Strain and Gender Differences in Expression of BK Potassium Channel Subunits in Mouse Adrenal Gland

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Abstract

Sexual dimorphisms in adrenal function and distinct behavioral patterns of aggression and "emotionality" in SJL and C57/BL6 mouse strains led to an intriguing question: are there strain- and/or gender-specific differences in the adrenal gland at the level of ion channels? In this study, we focused on the adrenal medulla, which is a critical component of the neuroendocrine system and responsible for modulating reactions to stress and regulation of various body processes including digestion, the immune system, mood, and energy usage. Secretion of epinephrine (EPI) from chromaffin cells of the adrenal medulla is tightly controlled by the electrophysiologial response generated from neural inputs. Several studies suggest that the intrinsic excitability of these cells is modulated by the number and kenetic properties of large-conductance calcium(2+)-activated potassium ion channels (BK channels). Therefore, we focused on BK channels to study strain- and gender-specific differences in the adrenal gland at the level of ion channels. Stress- and sex-steroids have been shown to regulate alternative splicing of the pore-forming subunit of BK channels (Slo-α) in several experimental contexts, and associated β-subunits confer on BK channels distinct sensitivities to acute effects of stress- and sex-steroids. Using a real-time PCR technique, we examined mRNA levels for total *Slo*, STREX (a stress-regulated splice-variant of *Slo*), β2, and β4 in two mouse strains, SJL and C57/BL6. The experimental groups were: SJL males, SJL females, and C57/BL6 males. SJL males expressed significantly higher levels of mRNA for total *Slo*, β2, and β4 than SJL females and C57/BL6 males. STREX levels were not significantly different between the groups, probably due to a small sample size, but on average, SJL males had higher levels of expression than the other two groups. These results suggest

that SJL females and C57/BL6 males are relatively similar in their BK channel expression in the adrenals, while SJL males show distinct expression patterns. From this study, we now have grounds for hypothesizing functional relationships between behavior and adrenal function.

Introduction

Sexual dimorphisms exist within the hypothalamic-pituitary-adrenal (HPA) axis of mice. Extensive experimental evidence suggests that there are gender differences in modulation of glucocorticoid synthesis (Wragg and Speirs, 1952; Kitay, 1963a, b; Sato, 1967; Touma et al., 2004; Malisch et al., 2007). Glucorticoids, such as cortisol and corticosterone, are a group of corticosteroids synthesized in the adrenal gland that control carbohydrate, protein, and fat metabolism. Studies show that female mice have greater basal concentrations of corticosterone in the blood, as well as greater cortiosteroidogenesis in adrenal slices *ex vivo* than males (Lesniewska et al., 1990). The HPA axis is a major part of the neuroendocrine system that controls reactions to stress and regulates various body processes including digestion, the immune system, mood, and energy usage. The intensity of physiological response to a stressor (eg. physical restraint) is higher in females. For example, females have a sharper increase in circulating glucocorticoids than males in response to physical restraint (Jones et al., 1998). Inflammation activates the HPA axis through cytokines, such as IL-1, IL-6, and tumor necrosis factor alpha (TNFα), which are thought to play a central role in stimulating corticotrophin releasing factor (CRH) production by the hypothalamus, thereby inducing glucocorticoid secretion by the adrenals (Besedovsky et al., 1991). Females have a

greater increase in corticosterone concentrations in response to inflammation than males (Imura et al., 1991). Studies also show that there are possible gender differences in the sensitivity to glucocorticoids. Overall, females seem to be more dependent on glucocorticoids than males to control inflammatory and immune responses (i.e. they are more sensitive to changes in glucorticoid levels) (Larbre et al., 1994). Glucocrticoid hormones produced in the adrenal cortex are known to regulate the phenotype and function of the adrenal medulla, for example stimulating the expression of PNMT, the enzyme responsible for converting norepinephrine to epinephrine in chromaffin cells (Kvetnansky et. al., 2006). The McCobb laboratory has shown that glucocorticoids, as well as androgens of adrenal and/or gonadal origin, control expression of BK potassium channel subunits, important determinants of chromaffin cell excitability (Lai and McCobb, 2002; Lovell et al., 2004). In addition to regulating molecular expression at the level of transcription and alternative splicing, steroids have also been shown to modulate BK channel gating acutely (King et al., 2006; Lovell et al., 2004; Lovell and McCobb, 2001). In this study, we were interested in gender differences in adrenomedullary function.

The most popular mammalian model is the mouse (*Mus musculus*) and in this study we focused on two widely used strains: SJL and C57/BL6. In addition to gender differences, we also studied strain differences in adrenal function due to interesting behavioral differences between these two strains. The frequency of inter-male aggression is noticeably higher in SJL than C57/BL6 (unpublished results from the McCobb Lab). In SJL males, there is a high rate of spontaneous fighting amoung males housed together, beginning at about 8 weeks of age (Page and Glenner, 1972). C57/BL6 mice tend to be

more passive than SJL and exhibit low "emotionality" according to their performance on various behavioral assays, like high open-field exploration and a short time of immobility in a forced swim test (Thompson, 1953). These behavioral differences are most likely correlated with natural differences in endocrince function, and particularly adrenal function. Aggression is part of the "fight-or-flight" response initiated by the sympathetic nervous system. Chromaffin cells of the adrenal medulla are the body's main source of the catecholamine hormones epinephrine (EPI) and norepinephrine (NE), which are intimately involved in the "fight-or-flight" reponse. For example, when EPI is secreted into the blood stream, it rapidly prepares the body for action in emergency situations by boosting the supply of oxygen and energy-giving glucose to the brain and muscles.

Sexual dimorphisms in adrenal function of mice and behavioral differences between SJL and C57/BL6 strains led to an intruiguing question: are there strain- and gender-specific differences in the adrenal gland at the level of ion channels? In this study, we focused on the adrenal medulla, which is composed mainly of hormone-producing chromaffin cells. Chromaffin cells are tightly controlled by the electrophysiologial response (action potentials) generated from neural inputs (McCobb et al., 2003). Several studies suggest that the intrinsic excitability of chromaffin cells is modulated by the number and kenetic properties of large-conductance calcium(2+)-activated potassium ion channels (BK channels) (Lovell and McCobb, 2001; McCobb et al., 2003). BK channels are also voltage-gated and play one very clear role in chromaffin cells: they bring about the rapid repolarization after an action potential. As a consequence of this repolarization, sodium (Na^+) ion channel inactivation is minimized and rapidly reversed. BK channels promote intense and sustained firing in response to stimulus input. This increases the

activity of the adrenal gland and in turn the synthesis of corticosteroids and catecholamines.

BK channels are encoded by the *Slo* gene. The α-subunits consist of seven transmembrane domains and four α-subunits cluster to form a water-filled, K^+ ion-selective pore (Lai and McCobb, 2002; Salkoff et al., 2006). BK channels have the largest single-channel conductance of all K^+ selective channels (Salkoff et al., 2006). Pituitary hormones, like adrenocorticotropic hormone (ACTH) and growth hormone (GH), regulate alternative splicing of *Slo* gene transcripts, which adds an extra range of flexibilty to their activity. Alternative splicing might be involved in modulating the long-term excitability of cells that are involved in stress-related responses. The accessibility of BK channels for rapid activation is substantially enhanced by inclusion of an optional exon, referred to as STREX, at one site in *Slo* transcripts. Inclusion of STREX enhances the repetitive firing ability of chromaffin cells, as compared with cells having channels lacking an insert at this splice site (referred to as the ZERO configuration) (Lovell and McCobb, 2001; Lovell et al., 2004). An additional level of modulation of BK channel activity is provided by the variable association of four related β-subunits—β1, β2, β3, and β4. Up to four β subunits, in various combinations, can be associated with one BK channel. This study focused on β2 and β4 subunits because they confer on chromaffin BK channels distinct sensitivities to acute effects of stress and sex steroids (King et al., 2006).

BK channels are clearly important to the electrophysiological properties of the adrenal medulla and the goal of this study was to understand strain- and gender-specific differences in the number and composition of BK channels in the adrenal medulla of SJL

and C57/BL6 mice, given the sexual dimorphisms in adrenal function and behavioral differences between these two mouse strains. This experiment used real-time quantitative PCR (qPCR) to measure differences in *Slo*, STREX, β2, and β4 mRNA expression levels.

Methods

Animals:

6 SJL males, 6 SJL females, and 6 C57/BL6 males were used in this experiment. Mice were 8 to 10 weeks of age (adults), and housed with littermates. This housing environment is known to be non-stressful to the mice. The mice did not have any prior aggressive experience.

RNA extraction and Reverse Transcription (RT)-PCR:

Mice were euthanized with CO, decapitated, and adrenals and pituitary removed and quickly frozen on dry ice. Total RNA was harvested with the Qiagen RNeasy Mini kit. RNA from each animal was quantified with a spectrophotometer, and not more than 1.7 μg was added to each 20-μl reverse-transcription reaction. RT reactions contained 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).

Creation of Calibration Series:

ABI PCR primers (Applied Biosystems) were used according to kit instructions to amplify β-actin mRNA extracted from mouse tissue. β-actin cDNA was cloned in a pGEM vector (Promega) according to kit instuctions. β2 and β4 RNA were amplified

using custom primers: β2 (forward) 5'-CAGTGGCCGGACCTCTTCATCT-3', (reverse) 5'-CCACACCCCCAGCCATCATAC-3'), β4 (forward) 5'-GGAAGCCGAAGACAAGAGCATC-3', (reverse) 5'-ACCACCGGCCAGAGGAAGC-3'). Products were cloned into TOPO TA vector (Promega) according to kit instructions. STREX and ZERO templates made for Xenopus oocyte expression were used (Xie and McCobb, 1998). All clones were confirmed by sequencing. Plasmids were grown up in large quantity (1 liter), and purified with Qiagen Maxiprep kits. For homogeneity, one large DNA stock was made for each of the 5 templates, concentrations of which were carefully determined by averaging 10 independent spectrophotometric readings each, and then diluting to 150 ng/µl.

Three template mixes were created with different ratios of selected templates. Each mix had10 pg/µl of β-actin, 10 pg/µl of *Slo*-α templates, and 10 pg/µl of Slo-β templates, however, ratios of STREX to ZERO and β2 to β4 differed between the 3 mixes. In mixes 1, 2, and 3, respectively, Slo-α was comprised of 15, 45, and 85% STREX (by weight), with the remainder being of the ZERO form. In the same mixes, the 10 pg of *Slo*-β template was comprised of 10, 55, and 85% β2, with remainder being β4. Six dilutions of each mix were made (yielding 7 total concentrations) with, respectively, 30, 10, 3, 1, 0.3, 0.1, and 0.3 pg/µl of total template. Thus 21 calibration data points could be generated for each of 5 templates. Template copy number was determined based on plasmid template sizes. Copy numbers in calibration series were spread over at least three orders of magnitude for each template.

Quantitative Real-Time PCR (qPCR):

Either 2 µl of RT product from tissue RNA or the appropriate amount of calibration template was transferred to each 10-µl real-time PCR reaction, along with TaqMan Universal PCR Master Mix (Applied Biosystems) according to kit instructions. For β-actin, *Slo*, and STREX, 'Assay-on-Demand' kits from Applied Biosystems were used. These contained FAM-Tamra probes. β2 and β4 primer-probe sets were developed in-house, using MGB probes (Applied Biosystems). β2 primer and probe sequences were as follows: (forward-primer) 5'-GACGAGAAAAGAAATATCTACCAGAAAAT-3'; (reverse-primer) 5'-CAGCCTTCAGAGCTGTCACAGT-3'; probe: 5'-ACCATGACCTCCTGGAC-3'. β4 primers and probes were: (forward-primer) 5'-CGCTCTTCATCTTCGGCTTCT-3'; (reverse-primer) 5'-CCGTGGCTTGCAGATCCT-3'; and probe: 5'-CTGGCTCAGTCCCGC-3'. 50 pmol of each primer and probe were included in the reactions. Tissue sample reactions were performed in triplicate. 105 wells on each 384-well plate were dedicated to calibration reactions. Reactions were run on an ABI PRISM 7900 real-time PCR machine.

Fluorescent probes were designed for qPCR. A probe binds to a specific sequence between the two conventional primers used to amplify the sequence. As the sequence is extended, the probe is broken up and fluoresces. The fluorescence is measured cycle-by-cycle using a special thermocycler designed with photosensing and recording electronics. The intensity of fluorescence reflects the rate of reaction. All qPCR data is obtained as a plot of log (reaction rate) vs. cycle number, with results expressed as threshold cycles (Ct). The threshold cycle is the cycle in which the reaction reaches a critical copy number that allows the reaction to proceed at an exponential rate. The Ct is dependent on the

original template copy number added to the reaction, so the Ct allows quantitative measurement of the template. β-actin is used as the housekeeping gene to standardize all assays.

β-actin was used as a housekeeping gene. Copy numbers for *Slo*, STREX, β2, and β4 were normalized to the experimentally obtained copy number for β-actin, our housekeeping gene, in each sample to correct for variation in RNA extraction and experimental conditions. Housekeeping genes are used as internal standards as they are supposed to indicate the rate of transcription of genes which are not affected by experimental conditions. Their levels are presumed to be proportional to the total amount of mRNA being examined. Accurate quantification of a true reference gene allows the normalization of differences in the amount of amplifiable cDNA in individual samples generated by: (1) different amounts of starting material, (2) the quality of the starting material and (3) differences in RNA extraction and cDNA synthesis, since the reference gene is exposed to the same preparation steps as the genes of interest (*Slo*, STREX, β2, and β4). In this study, differences in expression of subunits relative to beta-actin would imply strain- and/or gender-specific transcriptional regulation.

Quantitative Analysis:

Ct values (Cycle number at which probe fluorescence achieved detection threshold) was determined for each reaction. Standard curves were generated in Excel using a linear least-squares fit to the semi-log plot of standard Ct values as a function of the log of copy number. The respective curves were used to convert sample Ct values to copy number, and all percentages determined from these absolute copy number estimates.

Results

Calibrating BK channel real-time PCR (qPCR):

In order to avoid any complications with multiplex reactions, we used five independent real-time PCR assays run in parallel, with primer/probe sets targeting β-actin sequence, a constitutive exon in *Slo*, a stretch of sequence in the optional exon STREX, and in constitutive exons in β2 and β4. *Slo*, STREX, β2, and β4 mRNA was normalized to β-actin. Each 384-well qPCR plate contained the 21 calibration series mixes for each primer-probe set (a total of 105 calibration reactions per plate). Regression analysis was performed on the calibration series for each plate to determine the relationship between template number and Ct value for each primer-probe set (Fig. 3). As illustrated in Figure 3, the relationship between Ct (interpolated cycle number at which detection "threshold" was reached) and the log of copy number was very well fit with a line in all cases, with R^2 values ranging from 0.69 to 0.97 (only one R^2 value was below 0.9). Template copy number for each of the unknown reactions was then determined by back-extrapolation from measured Ct values, using the parameters from the regression. The independence of the five assays also allows one to measure the fidelity of the various ratio estimates at different absolute template amounts.

Differences in expression of BK channel subunits:

Significant differences in mRNA expression of total *Slo*, β2, and β4 were observed. The expression of *Slo* mRNA was significantly higher in SJL males as compared to SJL females and C57/BL6 males (Fig 1A; $p<0.05$). The average copy number ratios of *Slo* to β-actin for SJL males, SJL females, and C57/BL6 males were 0.051, 0.003, and 0.007, respectively. On average, SJL males expressed a 17-fold higher

level of *Slo* mRNA than SJL females, and a 7-fold higher level than C57/BL6 males. There was also a significant difference in *Slo* mRNA expression ($p<0.05$) between SJL females and C57/BL6 males. C57/BL6 males expressed a 2-fold higher level of *Slo* mRNA than SJL females, but this was a minor difference compared to the level of expression in SJL males. These results suggest that SJL males have much higher levels of the α-subunit in cells of the adrenal medulla, than the other two groups. In essence, this translates to a great abundance of BK channels (without the STREX exon) in SJL males, and relatively low levels of expression in SJL females and C57/BL6 males. Very similar patterns of expression were observed for the β-subunits. The expression of β2 mRNA was significantly higher in SJL males as compared to SJL females and C57/BL6 males (Fig 1C; $p<0.05$). The average copy number ratios of β2 to β-actin for SJL males, SJL females, and C57/BL6 males were 0.039, 0.005, and 0.005, respectively. On average, SJL males expressed an 8-fold higher level of β2 mRNA than the other animal groups. There was no significant difference in β2 mRNA expression ($p=0.05$) between SJL females and C57/BL6 males. The expression of β4 mRNA differed significantly between the three groups (Fig 1D; $p<0.05$). The average copy number ratios of β4 to β-actin for SJL males, SJL females, and C57BL6 males were 0.0004, 0.00002, and 0.00016, respectively. SJL males had the highest level of β4 expression. SJL males expressed a 2.5-fold higher level of β4 mRNA than C57/BL6 males and a 20-fold higher level than SJL females. C57/BL6 males expressed an 8-fold higher level of β4 mRNA than SJL females. The expression of STREX mRNA was not significantly different between the three groups (Fig 1B; $p=0.05$), but we suspect that this was due to a small sample size. The average copy number ratios of STREX to β-actin for SJL males, SJL females, and C57/BL6 males

were 0.007, 0.0007, and 0.0009, respectively. We were not able to make any firm conclusions about the level of STREX mRNA expression in SJL males due to a high level of variation within the group. However, the data suggests that SJL males express a higher level of STREX mRNA than both SJL females and C57/BL6 males. On average, SJL males expressed a 10-fold higher level of STREX mRNA than SJL females and a 7-fold higher level that C57/BL6 males. There was no significant difference in STREX mRNA expression ($p=0.05$) between SJL females and C57/BL6 males.

These results are quite striking and reveal that SJL females and C57/BL6 males have similar BK channel expression patterns, while SJL males have a distinct pattern from the other two groups. SJL females and C57/BL6 males are at the low end of the spectrum for expression of BK channel α- and β-subunits, while SJL males are at the high end of the spectrum.

In order to further explore the composition of BK channels in adrenals of these mouse groups, we determined the copy number ratios of STREX, β2, and β4 to total *Slo*. From Figure 2, we can see a pattern of sexual dimorphism emerging. On average, SJL females had the highest ratio of STREX/*Slo*, SJL males had an intermediate level, and C57/BL6 males had the lowest levels (Fig 2A). This ratio was significantly different between SJL females and C57/BL6 males ($p<0.05$). It is also important to note that the STREX splice-variant was a small percentage of the total *Slo* (13%-22%). For β2/*Slo*, the ratio was highest in SJL females and lowest in SJL males (significantly different between these two groups; $p<0.05$) as shown in Figure 2B. On average, the ratio was intermediate for C57/BL6 males (between SJL males and females), but it did not differ significantly from the other two groups due to a high degree of variation within the group. These

results suggest that SJL females are recruiting more β2 subunits per *Slo*-α subunit. For β4/*Slo*, the ratios were not significantly different between the three groups (Fig. 2C; p=0.05), but the general trend suggests that male mice express a higher percentage of β4, relative to total *Slo*, than females. STREX, β2, and β4 mRNA as percentages of total *Slo* show trends of sexually dimorphic expression.

Discussion

Mice have been become one of the most important mammalian models for biological research, especially in neurobiology. Sexual dimorphisms exist within the hypothalamic-pituitary-adrenal (HPA) axis of mice. Several studies suggest that there are gender differences in adrenal function of mice. In addition, behavioral studies characterize SJL mice to be a more aggressive strain that C57/BL6. Adrenal function, which is an extremely important component of the neuroendocrine system, is largely responsible for synthesis of molecules like glucocorticoids, EPI, and NE, which play a critical role in stress and aggression. The adrenal gland is also involved in immune and inflammatory responses. Given the gender differences in adrenal function, as well as strain differences in aggression between SJL and C57/BL6, it is important to study strain- and gender-specific differences in adrenal function at the molecular level. In this study, we looked at the level of ion channels and focused on a particular type called BK channels, which are known to play a critical role in modulating the adrenal gland.

There were two problems with this study. First, a sample size of six for each group (18 total mice) is relatively small and should be larger for a real-time PCR study, due to the inherent variability associated with the technique. Second, there is only one

housekeeping gene used. It is possible that β-actin mRNA is somehow changing in these animals due to the experimental conditions, and this change is different between the groups, which could also lead to the observed results. In future studies, sample sizes should be larger and more than one housekeeping gene should be used. Despite these two problems, we were able to determine some important differences in BK channel expression patterns in the adrenal glands of SJL males, SJL females, and C57/BL6 males.

In order to determine strain- and gender-specific differences in BK channel expression in adrenals, we first looked at the abundance of Slo-α transcripts, including the STREX-containing splice variant, relative to β-actin. In the adrenal gland, the expression of *Slo* mRNA was significantly higher in SJL males as compared to SJL females and C57/BL6 males. *Slo* mRNA expression was higher in SJL females than C57/BL6 males, but the difference was quite small compared to the extremely high level of expression in SJL males. A very similar pattern of expression was observed for STREX mRNA (although not significant due to a small sample size), where SJL males had a much higher expression level than the other two groups. These results suggest that SJL males have much higher levels of Slo-α transcripts, total and STREX-containing, than SJL females and C57/BL6 males. Similar patterns of expression were observed for the β2 and β4 subunits. The expression of β2 mRNA was significantly higher in SJL males as compared to SJL females and C57/BL6 males. SJL males also had higher levels of expression of β4 mRNA than the other two groups. It was very interesting to observe similarities in BK channel expression patterns in SJL females and C57/BL6 males, while the pattern of expression in SJL males was quite different from the other two groups. Yet, it is extremely difficult to predict how a specific pattern of BK channel expression will

determine adrenal function, and in turn, behavior. Inclusion of STREX enhances the repetitive firing ability of chromaffin cells, as compared with cells having channels lacking an insert at this splice site (Lovell and McCobb, 2001; Lovell et al., 2004). Association of the β2-subunit facilitates activation at negative voltages, but also slows deactivation kinetics, and confers inactivation (Wallner et al., 1999; Xia et al., 1999; Uebele et al., 2000; Orio et al., 2006), so it is difficult to predict its effect on endocrine function. β4 has multiple effects on BK gating, including a pronounced slowing of activation gating, and a steepening of the calcium-dependence (Brenner et al., 2000; Ha et al., 2004). Combining electrophysiology data with the results of this study, we now have grounds for hypothesizing functional relationships between behavior and the adrenal medulla. In future studies, it in necessary to explore strain- and gender-specific changes in BK channel expression in various environments and developmental stages to further understand the differences between these groups in adrenal function, which will also lead to a better understanding of behavior.

The causal link between BK channel expression in the adrenal gland and behavior is not clear. By having more BK channels, an animal has a more excitable and plastic adrenal gland. This may make the animal more aggressive, but this causal relationship is difficult to establish. It is also possible that the causal link is in the reverse direction, where greater aggressiveness leads to more BK channels. This study suggests that having more BK channels leads to aggressive behavior, since the more aggressive and "emotional" SJL male mice tend to have higher basal levels of BK channel subunits than SJL females and C57/BL6 males. More research is required on this subject to establish a firm causal link.

Figure Legends

Figure 1: Average copy numbers for genes of interest normalized to β-actin copy number and compared between SJL males, SJL females, and C57/BL6 males. A) total *Slo* copy number normalized B) STREX copy number normalized; C) β2 copy number normalized; D) β4 copy number normalized.

Figure 2: Ratio of average copy number for gene of interest to average total *Slo* copy number, and compared between SJL males, SJL females, and C57/BL6 males. A) Average STREX/*Slo* copy number ratio B) Average β2/*Slo* copy number ratio; C) Average β4/*Slo* copy number ratio.

Figure 3: Raw data showing regression analysis on the calibration series for each plate to determine the relationship between template number and Ct value for each primer-probe set. The relationship between Ct (interpolated cycle number at which detection "threshold" was reached) and the log of copy number was very well fit with a line in all cases. R^2 values for β-actin, *Slo*, STREX, β2, and β4 regression lines are 0.97, 0.97, 0.69, 0.97, and 0.96, respectively.

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