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Regulation of Alternative Splicing of Slo K^+ Channels in Adrenal and Pituitary during the Stress-Hyporesponsive Period of Rat Development

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Stress triggers release of ACTH from the pituitary, glucocorticoids from the adrenal cortex, and epinephrine from the adrenal medulla. Although functions differ, these hormone systems interact in many ways. Previous evidence indicates that pituitary and steroid hormones regulate alternative splicing of the Slo gene at the stress axis-regulated exon (STREX), with functional implications for the calcium-activated K^+ channels prominent in adrenal medullary and pituitary cells. Here we examine the role of corticosterone in Slo splicing regulation in pituitary and adrenal tissues during the stress-hyporesponsive period of early rat postnatal life. The sharp drop in plasma corticosterone (CORT) that defines this period offers a unique opportunity to test CORT's role in Slo splicing. We report that in both adrenal and pituitary tissues, the percentage of Slo transcripts having STREX declines and recovers in parallel with CORT. Moreover, addition of 500 nM CORT to cultures of anterior pituitary cells from 13-, 21-, and 30-d postnatal animals increased the percentage of Slo transcripts with STREX, whereas 20 μM CORT reduced STREX representation. Applied to adrenal chromaffin cells, 20 μM CORT decreased STREX inclusion, whereas neither 500 nm nor 2 μM had any effect. The mineralocorticoid receptor antagonist RU28318 abolished the effect of 500 nm CORT on splicing in pituitary cells, whereas the glucocorticoid receptor antagonist RU38486 blocked the effect of 20 μM CORT on adrenal chromaffin cells. These results support the hypothesis that the abrupt, transient drop in CORT during the stress-hyporesponsive period drives the transient decline in STREX splice variant representation in pituitary, but not adrenal. (*Endocrinology* 147: 3961–3967, 2006)

THE STRESS-HYPORESPONSIVE period (SHRP), prenatal in humans but conveniently postnatal in rats, represents a temporary down-grading of hypothalamo-pituitary-adrenal (HPA) responsiveness (1–3). As such, the SHRP provides a unique window for exploration of a potentially important interaction between HPA hormones and the structure and function of potassium channels encoded by the *Slo* gene. Slo channels (BK voltage- and Ca^{2+}-activated K channels) are critical determinants of action potential firing properties in catecholamine-secreting adrenomedullary chromaffin cells (4) and anterior pituitary corticotropes (5, 6).

Previous experiments suggested that the HPA, the endocrine stress axis, exerts a chronic modulatory influence over the neural stress axis, involving catecholamine secretion from the adrenal medulla. In the slower, endocrine cascade, hypothalamic release of CRH and arginine vasopressin stimulates synthesis and release of ACTH, which in turn stimulates synthesis and release of cortisol or corticosterone (CORT) from the adrenal cortex. CORT enables coping at many physiological levels. Adrenaline is a more immediate stress hormone; unlike CORT, adrenaline secretion from adrenomedullary chromaffin cells is triggered by action potentials generated in response to sympathetic neural input. That the intrinsic excitability of chromaffin cells is not fixed but malleable was suggested by pituitary ablation (hypophysectomy) experiments. Hypophysectomy was shown to alter the ability of chromaffin cells to fire repetitive action potentials in rapid succession (4). This change was attributed to a change in the gating properties of BK channels that resulted from a change in the relative abundance of two splice variants of Slo. Thus, the stress axis-regulated exon (STREX) variant was reduced relative to the variant lacking an insert at this site (ZERO) by hypophysectomy (7), and STREX channels were shown to open more easily than ZERO channels (7, 8). The STREX channel properties promote repetitive firing by speeding action potential repolarization and augmenting the after-hyperpolarization, facilitating Na^+ channel de-inactivation. Thus, by modulating the splicing decision, CORT was postulated to tune the intrinsic excitability of chromaffin cells to increase their ability to rapidly secrete epinephrine under provocation. Importantly, experiments to test the ability of CORT itself to promote STREX inclusion were inconclusive, perhaps because of the difficulty in raising CORT levels in the inner adrenal with sc CORT implants (7).

In the pituitary, as in the adrenal, STREX and ZERO variants of Slo are expressed to the virtual exclusion of other Slo variants at this site (5, 9) (Fig. 1). The STREX proportion in adult pituitary is similar to that in adrenal medulla and has been shown to be regulated by gonadal testosterone (10). Stress steroids have been implicated as acute modulators of BK function in corticotropes (11). In artificial expression systems, STREX has been shown to reverse the effect of protein

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Abbreviations: CORT, Corticosterone or cortisol; GR, glucocorticoid receptor; HPA, hypothalamo-pituitary-adrenal; hypox, hypophysectomized; MR, mineralocorticoid receptor; P3, postnatal d 3; SHRP, stress-hyporesponsive period; STREX, stress axis-regulated exon; ZERO, variant lacking an insert at the STREX site.

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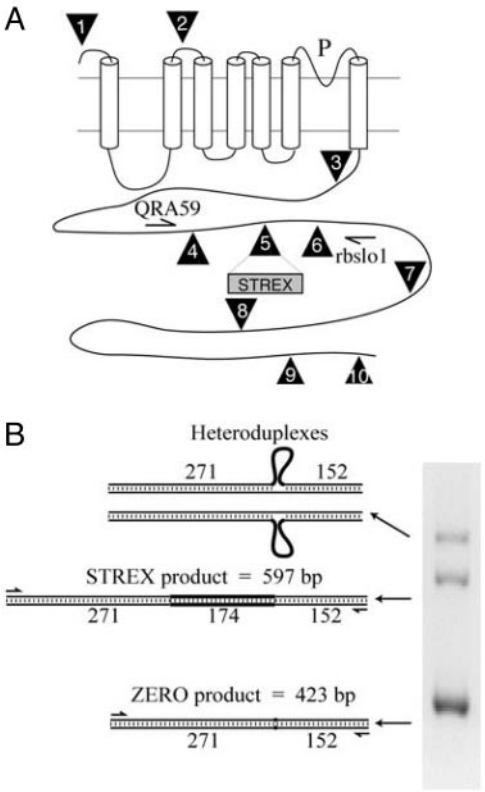

FIG. 1. A, Schematic of the Slo protein showing seven transmembrane-spanning domains (cylinders), 10 alternative splice sites, and binding sites of PCR primers used for amplification of variants at site 5 (QRA59 and RbSlo1); B, primers QRA59 and RbSlo1 simultaneously amplified transcripts with and without the 174-bp STREX at site 5 (no inserts were found at sites 4 and 6). Sequence outside the STREX itself that is shared by STREX and ZERO PCR products allows for formation of two heteroduplex forms (upper band) as well as respective STREX and ZERO product homoduplexes (10), as seen on a 3.5% agarose gel and illustrated to the *left*.

kinase A (PKA)-mediated phosphorylation on channel gating, resulting in inhibition of channel activity with STREX, as opposed to activation occuring in its absence (6, 12–14).

During the SHRP, CORT levels drop precipitously and then gradually recover during the ensuing 3 wk (15). This provides a unique opportunity to test whether CORT itself plays a role in the regulation of Slo splicing *in vivo*. Based on the original observation that hypophysectomy at 5 wk triggers an adrenal STREX decline that can be prevented by ACTH injection (7), we postulated that the postnatal CORT decline might be accompanied by a decline in the relative abundance of STREX relative to ZERO, with a gradual reelevation as CORT levels are restored. In the present report, we document concurrent decline and restoration of serum CORT levels and STREX splice-variant representation in both adrenal and pituitary tissues over the course of the SHRP. Moreover, by direct application of CORT to cell-cultured pituitary and adrenomedullary cells isolated at several developmental stages, we demonstrate that CORT can regulate Slo splice variant abundance, inhibiting STREX inclusion at high doses in both tissues and promoting STREX inclusion at lower doses in pituitary. We provide evidence that the classic nuclear receptor mineralocorticoid receptor (MR) may mediate the positive regulatory effect of CORT in pituitary, whereas glucocorticoid receptor (GR) may mediate negative effects in the adrenal. The data support the hypothesis that endogenous adrenal corticosteroids shape the intrinsic excitability of neuroendocrine cells during development.

Materials and Methods

Animals

Male Sprague Dawley rats were used for all experiments. Pups were shipped from Charles River Laboratories (Wilmington, MA) at postnatal d 3 (P3) with a female feeder (date of birth designated as P0). Litters were housed with their dams in polypropylene cages (11.5 × 21 × 8 in.) with a flooring of wood shavings and a grid top. Rat chow (Teklad 2016 rodent diet, 16% protein; Harlan, Indianapolis, IN) and tap water were provided *ad libitum*. The room was maintained under constant temperature (22 C) and 31% humidity with a 12-h light, 12-h dark cycle (lights on at 0700 h). All experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of Cornell University and the National Institutes of Health.

CORT and testosterone measurement

Between 1600 and 1800 h, pups were decapitated, blood collected, adrenals and pituitaries dissected, and samples frozen immediately in separate RNAse-free tubes on dry ice. Samples were kept at −80 C until RNA extraction. Trunk blood from each pup was collected in a precooled plastic vial kept on ice. Samples were kept at 4 C for 2 h and centrifuged at 4 C for 15 min at 700 × *g*. The supernatant was collected and kept at −80 C until RIA for CORT measurement (ICN Biomedicals, Costa Mesa, CA) or testosterone measurement (DPC Coat-A-Count total testosterone kit; Nippon DPC Corp., Chiba, Japan). The standard curve range of the CORT assays was 25–1000 ng/ml and for the testosterone assays was 0.04–16 ng/ml.

RNA extraction and RT-PCR

Total RNA was harvested using the QIAGEN RNeasy kit (QIAGEN, Valencia, CA). RNA from adrenal or pituitary glands was quantified with a spectrophotometer, and 1.7 mg was added to each 20-μl RT reaction (Superscript II reverse transcriptase and RNase out; Invitrogen, Carlsbad, CA). We used PCR primers targeting constitutive exons and bracketing splice site 5 (Fig. 1A) in the Slo gene to simultaneously amplify two splice variant transcripts occurring in the RNA (7, 10). The consistency of PCR was tested by dividing one RT sample into several duplicates and running PCR to determine the SD between duplicates. The sd was approximately 5% of measured values (see also Ref. 16).

Sense and antisense primers were QRA59 5′-CACATTGGAGTC-CATGTTGTC-3′ and RbSlo1 5′-AGTGCCTTCGTGGGCTGTCCTTC-3′. A 1.5-μl aliquot of the RT product was transferred to each 30 μl PCR. *Taq* DNA polymerase from Promega (Madison, WI) was used. After 3 min at 95 C, 30 cycles were run with 30-sec steps at 95, 55, and 72 C. After a final denaturation step of 3 min, denatured reaction products were allowed to reanneal for 30 min at 72 C.

Data analysis

Five microliters of PCR product were run on 3.5% agarose gels for 90 min at 30 V/cm. The ethidium-bromide-stained gel was UV transilluminated, and images were captured with a Cohu camera and an LG-3 digitizer controlled with modified NIH Image software (Scion, Frederick, MD). As described previously (10, 16), this protocol yields three bands, the bottom and middle of which are amplification products from the ZERO and STREX splice variant transcripts, respectively, in double-stranded homoduplex form (Fig. 1B). The top band is composed of the two heteroduplexes formed by indiscriminate reannealing between the STREX sense and ZERO antisense strands and the converse pairing. This three-band pattern could be reproduced by mixing independently generated STREX and ZERO products and then denaturing, reannealing, and running as described above.

To estimate relative STREX and ZERO copy numbers from band intensities, the STREX band intensity was corrected for its greater length

by multiplying by 0.709 (*i.e.* 423/597 bp). Half of the intensity value of the heteroduplex band was then added to each homoduplex value. The heteroduplex value was not corrected for length because the single-stranded DNA of the STREX loop would be expected to have a very low affinity for ethidium bromide. Representative samples were also run on denaturing polyacrylamide gels [1× Tris-borate-EDTA buffer (TBE), 8% polyacrylamide, and 40% deionized formamide] to eliminate heteroduplex formation (as described in Ref. 10). Results from these samples were consistent with results from agarose gel analysis. Estimates of the STREX percentage in PCR templates, as opposed to products, were made according to a calibration curve obtained by coamplification of known ratios of the templates, as described previously (10, 16).

All data are presented as means ± SEM. The n values represent the number of animals or tissues from at least as many animals. Data were analyzed either by ANOVA (general linear model, pairwise comparison) or two-sample *t* test with the level of significance set at $P < 0.05$.

Cell culture experiments

P13, P21, or P30 neonates were euthanized with CO_2. The anterior pituitary and adrenal medulla were dissected out. Anterior pituitaries were digested with collagenase (10 mg/2 ml Hanks' solution; Roche, Indianapolis, IN) for 15 min. The digested tissues were then triturated with fire-polished Pasteur pipettes in medium (RPMI 1640, 5% horse serum, and 2.5% fetal bovine serum). Adrenal medullae were digested with collagenase (4.5 mg/3 ml Hank's solution) for 30 min and then digested with trypsin (0.25%; Invitrogen) for 15 min. Digested adrenal medullae were triturated in medium (RPMI 1640, 10% horse serum, and 5% fetal bovine serum). All cells recovered in an incubator (37 C, 5% CO_2) for 24 h before treatment.

CORT and RU38486 were obtained from Sigma Chemical Co. (St. Louis, MO). RU28318 was a generous gift from Dr. Bruce McEwen. Culture medium was replaced with serum-free medium before the addition of steroid or antagonist. The cells were harvested with lysis buffer (QIAGEN RNeasy kit) 24 h after the addition of steroid or antagonist and immediately frozen on dry ice.

Results

Developmental parallels between CORT and STREX

Hypophysectomy at 5–6 wk of age in rats was shown previously to cause concurrent drops in CORT and the proportion of Slo transcripts in the adrenal medulla having the optional STREX (7). Because both could be prevented by ACTH injections, it was proposed that CORT itself might promote STREX inclusion into Slo. Immediately after birth, serum CORT levels in rat pups exhibit a naturally occurring drop and gradual recovery that serve as markers for the postnatal SHRP of development, during which many aspects of HPA function are suppressed (1, 3). Taking advantage of the natural CORT trajectory, we looked for concurrent changes in serum CORT and STREX splice variant representation in adrenal medulla and anterior pituitary tissues collected from different stages of neonatal rats. RIA results indicated plasma CORT levels of approximately 180 ng/ml (~540 nM) during the first 24 h after birth (Fig. 2A). Thereafter, CORT levels dropped steeply, reaching a trough at barely detectable levels within a week and remaining there through roughly P13. A gradual recovery brought levels to nearly half of P1 levels by P30, with full recovery (~170 ng/ml) by P90.

An RT-PCR protocol involving a single pair of primers that target constitutive exons flanking alternative splice sites 4–6 has been used extensively in this lab to measure the relative abundance of splice variants in adrenal and pituitary tissue (7, 10, 16). Both tissues express ZERO and STREX variants at site 5, to the virtual exclusion of other possible variants, and

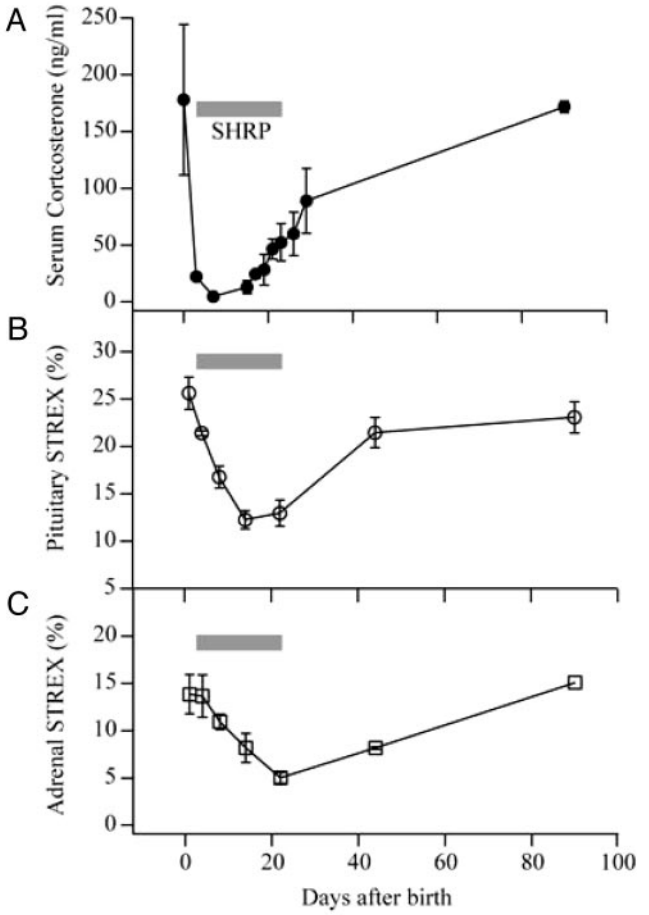

FIG. 2. Serum CORT levels and the relative abundance of Slo splice variants show parallel changes during rat postnatal development. A, CORT levels as determined by RIA from blood samples collected between 1600 and 1800 h for days shown. The SHRP is indicated by the *shaded horizontal bar*. Each data point represents the mean ± SEM for four animals. B, Postnatal changes in the percentage of total Slo amplification templates having the STREX [*i.e.* STREX/(STREX + ZERO) × 100], as estimated by RT-PCR amplification and electrophoresis from whole pituitary RNA (n = 4 animals per developmental stage). C, Postnatal changes in proportional representation of STREX templates in whole adrenal (n = 4 per stage).

no optional inserts at 4 or 6. The reproducibility of the quantitative method has been repeatedly tested; SD as a result of measurement error alone is approximately 2% (10, 16).

Strikingly, at birth, STREX variants of Slo represented 26% of total Slo transcripts (STREX plus ZERO) in whole pituitary and 14% in whole adrenal. These values were very similar to those in mature animals. Moreover, as predicted from the straightforward hypothesis that CORT promotes STREX inclusion, levels in both tissues dropped and then recovered gradually, roughly in parallel with CORT levels, although with some delay (Fig. 2, B and C). The lowest value measured for pituitary (12%) was at P13, whereas the lowest value measured for adrenal was at P21 (5%). As with the decline, recovery of STREX values were delayed relative to CORT, and that for pituitary was somewhat faster than adrenal.

Because both pituitary and adrenal glands are two glands in one (anterior and posterior pituitary, adrenal medulla and cortex), it is possible that STREX percentage changes reflect

changes in the proportional makeup of subregions of the tissues. To address this, the anterior pituitaries and adrenal medullae were separated from posterior pituitary and adrenal cortex, respectively, for separate analysis. Tissues were isolated at stages P2, P9, P14, P21, P44, and P90. The developmental patterns of decline and recovery seen with whole pituitary and adrenal were also seen with separated tissues (data not shown).

CORT effects on Slo splicing in anterior pituitary cells in culture: concentration dependence

To understand the effect of CORT on the alternative splicing during SHRP, anterior pituitaries were dissociated and cultured in vitrogen-coated petri dishes for 24 h before any treatment. Cells were then treated either with CORT (500 nM or 20 μM) or vehicle only in serum-free medium for 24 h. The 500 nm CORT increased STREX inclusion in cells isolated at P13. Thus, the STREX percentage in dishes to which CORT was added, normalized to parallel control dishes, to which only vehicle (dimethylsulfoxide) was added, was 130.6 ± 33.84% (mean ± SEM; $P = 0.005$; n = 12 sets of control and CORT-treated dishes). In contrast, 20 μM CORT decreased STREX inclusion compared with vehicle (to 78.02 ± 12.98%; $P = 0.021$; n = 4). Very similar results were obtained in separate experiments involving cells isolated at P21 and P30 (Fig. 3). Note that average values of nonnormalized percentages of STREX for control cells isolated at stage P13 were 19.4 ± 2.1 and 32.3 ± 1.8% for anterior pituitary and adrenal medulla, respectively. These figures were comparable to those from whole tissues at the same stage.

During the SHRP *in vivo*, serum CORT concentrations to which pituitary cells are presumably exposed were estimated to decline from approximately 500 nM to a low of 30 nm. Because 500 nM is within the range of positive effects of CORT seen *in vitro*, it is plausible that the decline in endogenous CORT during this period is partly or fully responsible for the drop in STREX transcript representation. It is important to note that serum CORT levels may provide a better estimate of concentrations to which pituitary cells are exposed than to those to which chromaffin cells are exposed. Because chromaffin cells are embedded within the tissue source of CORT, they are likely to be exposed at least episodically to levels much higher than those measured in systemic circulation, and perhaps as high as 100 μM (17).

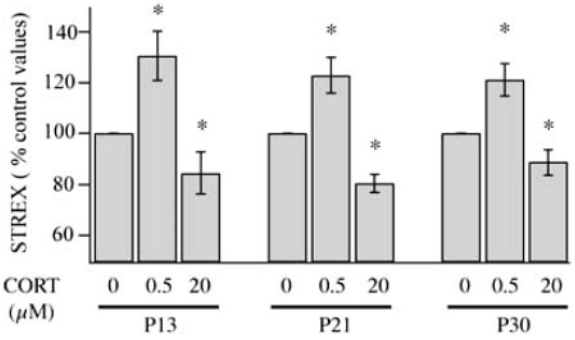

FIG. 3. Dose-dependent effects of 24-h exposure to CORT on Slo splice variant representation in rat anterior pituitary cells in cell culture. Anterior pituitary cells dissociated at P13, P21, and P30 were allowed to recover for 24 h in culture medium with serum and then treated for 24 h with either CORT (500 nM or 20 μM) or vehicle alone in serum-free culture medium. The 500 nm CORT increased STREX representation in all age groups (P13: $P < 0.005$, n = 12 pairs of dishes; P21: $P < 0.003$, n = 20; P30: $P < 0.004$, n = 12). By contrast, 20 μM CORT decreased STREX representation in all age groups (P13: $P = 0.021$, n = 4; P21: $P = 0.0001$, n = 17; P30: $P = 0.017$, n = 17). *, $P < 0.05$ compared with parallel control (vehicle only).

CORT effects on adrenal chromaffin cells in culture

Twenty-four-hour treatment with 20 μM CORT in serum-free medium decreased STREX representation in P13 adrenal chromaffin cells, as compared with parallel, vehicle-treated control dishes (to 88.4 ± 4.3%, $P = 0.015$, n = 8 pairs of dishes). This pattern was also seen with P30 cells (90.6 ± 2.4%; $P = 0.009$; n = 5), but no difference from controls was seen with P21 cells (98.5 ± 3.4%; Fig. 4). Unlike pituitary, lower doses of CORT did not increase but decreased STREX inclusion in chromaffin cells, to 94.1 ± 5.3% (n = 4) and 91.6 ± 2.7% (n = 3) for 0.5 and 2 μM doses, respectively. The results were significantly different from control only when the two doses were pooled (93.0 ± 3.0%; $P = 0.031$; n = 7). Thus, the effect of CORT on Slo splicing in developing chromaffin cells appears to be unidirectional and negative rather than positive.

MR and GR in Slo splicing regulation

Of the two nuclear receptors known to bind CORT, the MR has a higher affinity for CORT than the GR (18). To determine whether either is involved in Slo splicing regulation, antagonists for each were added in combination with CORT in cell culture experiments. In anterior pituitary cells, the positive effect of 500 nM CORT on STREX representation was blocked by the MR antagonist RU28318 (10 μM) (91.6 ± 10.0%; $P = 0.015$, as compared with 0.5 μM CORT alone; n = 8 pairs of dishes). Thus, the STREX percentage, rather than being elevated relative to that in control dishes, was not changed ($P = 0.43$; n = 8). RU28318 by itself had no effect on STREX representation (98.31 ± 3.89%; n = 6) (Fig. 5A). This suggests

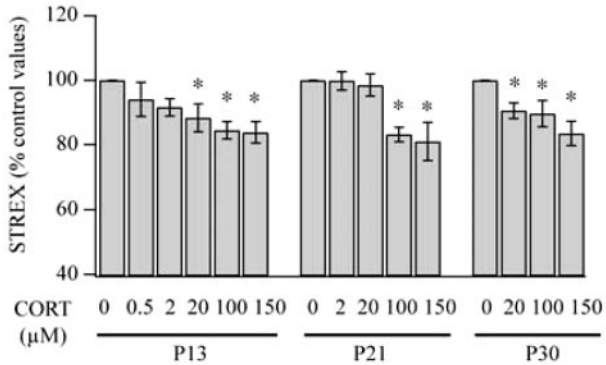

FIG. 4. Effects of CORT on splice variant representation in adrenal chromaffin cells in cell culture. Adrenal medullae were dissociated at three different stages, allowed to recover for 24 h, and exposed for 24 h to CORT in serum-free medium. The 20 μM CORT modestly decreased STREX representation in P13 ($P = 0.015$, n = 8 pairs of dishes) and P30 cells ($P = 0.009$, n = 5), but had no significant effect on P21 cells. The lower dose of CORT did not significantly reduce the STREX percentage. *, $P < 0.05$ compared with parallel controls (vehicle only).

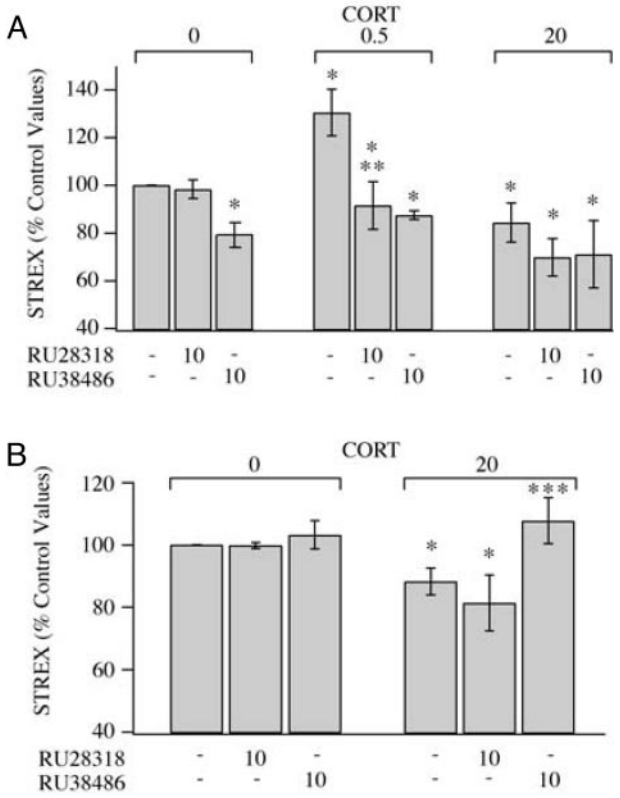

FIG. 5. Pharmacological analysis of the involvement of classic nuclear glucocorticoid receptors in Slo splicing regulation *in vitro*. A, For P13 anterior pituitary cells in culture, the positive effect of 0.5 μM CORT on STREX inclusion was blocked by cotreatment with the MR antagonist RU28318 ($P = 0.015$ as compared with 0.5 μM CORT alone, n = 8 pairs of dishes). RU28318 had no effect by itself (n = 6). The negative effect of 20 μM CORT was seen in the presence of either RU28318 or the GR antagonist RU38486 ($P = 0.47$ and 0.63, n = 5 and 5 each as compared with 20 μM CORT alone). However, the negative effect of RU38486 applied alone ($P = 0.03$, n = 6) leaves open the possibility that GR is involved in the negative regulatory effect of CORT, although the nature of the interaction of the drug with GR is unclear. B, For P13 adrenomedullary cells in culture, neither RU38486 nor RU28318 applied alone had any effect on STREX inclusion (n = 5 and 3). The negative effect of 20 μM CORT was blocked by cotreatment with the GR antagonist ($P = 0.017$ as compared with 20 μM CORT alone, n = 6) but not the MR antagonist (n = 8). *, $P < 0.05$ compared with parallel controls (vehicle only); **, $P < 0.05$ compared with CORT at 0.5 μM; ***, $P < 0.05$ compared with CORT at 20 μM.

that at low dose, CORT increases STREX inclusion by acting through MR. As with the MR antagonist, application of the GR antagonist RU38486 (10 μM) in combination with 0.5 μM CORT appeared to block the positive effect of low CORT (87.85 ± 1.82%; $P = 0.0032$; n = 4). However, application of RU38486 by itself gave even lower values (79.8 ± 8.3%; $P = 0.03$; n = 6), significantly lower than those of parallel dishes not treated with agonist or antagonist. Because RU38486 itself has a negative effect on STREX representation, it cannot be used to determine whether the positive effect of low CORT might be mediated via GR. Moreover, the negative effect of RU38486 by itself, because it is known to interact with GR, leaves open the possibility that GR activation is linked to lower rates of STREX inclusion.

In adrenal chromaffin cells, 20 μM CORT had a negative effect (described above) that was not seen with coapplication of the GR antagonist RU38486 (107.7 ± 7.4%; $P = 0.017$; n = 6) but was seen with coapplication of the MR antagonist RU28318 (81.4 ± 9.0%; $P = 0.75$; n = 8). Because RU38486 alone had no significant effect on STREX representation (nor did RU28318 alone; Fig. 5B), its apparent block of CORT's negative effect on chromaffin cells provides more straightforward support for the negative effect of CORT being mediated by GR in chromaffin cells.

Could gonadal androgens be involved in STREX splicing regulation?

Like CORT, the testosterone level is high at birth and drops steeply thereafter. Testosterone levels begin to rise later than CORT levels, beginning gradually after P20. To test whether testosterone might be involved in the recovery of STREX levels after the SHRP, animals were castrated at P16. Tissues were analyzed at P45, when STREX levels had almost reached adult levels in noncastrated animals. Although castration dramatically reduced testosterone levels without significantly altering CORT levels, STREX percentages were not affected in any of the tissues examined (adrenal cortex and medulla, anterior and posterior pituitary; Fig. 6). Thus, testosterone does not appear to regulate Slo splicing during neonatal development in these tissues.

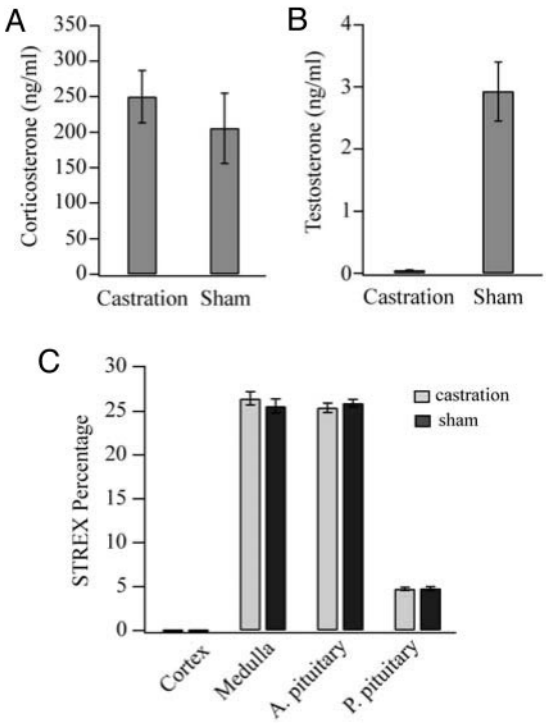

FIG. 6. Does gonadal testosterone play a role in Slo splicing regulation during development? Rats were either castrated or sham castrated at P16 and killed at P45. CORT and testosterone levels were measure in serum (A and B), and STREX percentages were measured in acutely isolated RNA from adrenal medulla, adrenal cortex, anterior pituitary, and posterior pituitary (C). No significant differences were observed between castrated and intact animals. n = 6 and 7 for castrated and sham treatments, respectively.

Discussion

The results presented here support the hypothesis that adrenal corticosteroids contribute to the regulation of expression of splice variants of a key ion channel expressed in anterior pituitary and adrenomedullary cells, with possible ramifications for intrinsic excitability and hormonal output (4, 7). Four types of data are presented: 1) a correlation between the natural pattern of decline and recovery of serum CORT levels during the SHRP and similarly biphasic changes in the relative abundance of the STREX variant of Slo, in both anterior pituitary cells and adrenal chromaffin cells, 2) evidence for positive and negative regulatory control of Slo splicing by CORT, 3) evidence for involvement of two types of glucocorticoid receptor, MR or GR, in splicing regulation, and 4) evidence against a role for testicular steroids at this stage.

Changes in Slo splicing in two neuroendocrine tissues during the SHRP

We report roughly simultaneous transient declines in the abundance of STREX-containing transcripts of Slo relative to ZERO transcripts (those having no insert at this site) in both adrenomedullary and anterior pituitary tissues. The concurrence of these changes with a transient drop in serum CORT and with other aspects of the SHRP of development argues for shared underlying mechanisms and perhaps developmental significance. These are the first data to implicate glucocorticoids in Slo splicing regulation in the pituitary, broadening the potential significance of steroid-BK channel link with respect to adaptable, multilevel neuroendocrine responses to stress.

Regulation of pituitary STREX splicing

During the first week of postnatal life in rats, serum CORT drops from approximately 500 nM to near zero and recovers only gradually over the ensuing month. In anterior pituitary cells, STREX representation drops during the same period by about 50%. STREX representation in anterior pituitary cells in dissociated cell culture can be elevated within 24 h by the addition of 500 nm CORT to the culture medium. This finding is consistent with the hypothesis that endogenous CORT *in vivo* plays a positive role in the decision to include the STREX and that bidirectional changes in endogenous CORT levels during the SHRP drive parallel changes in STREX representation. High concentrations of CORT (20 μM) have a negative effect on the relative abundance of STREX. Because this is a higher concentration than pituitary cells are likely to be exposed to, especially during the SHRP, its significance is unknown. As discussed below, this negative effect is pharmacologically distinct from the positive effect of 500 nm CORT.

Regulation of adrenal STREX splicing

The hypothesis that adrenal glucocorticoids promote inclusion of the optional STREX in chromaffin cells arose from the sharp drop in STREX (and CORT) after hypophysectomy at 5–6 wk of age and the ability of exogenous ACTH replacement to prevent it (7). However, the failure of CORT implants to prevent the STREX drop might be attributable to their failure to replicate normal CORT levels of the inner adrenal, which fluctuate to levels much higher than systemic levels. The concurrence of drops in CORT and adrenal STREX during the SHRP are very reminiscent of the hypophysectomy result and are consistent with a positive regulatory effect of CORT. Applied directly to early rat chromaffin cells *in vitro*, CORT had negative effects, even at low concentrations. The cell culture environment is by nature artificial and potentially nonpermissive, perhaps lacking cofactors required for maintaining steroid receptor or splicing factor expression, for example.

It is important to note that adult bovine chromaffin cells *in vitro*, like their rat counterparts described here, responded to direct application of CORT and dexamethasone (CORT analog) with an unexpected decrease in STREX inclusion. The current evidence for potentially negative effects of CORT on chromaffin cells is also consistent with the reduced STREX representation in adrenals of tree shrews subjected to intense chronic stress (19). In contrast to CORT, application of adrenal androgens increased STREX inclusion in bovine cells (16). This raises the possibility that other steroids under pituitary control, particularly adrenal androgens like dehydroepiandrosterone, could exhibit a developmental expression pattern like that of CORT, and perhaps promote STREX inclusion. This remains to be investigated.

GR and MR involvement

The positive effect of 500 nM CORT on pituitary STREX was blocked by RU28318, suggesting MR involvement. Differential effects of MR and GR activation are well known in hippocampal neurons and elsewhere (20–24). In the adrenal, the negative effect of CORT was blocked by the GR antagonist but not the MR antagonist. In the pituitary, the GR antagonist RU38486 failed to block CORT's negative effect and had a negative effect when applied in the absence of CORT. These results suggest interesting differences between the transduction pathways in the two cell types. The results support the idea that members of the classic nuclear receptor family may participate in splicing regulation, although their interaction with pharmacological agents and downstream transduction mechanisms cannot be assumed to be like those in transcriptional regulation.

Functional consequences of glucocorticoid regulation of STREX splicing

BK channels are very prominent in chromaffin cells, where a primary role is to facilitate repetitive firing by repolarizing rapidly after each spike, thus minimizing the refractory period. In heterologous expression systems, the STREX facilitates BK channel activation (7, 8). Consistent with this, chromaffin cells from hypophysectomized (hypox) and control rats exhibited robust differences in excitability, with hypox BK channels requiring stronger depolarizations to activate and hypox cells being much less effective at repetitive firing (4). On this basis, we would predict reduced chromaffin cell excitability during the SHRP, perhaps contributing to the suppression of stress-related behavior.

Both STREX and ZERO variants are abundantly expressed

in anterior pituitary, including in corticotropes, where their gating is modulated by PKA-dependent phosphorylation and glucocorticoid-sensitive phosphatase activity (5, 11). Intriguingly, the presence of the STREX changes the PKA response to a decrease in channel activity, rather than the increase seen with ZERO channels (6, 13, 14). The contributions of BK channels to excitability and secretion are not well characterized in corticotropes or most other pituitary cell types but can be surprising, as described, for example, in somatotropes, where their activation promotes GH secretion by enabling prolonged plateau potentials (25).

In summary, the natural developmental pattern is consistent with a positive role for CORT in relation to STREX splice variant expression in both adrenal and pituitary, as was originally hypothesized in the adrenal based on hypophysectomy experiments. *In vitro* experiments with directly applied, low-dose CORT exposure support such a role in pituitary cells but not adrenomedullary cells. High-dose experiments *in vitro* raise the possibility of an inverted U-shaped relationship between CORT and the Slo splicing decision in pituitary. Pharmacological reagents targeting nuclear MR and GR suggest a mechanism for such a dual relationship but also point to the need for additional reagents and approaches to unraveling what may be a complexly interactive regulation of cellular excitability in the neuroendocrine stress axis.

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G.L. and D.P.M. have nothing to declare.

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