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Subordination stress alters alternative splicing of the *Slo* gene in tree shrew adrenals

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Abstract

It was previously hypothesized that stress hormones regulate the alternative splicing of Slo potassium channels, thereby tuning the intrinsic excitability of adrenal chromaffin cells. Male tree shrews subjected to chronic stress by exposure to a dominant male develop robust symptoms with parallels to human depression. We report here that adrenals from males subjected to 4–6 weeks of subordination have a significantly smaller proportion of Slo transcripts with the optional STREX exon (STRess-axis regulated EXon) than unstressed male adrenals. Female adrenals (unstressed) had even lower levels than stressed males. These data suggest both behavioral regulation and sexual dimorphism in ion channel structure. We hypothesize that chromaffin cell excitability and sympathoadrenal function will be altered, and speculate that this may favor passive coping responses in subordinate males and females.

Keywords: Potassium channels; BK channels; Slo; HPA axis; Psychosocial stress; Alternative splicing; Steroids

Secretion of epinephrine (EPI) and norepinephrine (NE) from adrenomedullary chromaffin cells is tightly controlled by action potentials generated in response to neural inputs. A variety of evidence suggests that the intrinsic excitability of chromaffin cells is heavily influenced by the number and precise gating properties of BK Ca^{2+}- and voltage-gated K^+ channels encoded by the *Slo* gene (Lingle, Solaro, Prakriya, and Ding, 1996; Lovell and McCobb, 2001). Recent experiments have suggested that neither BK channel gating properties nor the intrinsic ability of chromaffin cells to fire repetitively are fixed, but are instead subject to chronic regulation on a time scale of days to weeks by neuroendocrine signals emanating from the pituitary (Xie and McCobb, 1998; Lovell and McCobb, 2001). Thus hypophysectomy has been shown to reduce the repetitive firing ability of chromaffin cells by roughly 50%, in large part attributable to a substantial reduction in the accessibility of Slo-encoded BK channels for activation. BK channels promote repetitive firing by bringing about a rapid but brief afterhyperpolarization following each spike, thereby expediting the reclamation of Na^+ and Ca^{2+} channels lost to an inactivated state during the spike. Alternative splicing of Slo gene transcripts has been postulated to be a level at which BK function is chronically regulated by pituitary hormones (Xie and McCobb, 1998; Lai and McCobb, 2002). 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. Channels with this exon configuration are therefore postulated to enhance 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). The STREX exon has also been shown to broaden the repertoire of second messenger-mediated responses of BK channels (Tian, Duncan, Hammond, Coghill, Wen, Rusinova, Clark, Levitan, and Shipston, 2001a; Tian, Hammond, Florance, Antoni, and Shipston, 2001b).

The negative impact of hypophysectomy on chromaffin cell excitability was first predicted from the observation that

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the representation of the STREX variant of Slo in chromaffin cells dropped by 50% within 2 weeks after hypophysectomy at 5–6 weeks of age, a drop that could be prevented by concurrent replacement injections of adrenocorticotropic hormone (ACTH). Direct application of steroid hormones to bovine chromaffin cells in culture further attests to a link between stress-related pituitary function and BK structure and function (Lai and McCobb, 2002). Hypophysectomy is obviously an invasive and complex, multiendocrine experimental perturbation, and thus these experiments demand more behaviorally realistic experiments. Because behavioral stress typically raises ACTH production in the pituitary, our first-order hypothesis would be that behavioral stress would have downstream consequences directly opposite those of hypophysectomy, elevating STREX representation, and ultimately enhancing excitability and evoked catecholamine secretion.

Emerging as one of the most potent stressors in several species, psychosocial stress can have neurochemical, anatomical, and functional consequences in the CNS and periphery (McEwen and Magarinos, 1997; Sapolsky, Romero, and Munck, 2000). A robust chronic social stress paradigm has been developed in laboratory-reared tree shrews (*Tupaia belangeri*), a highly territorial Southeast Asian insectivore phylogenetically closer to humans than rodents. Thus naïve males are exposed to a known dominant, with confrontation rapidly leading to a clear dominant–subordinate relationship. The initial encounter is followed by several weeks of constant proximity in adjacent cages separated by a wire mesh, with 1-h daily confrontations permitted by raising the mesh. This treatment produces a wide spectrum of symptoms in the subordinate male, many of which parallel those of severe human depression. Reductions in locomotor activity, territorial scent marking, autogrooming, and gonadal function (Fuchs, Flügge, Ohl, Lucassen, Vollmann-Honsdorf, and Michaelis, 2001) are accompanied by chronically elevated urinary cortisol (CORT) and catecholamines, adrenal hypertrophy, weight loss, sleep and circadian disturbances, reduced hippocampal volume and CA3 pyramidal cell dendritic arborization, impaired cognitive performance, and decreased hippocampal glucocorticoid and mineralocorticoid receptor expression, suggesting impaired negative feedback regulation of stress responses. Antidepressant drugs ameliorate most symptoms of subordinate tree shrews (Kramer, Hiemke, and Fuchs, 1999; Czeh, Michaelis, Watanabe, Frahm, de Biurrun, van Kampen, Bartolomucci, and Fuchs, 2001).

Recent advances in the accuracy of an RT-PCR method involving a single primer pair that spans the STREX site increases the power to resolve small, quantitative shifts induced by more natural perturbations than hypophysectomy (Mahmoud, Bezzerides, Riba, Lai, Lovell, Hara, and McCobb, 2002). In this study, we exploit the tree shrew subordination stress paradigm to determine whether Slo splicing is modulated by stress-axis perturbations less invasive and extreme than hypophysectomy, and especially, of a behavioral nature.

Materials and methods

Experimental stress and urinalysis

Experiments were performed with adult male tree shrews (5–24 months) from the German Primate Center breeding colony (Göttingen, Germany). Experimentation was conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/EEC), and approved by the Government of Lower Saxony, Germany. Animals were housed singly on a regular day/night cycle (lights 0800–2000 h) at 26°C, 55% relative humidity, with tree shrew diet (Altromin, Lage, Germany). During the 10-day control phase animals were individually housed. During the *stress* period, the opaque partition between neighboring cages of two males unknown to one another was removed. After establishment of a stable dominant/subordinate relationship (1–2 h), the two were separated by a transparent wire mesh. During subsequent *recovery* (10 days), the opaque partition was replaced. Females aged 2–40 months were housed and handled in the same manner as control males.

Animals were weighed and morning urine samples were collected daily. Basal HPA was determined by measuring free cortisol in urine with a scintillation proximity radioimmunoassay (Udenfriend, Gerber, Brink, and Spector, 1985) with anti-rabbit antibodies (Paesel and Lorei, Frankfurt, Germany) bound to fluoromicrospheres, and [^{3}H]cortisol as tracer (Amersham, Braunschweig, Germany). CORT was normalized to creatinine concentrations, determined with Beckman Creatinine Analyzer 2.

Urine catecholamines were extracted by cation-exchange chromatography with Bio-Rex 70 resin (Bio-Rad, Munich), separated by reversed-phase HPLC, and quantified colorometrically (Fuchs, Johren, and Goldberg, 1992). *t* tests assuming unequal variances were used for statistical comparisons (Flügge, Kramer, and Fuchs, 2001), except where otherwise indicated.

RT-PCR for splice variants

Extracted total RNA (Qiagen RNEasy) (2 μg) was used in 20-μl RT reactions primed with oligo-dT. RT product (2 μl) was used in 24-μl PCR reactions primed with RbSlo1 5′ AGTGCCTTCGTGGGTCTGTCCTTC 3′ and QRA59 5′ CACATTGGAGTCCATGTTGTC 3′ (antisense). Following a 3-min denaturation at 95°C, 30 cycles were run, with 30 s each at 94, 55, and 72°C. Negative controls with water as template were run in parallel.

PCR products were electrophoresed in denatured form using carefully tested quantification procedures (Mahmoud et al., 2002). Rigorous denaturation was required to elimi-

nate formation of heteroduplexes between STREX and ZERO products. PAGE gels (8%, 19:1 acrylamide-bisacrylamide) contained 40% formamide by volume. Denaturing loading buffer (DLB) (2×) was comprised of 80% formamide, 20% water (volume:volume), 10% sucrose (10 g/100 ml), and 20 mM EDTA. A constant sample volume of DNA was diluted 1:1 in DLB, boiled 1 min, and loaded on a vertical gel submerged in near-boiling TBE buffer. Gels ran 80 min at 240 V. DNA was visualized with SYBRGold dye (Molecular Probes) and images were acquired with a Molecular Dynamics STORM-840 imager. Fully denatured gels revealed only STREX and ZERO bands, with very low background staining (Fig. 1B).

Band intensities were determined as illustrated (Fig. 1), using an IGOR (Wavemetrics) procedure written in-house ("Band-Buster"). To convert to relative copy numbers, STREX intensities were corrected for greater length, and dye binding capacity, by multiplying by 0.709. Data were corrected for dye saturation after calibration with known absolute amounts of STREX and ZERO run alongside unknowns (Mahmoud et al., 2002). Most samples were run two or more times to confirm reproducibility, and results were averaged. Estimates of the abundance of STREX relative to total (STREX + ZERO) in PCR templates, as opposed to products, were made by converting according to the previously determined formula: $Y = -0.258*(\ln(1.35/(X + 0.105) - 1) - 2.50)$, where Y is the fraction of STREX in the template, and X is the fraction of STREX in the product (Mahmoud et al., 2002). Saturation and template corrections had little effect on statistical outcomes. STREX percentages were analyzed with single-factor ANOVA except where indicated, and statistical significance was defined by individual and family error rates below 0.05 with Tukey's conservative pairwise comparison (Minitab).

Results

Effects of stress on body weight and urinary hormones

To measure effects of subordination stress on male tree shrews, body weight and urine levels of cortisol, NE, and EPI were measured daily or near daily for 17 animals (representing a subset of the 33 males described below) during a 10-day pretreatment period, and during a 28-day treatment period that consisted of either stress by constant proximity and 1 h daily unrestricted access to a dominant male (11 animals), or a continuation of the nonstressful individual housing situation (6 animals). Five of the 11 stressed animals were allowed a 10-day recovery period in individual housing following a prolonged stress period. For simplicity, statistics reported represent pairwise comparisons between two periods for the same individuals. ANOVA was applied to three treatment groups for the 5 animals experiencing all three, with Tukey's conservative post hoc comparisons identifying the same treatment differences, with the family error set at 0.05. Body weights of the 11 stressed animals decreased to 95.2 ± 0.9% of their respective pre-stress weights by the end of 4 weeks of stress (t test assuming unequal variances; $P = 0.0002$). The 6 nonstressed control animals gained 2.3 ± 1.6% of body weight over this period. For the 5 animals given 10 days to recover, body weights returned to 102% of pre-stress values by the end of recovery ($N = 5$). Hormone output comparisons were made by averaging urine levels over the last 10 days of the period. In stressed animals, cortisol levels were elevated to an average of 215 ± 22.4% of pretreatment levels during the last 10 days of the stress period ($P < 0.0001$; see Table 1 for absolute levels). Over the same time period, parallel nonstressed controls had urinary cortisol levels that were 97.8 ± 3.7% of pretreatment values. During the 10-day recovery, cortisol levels of the 5 animals dropped from 237 to 132% of their pre-stress levels ($N = 5$). Recovery values were not significantly different from pretreatment values. NE and EPI excretion also increased during stress, reflecting the activation of sympathetic nervous and adrenomedullary systems. Stressed animals excreted on average 35 ± 12.0% more EPI during the last 10 days of the stress period compared to the pre-stress period in the same animals ($N = 11$; $P = 0.009$), whereas excretion from nonstressed animals decreased by 32.3 ± 7.7% ($N = 6$) over the comparable time period. During the 10-day recovery period, levels dropped only slightly, from 150 to 140% of pre-stress levels in this subset of animals ($N = 5$). NE excretion increased to 235 ± 23.2% of pretreatment levels during stress ($N = 11$; $P = 0.0006$), whereas it dropped by 21.4 ± 3.5% for parallel control animals. NE levels dropped from 241 to 171% of pre-stress levels during the 10-day recovery period ($P = 0.009$).

Stress effects on Slo splicing

Whole adrenal glands from 14 adult males (5–24 months, mean ± SD = 13.1 ± 4.3 months) subjected to 4–6 weeks of subordination were analyzed for Slo splice variant expression, and compared with adrenals from 14 unstressed males of similar age (mean 10.9 ± 3.4 months). Adrenals from 5 additional males given 10 days in solitary housing to recover from chronic stress were analyzed. Adrenals from 9 nonstressed females (2–40 months; mean ± SD = 7.22 ± 12.3 months) were also analyzed. Prior to sacrificing, all stressed males exhibited symptoms of stress, including behavioral withdrawal, reduced locomotor activity, scent marking, and aggressive behavior, in addition to the aforementioned physiological markers (Fuchs et al., 2001).

After reverse transcription, PCR primers targeting constitutive flanking exons were used to amplify splice variants of the Slo gene (Fig. 1). Amplification products were of the same sizes as those amplified from rat and bovine tissues with these primers (Lai and McCobb, 2002; Mahmoud et al., 2002). Samples were subcloned and sequenced, con-

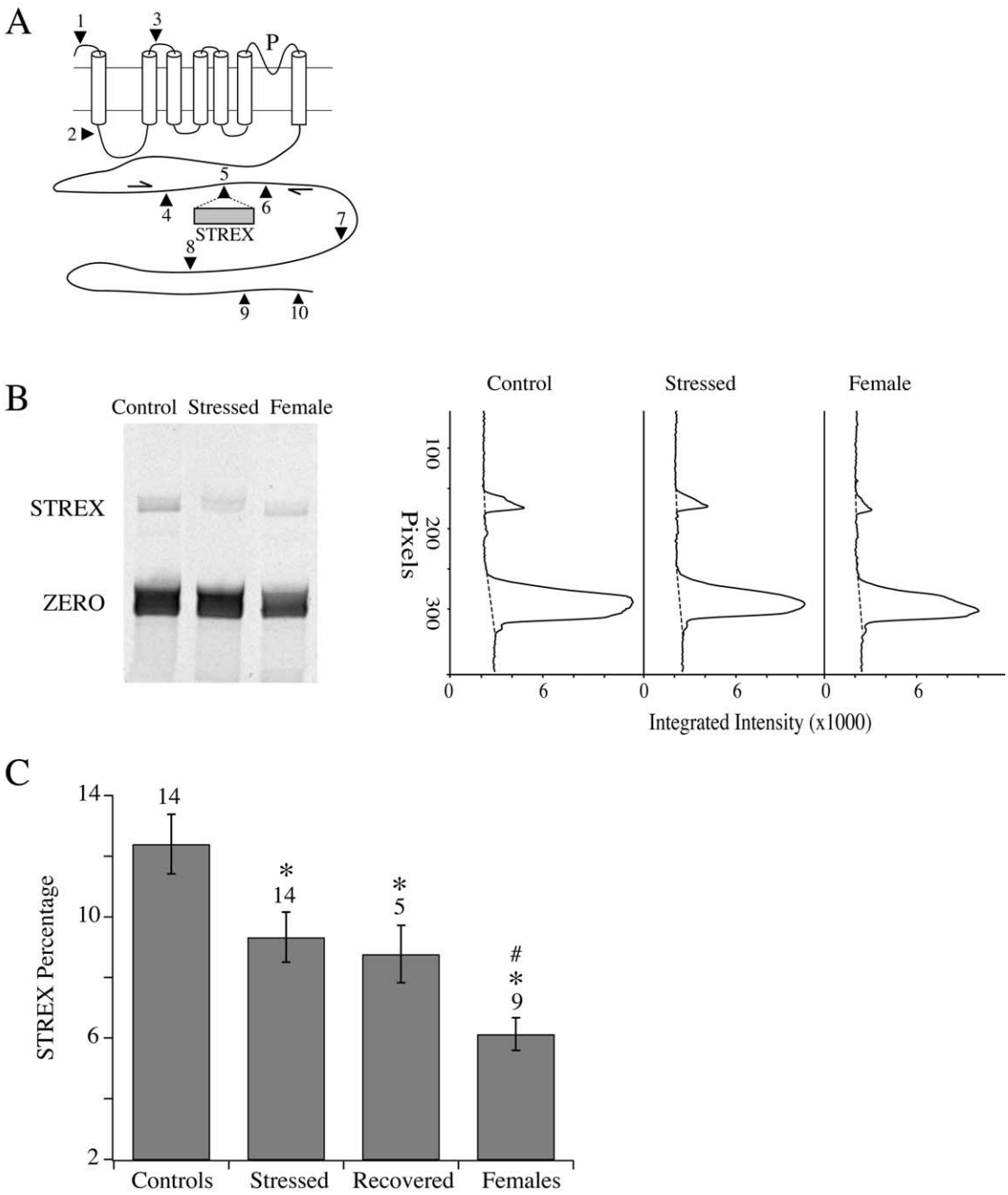

Fig. 1. Quantitative differences in splicing of Slo K^+ channels in tree shrew adrenals associated with chronic subordination stress and gender. (A) Slo schematic, showing splice site 5 and flanking primer sites, where 597-bp STREX and 423-bp ZERO (with and without the 174-bp STREX exon, respectively) RT-PCR products are amplified from adrenal chromaffin cells. (B) Representative RT-PCR products from adrenals of control and stressed male and nonstressed female tree shrews run on a denaturing PAGE gel. Band intensities were determined by integrating plots of horizontally summed gray-scale values versus vertical position. (C) Mean ± SEM values for estimated percentages of Slo transcripts containing STREX. Sample sizes are indicated above the bars. Post hoc tests indicate that the percentage of Slo transcripts containing STREX was significantly lower (*) in stressed males vs unstressed control males, stressed and recovered males vs controls, and unstressed females vs control males. Unstressed females were significantly lower than stressed males as well (#).

firming their identification as STREX and ZERO variants. Band intensities were measured blindly, without reference to treatment group, from denaturing PAGE gel images. Intensities were converted to estimates of STREX percentages in template RNA as described above.

A single-factor ANOVA test was applied to estimates of STREX percentages from 14 control males, 14 stressed males, 5 stressed with recovery, and 9 control females. With 3 and 38 degrees of freedom for between group and within group variation, the *F* statistic was 8.93, giving an overall *P*

Table 1
Effects of subordination stress on body weight and urinary output of cortisol, norepinephrine, and epinephrine

Treatment period	Body weight	Cortisol	Adrenaline	Noradrenaline
Pretreatment	207 ± 3.4	118 ± 8.9	35.6 ± 4.8	75.3 ± 7.8
Stress	197 ± 4.9	248 ± 26.0	50.6 ± 6.1	177.2 ± 27.9
Recovered	216 ± 8.4	162 ± 14.9	46.6 ± 10.0	167 ± 27.2

Note. Mean values (±SEM) were obtained by averaging over control, stress, and recovery periods of 10, 42, and 10 days. Values during stress were significantly different from pre- and post-stress values in corresponding animals, with the exception of post-stress epinephrine values (see text).

value of 0.00021. Values for the 14 unstressed control males ranged from 6.47 to 19.5%, with a mean (±SEM) of 12.4 ± 0.98% (Fig. 1C). Values from 14 stressed males ranged from 5.60 to 14.9% (mean = 9.33 ± 0.83%). This was significantly lower than unstressed controls, as determined using the post hoc Tukey's pairwise comparison (with family and individual error rates of $P < 0.05$). For 5 stress-recovery animals, STREX ranged from 6.72 to 11.9% (mean = 8.77 ± 0.95%). This was significantly below control, but not different from stressed animals. This suggests that any restoration of STREX levels following stress requires more than 10 days.

Correlating STREX and endocrine variables

Adrenal and body weights from 11 control and 11 stressed males were measured at necropsy. Adrenals from stressed animals were 6% heavier than controls ($P = 0.245$ (not significant); Fig. 2A). Body weights of stressed animals were 13.4% less than controls ($P < 0.0001$). Consequently, adrenal to body weight ratios were 21.8% higher in stressed animals than in controls. Testes were compared for 6 animals in each group, and were much reduced in all stressed individuals, weighing 39% as much as control testes ($P < 0.0003$). Disregarding treatment groups, no significant correlations were found between organ or body weights and STREX measured from corresponding animals. However, urinary cortisol levels from individual males, measured by averaging over the 10 days prior to sacrifice, did correlate roughly with STREX percentages from the respective individuals (Fig. 2B; coefficient of regression $R = -0.813$, $P = 0.095$). Catecholamine levels over this period, which were positively correlated with cortisol, did not correlate as well with STREX values.

Sex differences

Adrenal STREX percentages for 9 female tree shrews ranged from 2.64 to 8.34% (mean = 6.13 ± 0.54%). This was very significantly below control males, and significantly below stressed males as well. Though females tended to be younger than males at sacrifice, there was no relationship between age and STREX values for either males or females. Four 2-month-old females had from 2.64 to 7.01% STREX, while the one relatively old female (40 months) had a value of 4.36% STREX. Moreover, for a subset of 4 females and 4 males ranging from 4 to 6.5 months of age only, female values were still significantly lower, despite the fact that 3 of the 4 males were stressed (means were 7.05 ± 0.45 and 9.98 ± 1.04 for females and males, respectively; $P = 0.03$; t test assuming unequal variances).

Discussion

In this paper we present results of the first behavioral experiments to test a proposed link between HPA axis function and variable splicing of the Slo gene, encoding a potassium channel that can facilitate rapid repetitive firing of adrenal chromaffin cells. In rats, the relative representation of transcripts with the optional STREX exon drops by 50% during the 2 weeks following hypophysectomy (at 5–6 weeks), an effect that can be prevented by ACTH replace-

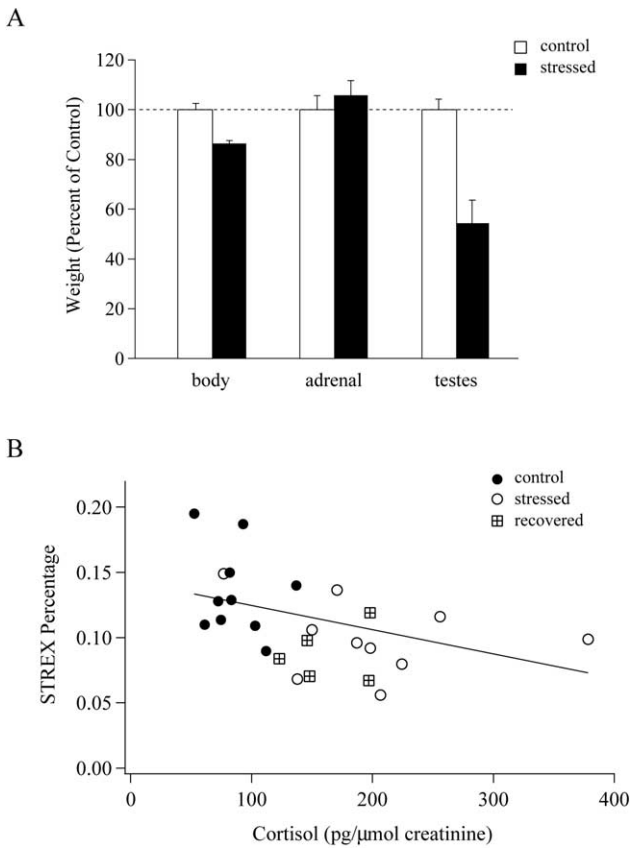

Fig. 2. Endocrine correlates. (A) Adrenal weights of stressed animals were not significantly different than controls, though body weights were significantly smaller, and testes much smaller. STREX percentages did not correlate with individual weights from corresponding animals (not shown). (B) Individual STREX percentages did correlate roughly with individual urinary cortisol levels averaged over the 10 days prior to sacrifice.

ment injections (Xie and McCobb, 1998). The present experiments exploit the robust, depression-like symptoms induced in male tree shrews forced to live for 4–6 weeks adjacent to a dominant male, with an intervening screen barrier that was lifted for 1 h per day. Based on the rat studies, we naively predicted that such stress would, by chronically elevating HPA function, affect Slo splicing in a direction opposite that of hypophysectomy. Instead we report a similar, though more modest, 25% drop in the representation of the STREX-containing isoform. In addition, adrenals from 9 female tree shrews were analyzed in parallel to the males, and revealed an unexpected sex difference; female STREX levels were half those of unstressed males, and significantly lower than those of stressed males as well.

Differences of the magnitude described here are well within detection limits, as previously tested with independent measures from 10 pieces of a single bovine adrenal medulla, yielding a standard deviation of only 2.7% of the total 100% range of STREX representation (Mahmoud et al., 2002). Duplicate measures on most of the tree shrew samples tested here further confirmed the reliability of the measurement, and attest to significant interindividual variation in stressed and unstressed treatment groups alike. Our RNA template was extracted from homogenized whole adrenals; thus our results represent an average change over a variety of cell types. Chromaffin cells are likely to vary in Slo expression, splice variant representation, and sensitivity to steroid regulation (Solaro, Prakriya, Ding, and Lingle, 1995; Lovell, James, and McCobb, 2000; Lovell and McCobb, 2001). Our results may therefore underestimate changes in STREX representation in any more-responsive subset(s) of cells. Additionally, disproportionate changes in cell number or Slo expression in chromaffin subsets, or cortex relative to medulla, could contribute to the average changes reported here.

Effects of splicing variation at the STREX site on channel gating and channel modulation imply functional consequences for stress-related changes in the splicing pattern. The STREX decline in rat chromaffin cells following hypophysectomy was accompanied by a roughly 30-mV positive shift in the depolarizing voltage needed to activate the BK channels, and a 50% reduction in the maximum firing rate of the cells (Lovell and McCobb, 2001). These changes were actually greater than predicted on the basis of comparisons between STREX and ZERO cloned variants heterologously expressed in *Xenopus* oocytes (Saito, Nelson, Salkoff, and Lingle, 1997; Xie and McCobb, 1998; Lovell and McCobb, 2001). Clone comparisons also indicate that modulatory response of Slo channels, including several levels of kinase-, phosphatase-, and/or steroid-mediated modulations, can be qualitatively and quantitatively altered by STREX inclusion (Nara, Dhulipala, Wang, and Kotlikoff, 1998; Tian et al., 2001a).

Altered chromaffin BK channel gating and cell excitability observed with hypophysectomized rats specifically predict that chromaffin cells from stressed male tree shrews, as well as those from females, will exhibit lower maximal rates of repetitive firing and catecholamine secretion (in response to identical autonomic input stimuli) than control male equivalents. Not surprisingly, subordination stress raised urinary NE and EPI levels in male tree shrews. However, EPI levels, which better reflect adrenomedullary than total sympathetic output, were elevated only transiently (Fuchs, Johren, and Flügge, 1993). One might speculate that a slow decline in STREX inclusion contributes to a compensatory down-scaling of adrenomedullary responsiveness. Rapid sympathoadrenal responses have been linked to proactive coping and dominant status in baboons, lizards, and other vertebrates (Sapolsky, 1986; Sgoifo, de Boer, Haller, and Koolhass, 1996; Korzan, Summers, Ronan, and Summers, 2000). Thus stress- or gender-related STREX reduction could represent part of an adaptive bias toward passive, withdrawing responses to threats, as opposed to active/aggressive counterattacks (Koolhaas, Korte, De Boer, Van Der Vegt, Van Reenen, Hopster, De Jong, Ruis, and Blokhuis, 1999).

Multiple steroid hormones are likely to be involved in Slo splicing regulation. With hypophysectomy in rats both CORT and STREX dropped, and both drops were prevented by ACTH injection. A positive link between CORT and STREX was suggested. However, recent experiments in which CORT and DEX were applied directly to bovine chromaffin cells in culture found the opposite relationship; this is more consistent with the inverse correlation in male tree shrews described here. Contrasting with the direct CORT effects, adrenal androgens DHEA and androstenedione, as well as testosterone, positively affected STREX inclusion in bovine cells (Lai and McCobb, 2002). Though DHEA levels are elevated by stress and ACTH, there is evidence that sustained stress, chronic illness, and aging depress DHEA synthesis, particularly relative to CORT (Cutler, Davis, Johnsonbaugh, and Loriaux, 1979; Parker, Gral, Perrigo, and Skowksy, 1981; Albertson, Hobson, Burnett, Turner, Clark, Schiebinger, Loriaux, and Cutler, 1984; Griffing, Allen, Pratt, and Melby, 1985; Hung and LeMaire, 1988; Parker, 1991; Oberbeck, Benschop, Jacobs, Hosch, Jetschmann, Schurmeyer, Schmidt, and Schedlowski, 1998). Effects of chronic subordination stress on tree shrew adrenal androgen production are unknown, but testicular morphology and function are dramatically depressed (Fischer, Heinzeller, and Raab, 1985; Flügge, Kramer, Rensing, and Fuchs, 1998; Flügge et al., 2001). The convergence of stressed males and females on a low-STREX phenotype is consistent with negative and positive effects of glucocorticoids and androgens, respectively, on STREX inclusion. This reinforces the idea that adrenomedullary BK channels represent an important node of interaction between sex and stress steroids, and motivates further research on their relevance to autonomic components of coping strategies.

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