CCACACCCCCAGCCATCATAC-3'].

Quantitative Real-Time PCR (qPCR)

1 μL of cDNA template was loaded into each well of a 384-well plate, in addition to 0.5 μL of each 10 μM primer of the primer pair of the appropriate assay, 3 μL of

nuclease-free water, and 2.5 μL of a 2X SYBR GreenER PCR Master Mix (Applied Biosystems). The qPCR experiments were performed in an Applied Biosystems Prism 7900-HT thermocycler connected to a computer running SDS software. For the subsequent experiment on adult and very young mice, 5 μL of 2X SYBR GreenER PCR Master Mix were used in the same reaction volume. The experiments followed a standard program optimized for the SYBR Green marker: 50°C for 2 minutes, 90°C for 10 minutes, 40 repeats of (90°C for 15 seconds, 60°C for 1 minute), 95°C for 15 seconds, and 60°C for 15 seconds. Quantitative results from the qPCR experiments were obtained in Applied Biosystems' Sequence Detection System (SDS) software as Ct values, representing the cycle at which an optical scanner within the thermocycler detected a level of fluorescence beyond an automatically determined threshold. The absolute copy number corresponding to a particular Ct value was determined through regression analysis using a standard curve for each gene assayed. In order to create standard curves of known copy number, a serial dilution series was created for each gene, encompassing dilution by a factor of 2.9505 at each transfer between a series of 11 tubes, for a series spanning 100 to 500,000 copies. The series for each gene was created from a PCR product generated from gene-specific primer amplification of a cDNA template, which was subsequently quantified using a UV NanoDrop spectrophotometer. In addition to BK-associated genes, SDHA was included as an assay as a housekeeping gene – a gene thought to demonstrate sustained expression through multiple treatments. Copy numbers for BK-associated genes were normalized to SDHA to control for RNA extraction efficiency in each animal.

For the 36 adult mice, 24 of the mice were from Ahmed (2007). These mice were assayed for Slo, STREX, β2, and β-Actin using TaqMan primer-probe mixes. Slo, STREX, and β-Actin were assayed using Assay-on-Demand kits (Applied Biosystems), while β2 was assayed using the following probe sequence: 5'-ACCATGACCTCCTGGAC-3'. In qPCR runs on these groups, estimated copy numbers were obtained from standard curves from calibration series each consisting of 21 points of known concentrations of templates for a certain gene and varying percentages of STREX relative to ZERO (an alternative splice variant without STREX). Template DNA for Slo-α, Slo-β, and β-Actin was generated from *Xenopus* oocytes expression by TOPO TA vectors (Promega) with for each gene. Plasmid DNA purified with a Qiagen Maxi Prep kit for each gene was then quantified with a spectrophotometer. Three mixes were generated by varying the percentages by weight of STREX and ZERO (BK channels without a STREX insert) in Slo-α, and varying the percentages by weight of β2 and β4 in Slo-β: mix 1 had 15% STREX and 10% β2, mix 2 had 45% STREX and 55% β2, and mix 3 had 85% STREX and 85% β2. A 21-point calibration series was generated from diluting each of the three mixes to obtain 7 known concentrations differing by a factor of 3, from 30 pg/μL to 0.3 pg/μL.

Data Analysis

Raw data from each experiment was exported from SDS into Microsoft Excel, in which the regression and graphical analyses were performed. For pooled qPCR data, copy numbers were combined in Microsoft Excel with those from the experiments in Dias (2008), and statistical analysis was performed in Minitab. For 16 of the 35 mice in

the pooled data sample, copy numbers for Pan BK – a segment within the Slo gene – was used in place of Total BK copy numbers.

A general linear model (GLM) was used to examine differences between adolescent control males, stressed males, control females, and stressed females in the following gene copy number ratios: β2 to SDHA, β3 to SDHA, β4 to SDHA, Slo to SDHA, STREX to SDHA, β2 to Slo, β3 to Slo, β4 to Slo, and STREX to Slo. The use of GLM allowed for the simultaneous computation of one-, two-, and three-way ANOVA comparisons of independent factors. One-way comparisons looked for differences in copy number ratios with respect to a single factor, while two-way comparisons looked for a relationship between two independent factors; a three-way comparison; a three-way comparison looked for an influence of a third independent factor on any possible two-way comparison. In addition to gender and stress as individual factors, the experiment from which each data point originated was included as a third factor, to test for the effect of non-biological variation between assay plates in combining data from multiple qPCR plates into a pooled sample. In a three-way comparison, inter-plate variation was not found to significantly influence two-way comparisons of gender and stress in any group, meaning if gender and stress were to interact. This means that, were gender and stress to have shown a two-way interaction between one another, this comparison would not have been affected by inter-plate variation. Two-way comparisons were computed for every possible combination of two factors from the three available factors.

To account for variation between assay plates, the ratios of copy number for a particular gene relative to the copy number for a housekeeping gene were ranked on each plate. This was possible as each assay plate contained similar numbers of test groups:

males and females on plates comparing adults; and control males, stressed males, control females, and stressed females on plates comparing adolescents. Rankings were converted into percentiles based on the number of mice on a plate, such that a mouse with the greatest copy number for a gene would have the highest percentile score. Percentile scores for mice from all three adult plates were combined into an adult data pool, and percentile scores for mice from all three adolescent plates were combined into an adolescent data pool. These pooled data sets were, in turn, used in non-parametric Mann-Whitney tests.

Results

Quantitative Real-Time RT-PCR for BK-associated genes in mouse adrenal medullae

Quantitative real-time PCR allowed for the assessment of the mRNA expression for Slo, STREX, β2, β3, and β4 by running simultaneous assays for each. In order to test gender, stress, and age as independent variables influencing the expression of these genes, two experiments were conducted. The first experiment comprised a sample of male and female adolescent mice, divided into a group of chronically stressed subjects and a group of control subjects not having been subjected to chronic stress; control and stressed groups both included male and female mice. The second experiment consisted of adult male and adult female subjects. Absolute copy numbers for each gene were normalized to that of the "housekeeping" gene unaffected by manipulation of gender or stress conditions. In these experiments, SDHA was used for this purpose. Standardization with respect to a housekeeping gene corrects for differences in RNA extraction yield from dissected tissues and allows for comparison of mRNA expression

results across different experiments. An example of a standard curve used to obtain estimates of absolute copy numbers are depicted in Figure 1, and copy numbers from qPCR are summarized in Tables 1-4.

Statistical analysis of pooled qPCR data sets

A pooled data sample of copy number ratios from 35 adolescent SJL mice was assembled from three qPCR experiments. Two-way comparisons between gender and stress were not significant in any copy number ratio. Two-way comparisons found inter-plate variation to significantly influence stress comparisons ($p=0.05$) for the following ratios: Slo to SDHA, STREX to Slo, β3 to STREX, β4 to STREX, β2 to β4. Two-way comparisons found inter-plate variation to significantly influence gender comparisons ($p=0.05$) for the following ratios: β2 to SDHA, β3 to SDHA, β4 to SDHA, Slo to SDHA, STREX to SDHA, β3 to Slo, β4 to Slo, STREX to Slo, β2 to STREX, β3 to STREX, β4 to STREX, β2 to β4. Due to the relatively large number of comparisons affected by inter-plate variation, a non-parametric approach was used, applying the Mann-Whitney U (Wilcoxon Rank-Sum) Test. For adolescent mice, the test was applied to a pooled data set of percentile scores based on ranking mice by copy number relative to SDHA relative to other mice on their respective experimental assay plates for each gene (see Methods).

For adult mice, percentile scores for ranked copy number for genes of interest relative to housekeeping genes were pooled from three qPCR runs for a total sample size of 36 adult SJL mice. Statistical tests on the pooled data set were conducted using the non-parametric Mann-Whitney U Test.

Gender differences in BK-associated gene expression

Among non-stressed adult mice, males and females were significantly different in Slo percentile scores (2-tailed Mann-Whitney U, W=433, N1=19, N2=16, p=0.0027), with males showing 3.8-fold higher expression than females in copy numbers normalized by SDHA. Among adults, males and females were significantly different in STREX percentile scores (2-tailed Mann-Whitney U, W=404.0, N1=18, N2=16, p=0.0022), with males showing 1.3-fold higher expression than females in copy numbers normalized by SDHA. Adult males and adult females were significantly different in β2 percentile scores (2-tailed Mann-Whitney U, W=400.5, N1=18, N2=16, p=.0033), with males showing 4.1-fold higher expression than females in copy numbers normalized by SDHA. In adults, percentiles scores for the ratio of β2 to Slo were significantly different between males and females, with females having a 3.1-fold higher ratio than males in copy number ratios (2-tailed Mann-Whitney U, W=214.0, N1=18, N2=15, p=0.0009). Percentile scores for adult gender comparisons are depicted in Figure 2, and copy number relative to SDHA is depicted in Figure 3.

In non-stressed adolescent mice, no significant gender differences were found in Slo percentile scores among control or stressed mice, although in both groups males showed qualitatively higher expression of Slo compared to females. This trend can be seen in graphs for adolescent gender comparisons depicted by percentile score in Figure 4, and by copy number relative to SDHA in Figure 5. Adolescent mice showed no significant gender differences in STREX percentile scores among control or stressed mice groups, although in both groups males showed qualitatively higher expression of STREX compared to females. Among control adolescent mice, males showed

significantly higher percentile scores for β2 compared to females (2-tailed Mann-Whitney U, W=118.0, N1=10, N2=8, p=0.0451), reflecting a 3.5-fold difference in copy numbers normalized by SDHA. A similar trend was observed in stressed adolescent mice, although the difference was not statistically significant. No significant gender differences were found in the percentile scores of β3 among adolescent control or stressed mice, though males showed qualitatively higher expression of β3 compared to females. For β4 in adolescent mice, males showed higher (but not significantly higher) expression of β4 compared to females among controls, and stressed males showed significantly higher percentile scores than stressed females (2-tailed Mann-Whitney U, W=96.0, N1=9, N2=7, p=0.05) by a factor of 6.5 in copy numbers normalized by SDHA. Adolescent mice showed no significant gender difference in the ratios of β2 to Slo, β3 to Slo, β4 to Slo, or STREX to Slo.

Age differences in BK-associated gene expression

Age comparisons in the expression of Slo, β2, and STREX mRNA are shown in Figure 6. Adult males and adolescent males were significantly different in Slo expression relative to SDHA (2-tailed Mann-Whitney U, W=348.0, N1=19, N2=9, p=0.001), with adults showing 5.1-fold higher expression than adolescents. Adult females and adolescent females were found not to differ significantly in Slo expression relative to SDHA, although adults showed qualitatively higher expression than adolescents. No significant difference was observed between adult and adolescent mice of either gender in the expression of STREX relative to SDHA. Adult males and adolescent males were found to differ significantly in the expression of β2 relative to SDHA (2-tailed Mann-

Whitney U, W=218.0, N1=18, N2=10, p=0.05), with adolescent mice showing 2.8-fold higher expression than adults. Adult females and adolescent females were found to differ significantly in the expression of β2 relative to SDHA (2-tailed Mann-Whitney U, W=154.0, N1=16, N2=8, p=0.01), with adolescent mice showing 3.2-fold higher expression than adults.

Within both male and female groups, adult and adolescent mice were found to be significantly different in the ratio of β2 to Slo, with adolescents showing higher ratios [Males: (2-tailed Mann-Whitney U, W=178.0, N1=18, N2=9, p=0.0002), Females: (2-tailed Mann-Whitney U, W=145.0, N1=15, N2=8, p=0.03)]. Adolescent males showed a higher β2 to Slo ratio than adult males by a factor of 28.1, and adolescent females showed a higher ratio than adult females by a factor of 20.6 No significant age differences were found in the ratio of STREX to Slo.

Stress-induced differences in BK-associated gene expression

All stressed mice were adolescents. Qualitatively, all subunits showed increased mRNA expression due to stress, which can be seen both in percentile scores in Figure 7, and in copy numbers relative to SDHA in Figure 8. GLM analysis found significant differences due to stress in the ratios of β2 to SDHA (p=0.01), β4 to SDHA (p=0.05), and STREX to SDHA (p=0.01), and no significant differences in the other groups (p=0.05). GLM analysis similarly found significant differences due to stress in the ratios of β2 to Slo (p=0.05) and β4 to Slo (p=0.05) but no significant differences in the other groups (p=0.05). All subsequent data analysis was performed using Mann-Whitney tests.

Stressed animals did not show any significant differences in the expression of Slo relative to SDHA compared to controls, although stressed mice of both genders showed qualitatively higher expression of Slo mRNA than in controls. For STREX, both male and female mice had significantly higher STREX percentile scores in stressed groups compared to controls [Males: (1-tailed Mann-Whitney U, W=78.0, N1=10, N2=9, p=0.0396), Females: (2-tailed Mann-Whitney U, W=44.0, N1=8, N2=7, p=0.0240)]. Stressed males on average had 3.7 times as many STREX transcripts as male controls, and stressed females on average had 3.3 times as many STREX transcripts as female controls in copy numbers normalized by SDHA. Stress was also found to have an effect on β2 percentile scores, as stressed males were significantly different from control males (2-tailed Mann-Whitney U, W=73.0, N1=10, N2=9, p=0.0305) and stressed females were significantly different from control females (2-tailed Mann-Whitney U, W=44.0, N1=8, N2=7, p=0.0240). Stressed males showed 3.5-fold increases in β2 expression compared to control males, and stressed females showed a 2.6-fold increase in β2 expression compared to control females in copy numbers normalized by SDHA. In both males and females, stressed animals showed higher percentile scores for β2 compared to controls. For β3 relative to SDHA, stressed females showed higher but not significantly different expression of β3 compared to controls, while stressed males showed higher percentile scores than their control counterparts, reflected by a 5.1-fold difference in copy numbers normalized by SDHA (2-tailed Mann-Whitney U, W=69.0, N1=10, N2=9, p=0.0128). For β4, stressed males showed significantly higher percentile scores compared to control males (2-tailed Mann-Whitney U, W=74.0, N1=10, N2=9, p=0.0373). No significant

differences were observed between stressed females and control females in β4 percentile scores, although stressed animals showed qualitatively higher expression than controls.

No significant stress differences were found in the ratios of β2 to Slo, β3 to Slo, β4 to Slo, or STREX to Slo. Stoichiometric ratios of subunit expression relative to Slo are interesting from the perspective of BK channel assembly, as functional differences may correspond to different subunit compositions in the channel (Lovell et. al 2001, Lovell et. al 2004).

Discussion:

The finding of significant gender differences in the expression of Slo, STREX, and β2 among adult SJL mice in Ahmed (2007) raised the possibility of gender-based differences in chromaffin cell excitability. Thus, replicating these findings in a larger sample size represents an important step in the exploration of this phenomenon, as evidence for gender differences on the mRNA expression level could suggest future electrophysiological investigations to see if these differences truly influence excitability. This study replicated the finding that males show significantly greater abundances of Slo, β2, and STREX compared to females in the adult SJL mouse adrenal medulla.

After observing gender dimorphisms among adult mice, it was important from a developmental perspective to understand whether similar dynamics in expression were present within adolescent mice. Similarity with adult mice in terms of gender differences would suggest the sexes begin to differ at a prepubescent age, whereas dissimilarity with adult mice would lend evidence to the idea of gender differences arising during puberty. In this investigation, although similar qualitative trends were observed in prepubescent

mice as in adult mice for Slo, STREX, and β2, with higher male expression compared to females, only β2 demonstrated a statistically significant difference among adolescents. The presence of similar qualitative trends in BK channel expression across gender in adolescents as in adults, and a significant gender difference only in the expression of β2, suggests differences in the time course of development of the expression patterns of these genes. While β2 expression appears to mature earliest, STREX and Slo appear to show a delayed onset. The absence of significance in Slo and STREX gender comparisons in adolescents results may suggest a role for puberty in the development of the gender dimorphisms observed in adults in the expression of these genes. Alternatively, however, the presence of the same general trend of higher expression in males compared to females in Slo and STREX, and the significantly higher expression of β2 in males, suggests that some degree of gender difference is already present before puberty, and that only relatively minor adjustments are required for completion of development in BK channel subunit expression. Ultimately, a larger sample size of unstressed adolescent mice is needed to prove whether the slightly greater levels of Slo and STREX mRNA expression in males compared to females is reproducible without reaching significance, or if the trend suggests a difference that would be revealed to be significant with more data.

The role of stress in influencing BK-associated gene expression was another area of focus in this investigation. Elucidating molecular consequences of the adolescent stress experience and how these differ from those in adults is a vital goal in understanding how the stress response develops. As reviewed in Romeo and McEwen (2006), the HPA axis is highly plastic during puberty, and adolescent and adult mice show differences in the time length of ACTH and CORT elevation and in neuronal

activation patterns, in response to both acute and chronic stress. Given the finding by Chatterjee et. al (2009) of significant upregulation of Slo and STREX in the adrenal medullae of chronically stressed young male SJL mice, we hypothesized that prepubescent animals would be more sensitive to stress and thus show larger differences. In this investigation, GLM analysis and Wilcoxon tests both found greater expression of STREX and B2 in stressed prepubescent mice compared to controls, and no significant differences in Slo expression due to stress experience. While the STREX result replicated Chatterjee et. al (2009)'s finding in a prepubescent subset, the differences for β2 and Slo may be explained in part by this investigation's findings of age-dependent expression differences in these genes, as β2 and Slo expression were both found to be significantly different between adolescents and adults. It is also worth noting, however, that the mechanism of stress in Chatterjee et. al (2009), cagemate rotation, was different from that used in this investigation, forced swim, and that Chatterjee et. al (2009) used only male mice; these differences may account for additional variation in comparing results.

The significantly greater abundance of β2, relative to both SDHA and to Slo, among adolescent mice compared to adults may have implications on excitability as a function of age. Chatterjee et. al (2009) found stress to have a greater effect on BK subunit expression in 6 week old mice compared to 9 week old mice, Dias (2008) found stressed adolescent mice to show a significant increase only in β2 expression, and Elhamdani et. al (2002) found an age-dependent decline in adrenal chromaffin cell excitability in humans. Noting the β2 subunit's role in modulating the BK channel's response to steroids such as DHEA (King et. al 2006), these findings suggest younger

mice may have more excitable adrenal chromaffin cells due to a greater sensitivity to stress steroid hormones, facilitated by the β2 subunit. Alternatively, greater abundance of β2 could alter channel function in younger animals independently of steroids. At the same time, however, our finding of lower abundances of Slo-α and STREX transcripts in adolescents compared to adults would suggest lower excitability for adolescents. Large variation was observed in STREX among adults, and the qPCR results reflected questionable stoichiometry of STREX transcripts, particularly in comparison to Ahmed (2007) and Chatterjee (2009). Together, these results highlight the importance of conducting additional experiments to explore age differences, and the possible role for puberty in adjusting channel properties, at the molecular and electrophysiological level.

All data in this experiment were obtained from quantitative real-time RT-PCR. Q-RT-PCR has been used extensively for several years in identifying expression of BK-associated genes (Mahmoud et. al 2002). Yet, q-RT-PCR remains a relatively nascent experimental technique and remains to be fully optimized as a reliable research tool for accurately quantifying gene expression (Rutledge 2004, Bustin 2002). Experiments using q-RT-PCR, particularly the standard curve-based method for obtaining results used in this investigation, are susceptible to inaccuracies due to variation do not utilize all available information from q-RT-PCR machine output of experimental results (Rutledge 2004). The GLM finding of a significant effect of inter-plate variation reflects drawbacks to the standard curve method, as estimates of copy number for reactions on a single plate are normalized relative to other reactions on the same plate but may differ in absolute values between replicates on different plates. To this effect, the method of ranking copy numbers for a particular gene and assigning percentile scores to mice relative to others on

a plate allowed for the ability to combine data from multiple assay plates without exposure to this problem. A comparison of STREX graphs in Figures 2 and 3, for instance, illustrates the benefit of the percentile method in allowing the possibility of making comparisons by reducing variation within groups consisting of data from multiple assay plates. Until q-RT-PCR techniques are improved so as to allow for direct comparison of copy number between plates, such measures are necessary for generating sufficient sample sizes to draw conclusions based on the expression of multiple genes, despite restrictions such as requiring similar numbers of subjects from each test group on each plate.

Together, these results suggest robust differences in chromaffin cell excitability between male and female mice, as well as between unstressed and chronically-stressed mice. Greater abundances of BK channel subunit mRNAs in males compared to females suggest fundamental differences in stress experience with respect to gender, encompassing differences in sympathetic nervous system activation and stress hormone release. The responsiveness of BK channels to androgens, such as in promotion of channel activity through STREX activation by testosterone (Mahmoud and McCobb 2004), creates a particularly intriguing story of interconnection if the implication that male chromaffin cells are more excitable is proven through subsequent research. Similarly, the finding of increased expression of all BK channel subunits in response to chronic stress illustrates molecular consequences of chronic stress that are particularly interesting within the context of previous results relating ACTH availability and STREX abundance and demonstrating changes in chromaffin cell excitability after hypophysectomy (Lovell et al 2001, Xie and McCobb 1998). Persistent elevation in BK

channel subunit expression in chronically stressed animals can render chromaffin cells continuously susceptible to repetitive action potential firing, resulting in chronically high levels of epinephrine and norepinephrine in the blood. The possible effects of gender- and stress-dependent changes in BK subunit composition on channel gating properties and, in turn, catecholamine secretion patterns, introduce a myriad of dimensions of interconnection and regulation that remain to be elucidated.

The findings in this investigation therefore raise a number of testable questions moving forward, primarily whether differences in mRNA expression are reflected in protein translation and activity. The discovery of changes in channel electrophysiology accompanying the evidence provided for differences in mRNA expression across gender, stress, and age would represent an important step forward in understanding HPA axis function.

References:

Ahmed, S. (2007) Strain and gender differences in expression of BK potassium channel subunits in the mouse adrenal gland. Cornell University Honors Thesis.

Bustin, S.A. Quantification of mRNA Using Real-Time Reverse Transcription PCR: Trends and Problems. *J Mol Endocrin* 29: 23-29, 2002.

Chatterjee O, Taylor LA, Ahmed S, Nagaraj S, Hall JJ, Finckbeiner SM, Chan PS, Suda N, King JT, Zeeman ML, McCobb DP (2009). Social stress alters expression of large conductance calcium-activated potassium channel subunits in mouse adrenal medulla and pituitary glands. *J Neuroendocrinol.* 21: 167-176.

Chen MX, Gorman SA, Benson B, Singh K, Hieble JP, Michel MC, Tate SN, Tresize DJ (2004)
Small and intermediate conductance Ca^{2+}-activated K+ channels confer distinctive patterns of distribution in human tissues and differential cellular localisation in the colon and corpus cavernosum. Naunyn Schneidebergs *Arch Pharmacol.* 369(6):602-15.

Dias, C. (2008) The role of sex differences and swim stress in the young mouse adrenal. Cornell University Honors Thesis.

Elhamdani, A., Palfrey, C., & Artalejo, C. (2002). Ageing changes the cellular basis of the "fight-or-flight" response in human adrenal chromaffin cells. *Neurobiology of aging.* 23 (2), 287-93.

King JT, Lovell PV, Rishniw M, Kotlikoff MI, Zeeman ML, McCobb DP (2006) Beta2 and
Beta4 subunits of BK channels confer differential sensitivity to acute modulation by steroid hormones. *J Neurophysiol.* 95(5):2878-88.

Kudielka, B., & Kirschbaum, C. (2005). Sex differences in HPA axis responses to stress: a review. *Biological psychology.* 69 (1), 113-32.

Lai GJ, McCobb DP (2002) Opposing actions of adrenal androgens and glucocorticoids on
alternative splicing of Slo potassium channels in bovine chromaffin cells. *Proc Natl Acad Sci USA.* 99:7722–7727.

Lai GJ, McCobb DP (2006) Regulation of alternative splicing of slo k+ channels in adrenal and
pituitary during the stress-hyporesponsive period of rat development. *Endocrinology* 147 (8): 3961-3967.

Lovell, P. V. and McCobb, D. P. (2001). Pituitary Control of BK Potassium Channel

Function and Intrinsic Firing Properties of Adrenal Chromaffin Cells. *J. Neurosci.* 21: 3429-3442.

Lovell PV, King JT, McCobb, DP (2004) Acute modulation of adrenal chromaffin cell BK
channel gating and cell excitability by glucocorticoids. *J Neurophysiol* 91: 561-570.

Mahmoud SF, McCobb DP (2004)Regulation of slo potassium channel alternative splicing in the
pituitary by gonadal testosterone. *J Neuroendocrinology.* 16 (3) 237-243.

Mahmoud, S., Bezzerides, A., Riba, R., Lai, G.-J., Lovell, P., Hara, Y. (2002). Accurate quantitative RT-PCR for relative expression of Slo splice variants. *Journal of neuroscience methods.* 115 (2), 189-98.

McCobb DP, Hara Y, Lai GJ, Mahmoud SF and Flugge G (2003) Subordination stress alters
alternative splicing of the Slo gene in tree shrew adrenals. *Hormones and Behavior.* 43: 180-186.

McCormick CM, Roberts D, Kopeikina K, Kelsey JE (2005) Long-lasting, sex- and age-specific effects of social stressors on corticosterone responses to restraint and on locomotor
responses to psychostimulants in rats. *Horm Behav.* 48 (1): 64-74.

Romeo, R., & McEwen, B. (2006). Stress and the adolescent brain. *Annals of the New York Academy of Sciences.* 1094: 202-14.

Rutledge, R.G (2004). Sigmoidal curve-fitting redefines quantitative real-time PCR with the prospective of developing automated high-throughput applications. *Nucleic Acids Res.* 32(22):178-186.

Salkoff, L., Butler, A., Ferreira, G., Santi, C., & Wei, A. (2006). High-conductance potassium channels of the SLO family. *Nature Reviews Neuroscience.* 7 (12), 921-31.

Shipston MJ, Armstrong DL 1996 Activation of protein kinase C inhibits calcium-activated potassium channels in rat pituitary tumour cells. *J Physiol (Lond)* 493:665-672

Solaro, C., Prakriya, M., Ding, J., & Lingle, C. (1995). Inactivating and noninactivating Ca(2+)- and voltage-dependent K+ current in rat adrenal chromaffin cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience , 15* (9), 6110-23.

Tian L, Hammond MS, Florance H, Antoni FA, Shipston MJ 2001 Alternative splicing determines sensitivity of murine calcium-activated potassium channels to glucocorticoids. J Physiol 537:57-68.

Tian L, Coghill LS, McClafferty H, MacDonald SH, Antoni FA, Ruth P, Knaus HG, Shipston MJ 2004 Distinct stoichiometry of BKCa channel tetramer phosphorylation specifies channel activation and inhibition by cAMP-dependent protein kinase. Proc Natl Acad Sci U S A

Xie J, McCobb DP (1998) Control of alternative splicing of potassium channels by stress hormones. Science. 280 (5362): 443-446.

Figures and Tables:

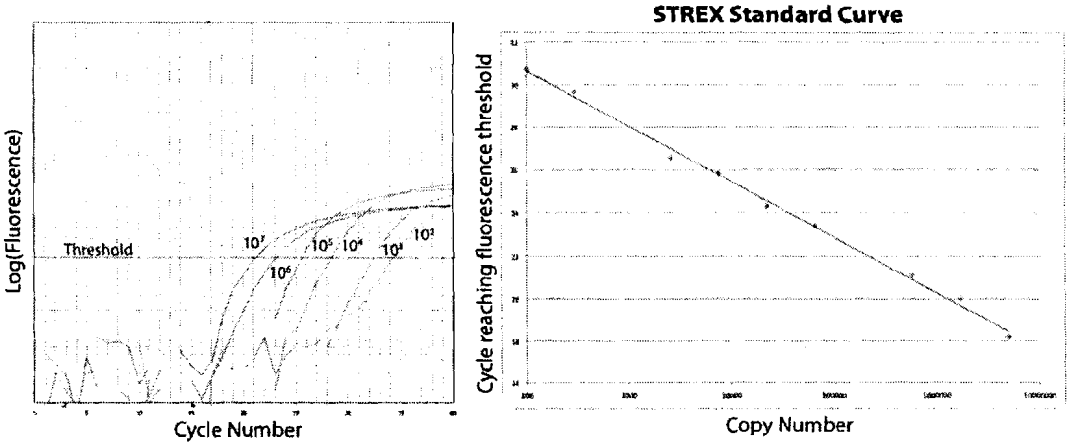

Figure 1:
Left: Fluorescence level versus qPCR cycle for STREX. Each curve corresponds to an order of magnitude in copy number and a point on the graph shown on the right.
Right: STREX standard curve regression analysis based on 11-point calibration series, used to determine copy number. Fluorescence amount corresponds to the concenration of double-stranded DNA.

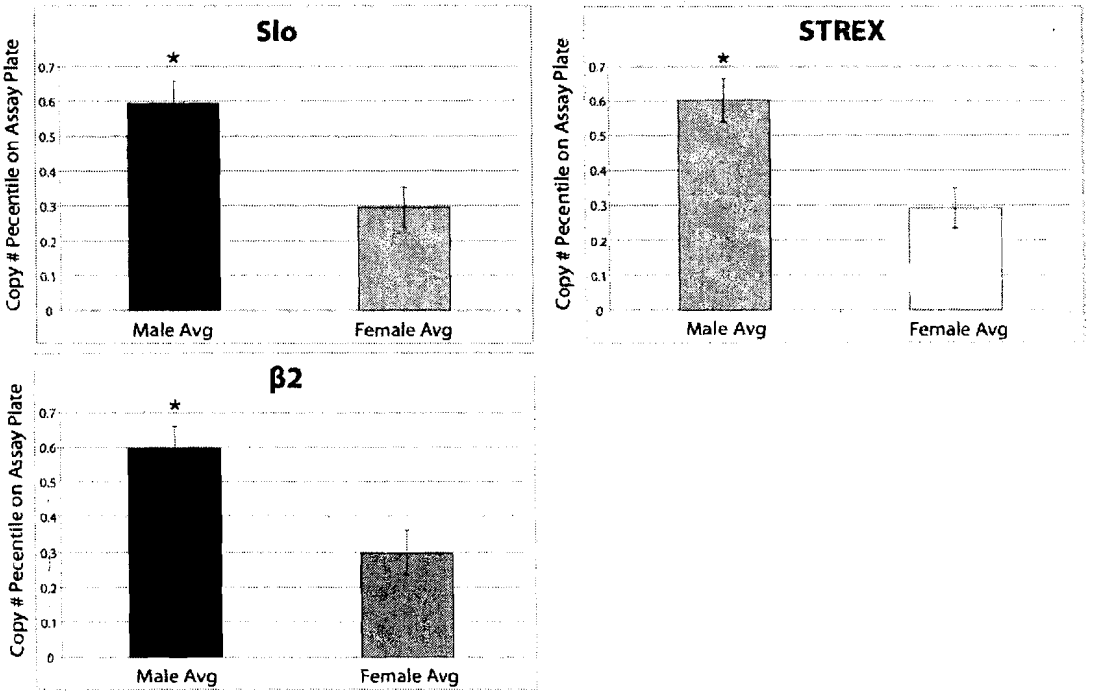

Figure 2: Percentile scores for non-stressed adult mice.

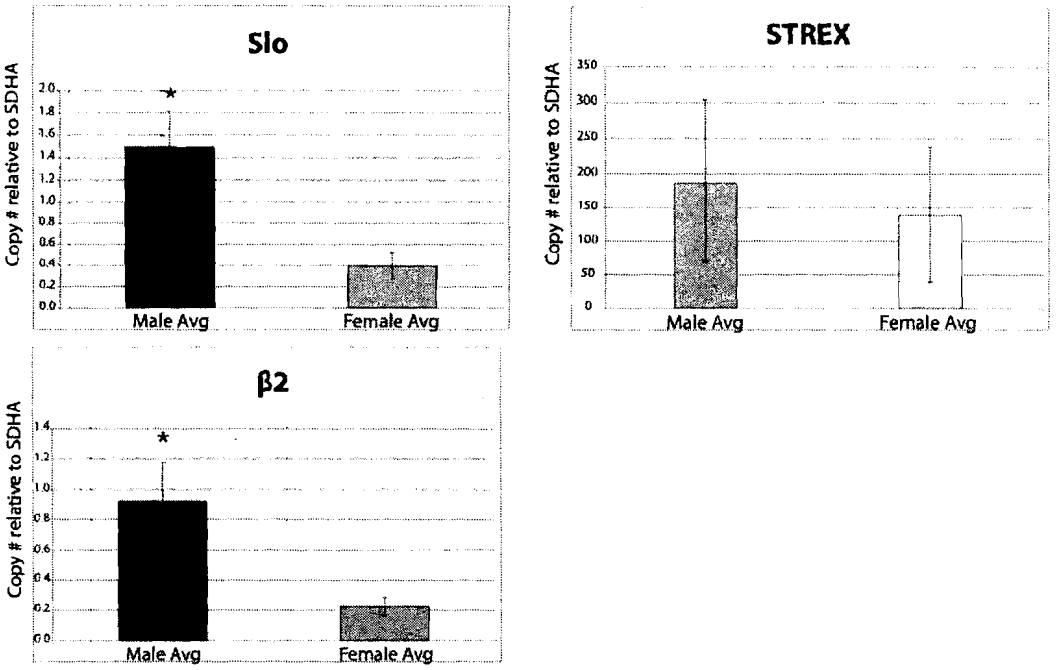

Figure 3: Expression of BK channel subunits in non-stressed adult mice, given as copy number relative to SDHA.

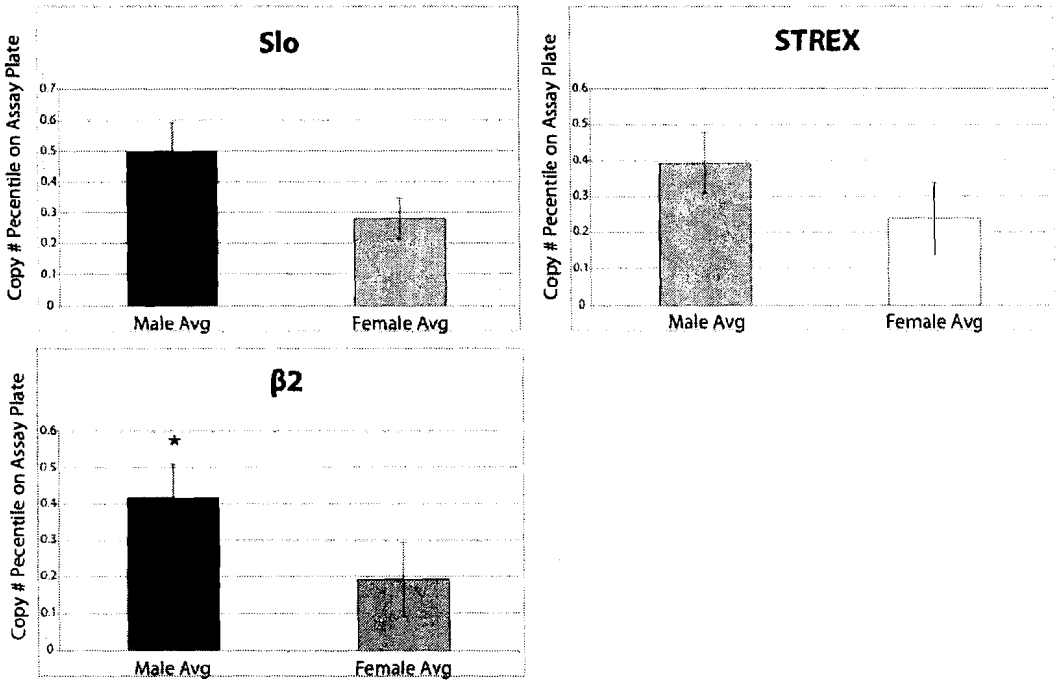

Figure 4: Percentile scores for non-stressed adolescent mice.

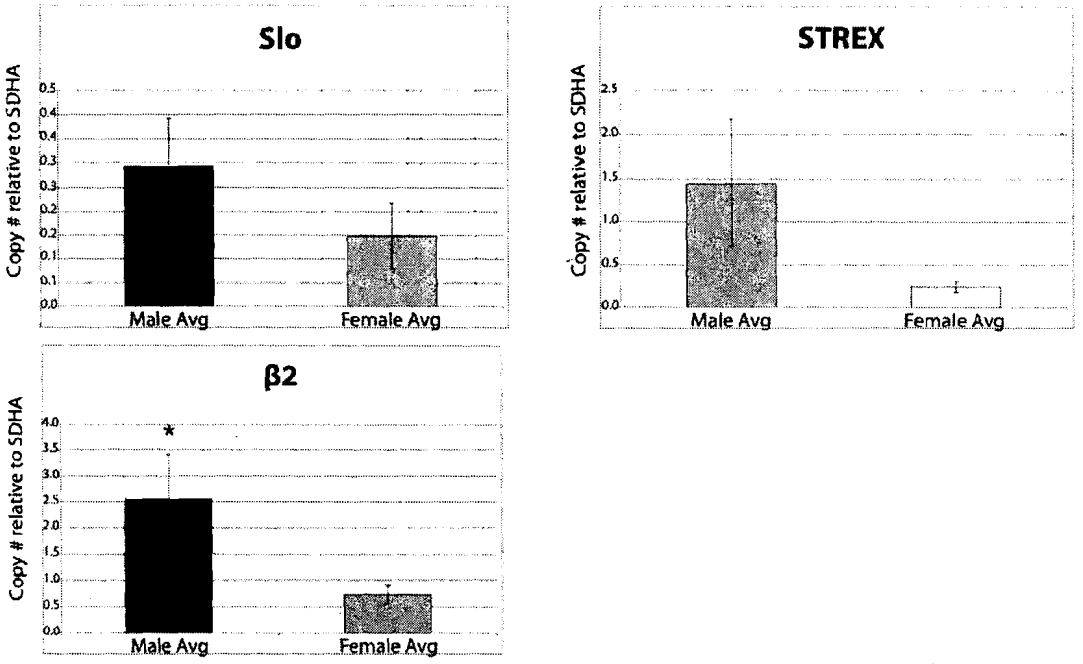

Figure 5: Expression of BK channel subunits in non-stressed adolescent mice, given as copy number relative to SDHA.

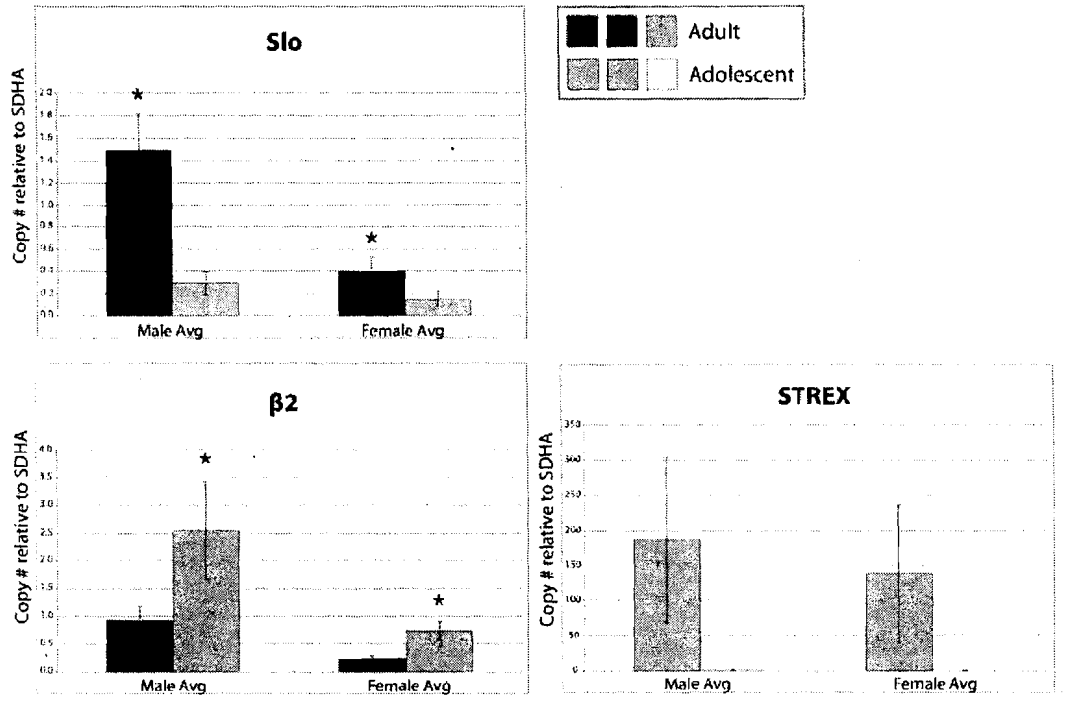

Figure 6: Age comparison in BK channel expression, with copy number relative to SDHA.

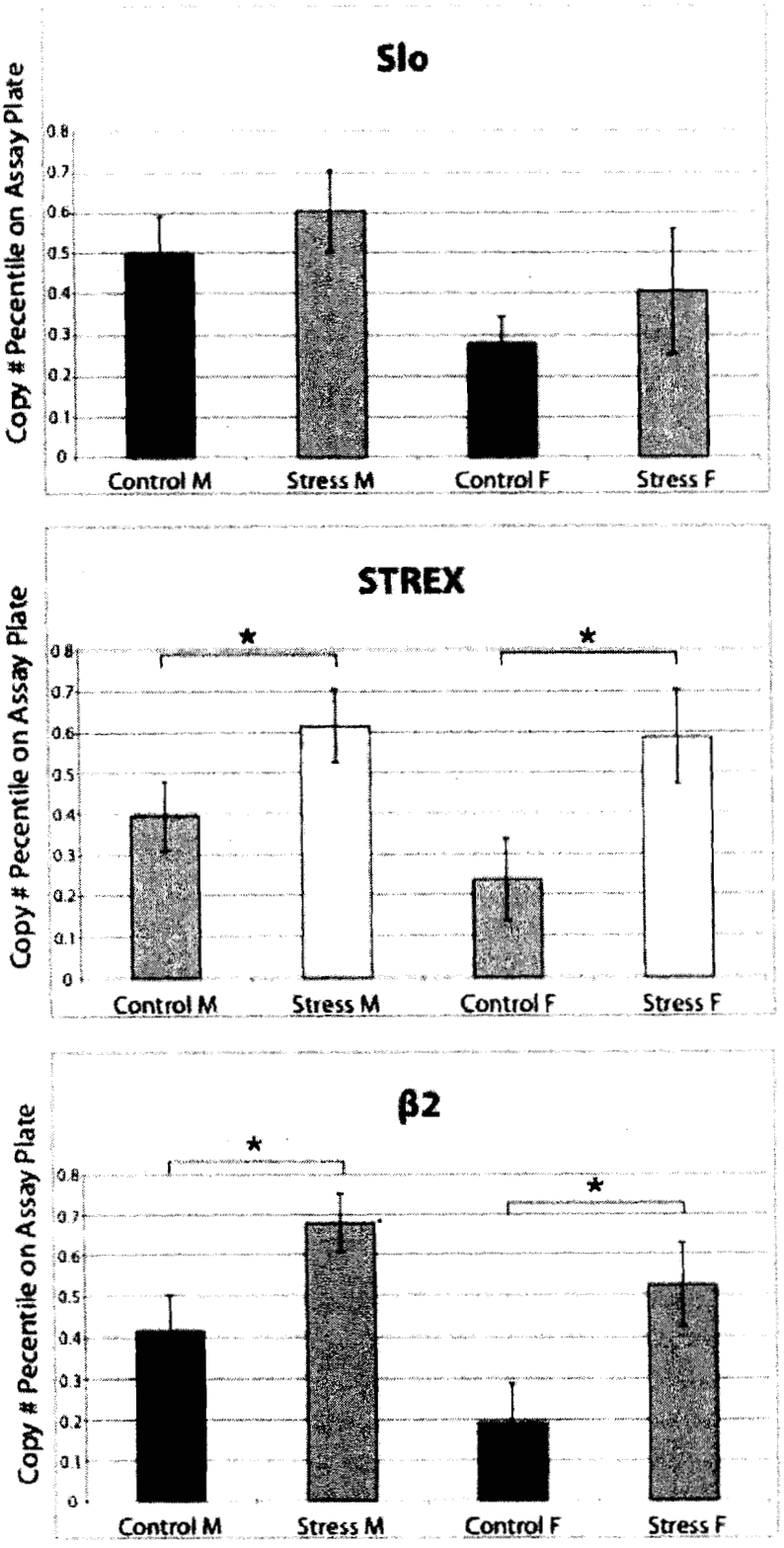

Figure 7: Percentile scores for stressed and non-stressed adolescent mice.

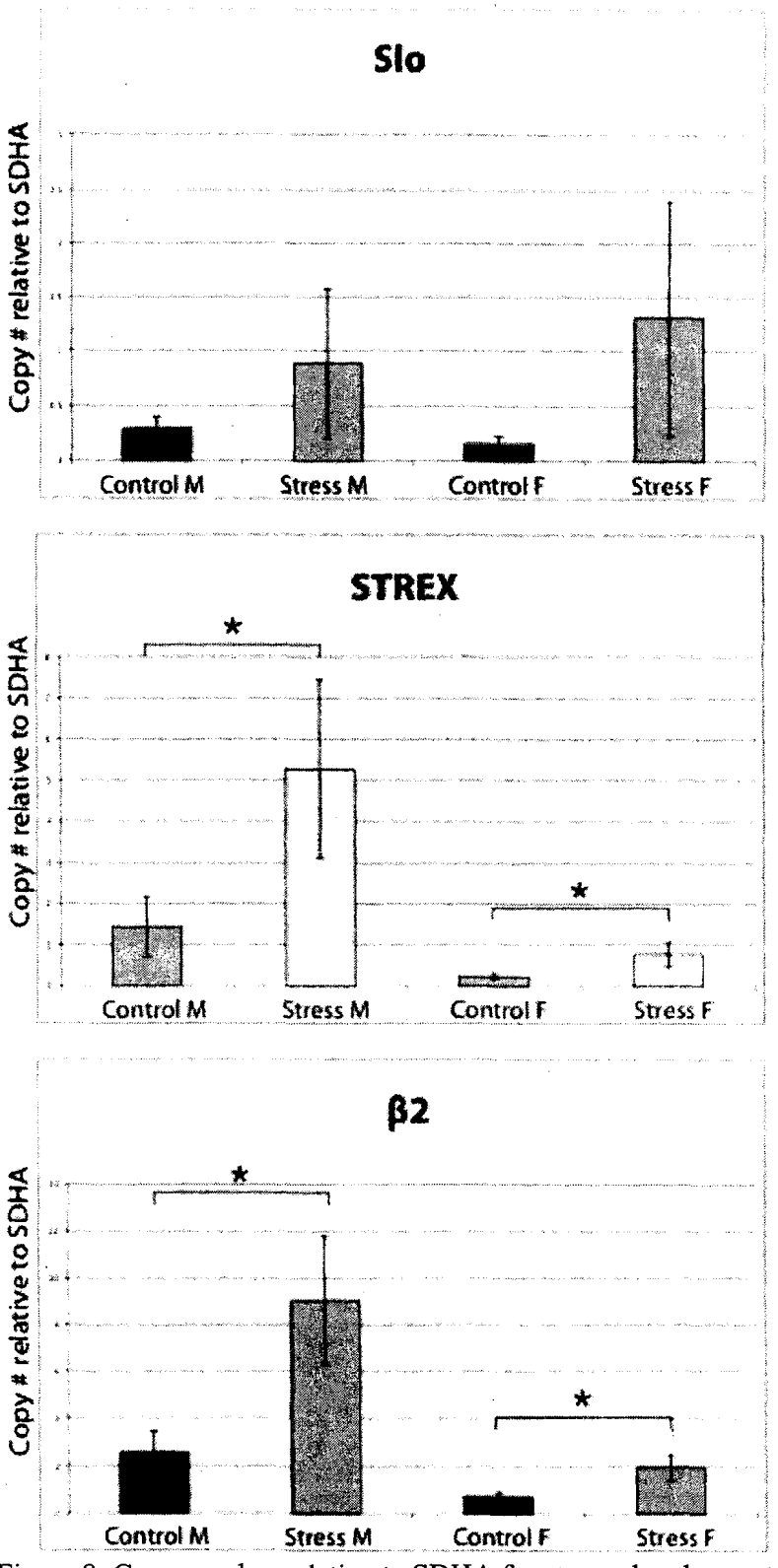

Figure 8: Copy number relative to SDHA for stressed and non-stressed adolescent mice.

	β2/SDHA	β3/SDHA	β4/SDHA	Slo/SDHA	STREX/SDHA	N# (33)
Control M Avg	2.546± 0.880	0.452± 0.207	0.278± 0.124	0.293± 0.100	1.440± 0.730	10
Stress M Avg	9.026± 2.741	2.302± 0.934	0.558± 0.212	0.888± 0.687	5.274± 2.157	8
Control F Avg	0.734± 0.167	0.186± 0.065	0.036± 0.020	0.149± 0.068	0.238± 0.064	8
Stress F Avg	1.936± 0.532	0.382± 0.131	0.086± 0.033	1.313± 1.087	0.779± 0.305	7
Significance	S, GC, GS	S, GC, GS	N	N	S, GC, GS	

Table 1: Adolescent ratios of mRNA expression to SDHA, including standard error. S: Significant stress difference within at least one gender. GC: Gender difference among controls. GS: Gender difference among stressed animals. N: No significant differences.

	B2/Slo	B3/Slo	B4/Slo	STREX/Slo	N#
Control M Avg	18.311± 6.862	2.305± 0.888	1.447± 0.570	7.238± 3.050	10
Stress M Avg	62.152± 25.727	11.337± 6.195	2.132± 0.812	24.787± 11.278	8
Control F Avg	42.244± 26.189	15.061± 10.580	1.119± 0.428	8.138± 3.694	10
Stress F Avg	191.094± 142.126	5.541± 2.647	1.181± 0.653	30.247± 16.532	7
Significance	S	S	N	S	

Table 2: Adolescent ratios of adolescent mRNA expression to Slo mRNA expression, including standard error. S: Significant stress difference within at least one gender. GC: Gender difference among controls. GS: Gender difference among stressed animals. N: No significant differences.

	B2/STREX	B3/STREX	B4/STREX	B2/B4	N#
Control M Avg	4.195± 1.361	0.348± 0.041	0.151± 0.042	54.031± 24.252	10
Stress M Avg	4.931± 1.846	0.492± 0.124	0.111± 0.019	94.388± 56.711	8
Control F Avg	4.112± 1.098	1.306± 0.627	0.167± 0.089	87.254± 27.618	10
Stress F Avg	3.927± 1.107	0.709± 0.286	0.062± 0.023	295.179± 170.119	7
Significance	N	N	N	N	

Table 3: Adolescent ratios of mRNA expression to STREX mRNA expression, including standard error. S: Significant stress difference within at least one gender. GC: Gender difference among controls. GS: Gender difference among stressed animals. N: No significant differences.

	Avg Slo/SDHA	Avg STREX/SDHA	Avg β2/SDHA	Avg STREX/Slo	Avg β2/Slo	Avg β2/STREX	N# (36)
Avg Males	1.493± 0.324	186.504± 118.035	0.922± 0.251	141.263± 78.027	0.651± 0.118	6.665± 2.922	19
Avg Females	0.395± 0.127	138.399± 98.783	0.226± 0.061	194.967± 157.430	2.050± 0.389	0.676± 0.208	17
Significance	S	NS	S	NS	S	S	

Table 4: Adult ratios of mRNA expression, with standard error. Significant gender differences in a ratio are indicated by S: Significant and NS: Not Significant.

Supplementary Information

Expt	Researcher	Year	# Total Mice	# SJL Males	# SJL Females	Housekeeping Gene Used	Detection Method
1	S. Ahmed	2006	10	5	5	β-Actin	Primer/Probe
2	S. Ahmed	2007	12	6	6	SDHA	Primer/Probe, SYBR Green
3	C. Sasannejad	2009	14	8	6	SDHA	SYBR Green

Table 5: Experiments on adult mice.

Expt	Researcher	Year	# Total Mice	# Control SJL Males	# Stress SJL Males	# Control SJL Females	# Stress SJL Females	HK Gene Used	Detection Method
1	C. Dias	2007	16	5	6	3	2	SDHA	SYBR Green
2	C. Dias	2008	8	2	2	2	2	SDHA	SYBR Green
3	C. Sasannejad	2008	11	3	2	3	3	SDHA	SYBR Green

Table 6: Experiments on adolescent mice.