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11β-HYDROXYSTEROID DEHYDROGENASE TYPE 2 PROTECTS THE NEONATAL CEREBELLUM FROM DELETERIOUS EFFECTS OF GLUCOCORTICOIDS

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Abstract—11β-Hydroxysteroid dehydrogenase type 2 is a glucocorticoid metabolizing enzyme that catalyzes rapid inactivation of corticosterone and cortisol to inert 11-keto derivatives. As 11β-hydroxysteroid dehydrogenase type 2 is highly expressed in the developing brain, but not in the adult CNS, we hypothesized that it may represent a protective barrier to the deleterious actions of corticosteroids on proliferating cells. To test this hypothesis we have investigated the development and growth of the cerebellum in neonatal C57BL/6 mice and mice lacking 11β-hydroxysteroid dehydrogenase type 2 ($^{-/-}$). 11β-Hydroxysteroid dehydrogenase type $2^{-/-}$ mice had consistently lower body weight throughout the neonatal period, coupled with a smaller brain size although this was normalized when corrected for body weight. The cerebellar size was smaller in 11β-hydroxysteroid dehydrogenase type $2^{-/-}$ mice, due to decreases in size of both the molecular and internal granule layers. When exogenous corticosterone was administered to the pups between postnatal days 4 and 13, 11β-hydroxysteroid dehydrogenase type $2^{-/-}$ mice were more sensitive, showing further inhibition of cerebellar growth while the wildtype mice were not affected. Upon withdrawal of exogenous steroid, there was a rebound growth spurt so that at day 21 postnatally, the cerebellar size in 11β-hydroxysteroid ehydrogenase type $2^{-/-}$ mice was similar to untreated mice of the same genotype. Furthermore, 11β-hydroxysteroid dehydrogenase type $2^{-/-}$ mice had a delay in the attainment of neurodevelopmental landmarks such as negative geotaxis and eye opening. We therefore suggest that 11β-hydroxysteroid dehydrogenase type 2 acts as to protect the developing nervous system from the deleterious consequences of glucocorticoid overexposure.

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Abbreviations: BrdU, bromodeoxyuridine; cDNA, complementary DNA; DAB, diaminobenzidine; EGL, external granule layer of the cerebellum; GFAP, glial fibrillary acidic protein; HPA, hypothalamic–pituitary–adrenal; IGL, internal granule layer of the cerebellum; ML, molecular layer of the cerebellum; MR, mineralocorticoid receptor; P, postnatal day; TdT, terminal-deoxynucleotidyl transferase; TUNEL, terminal-deoxynucleotidyl transferase dUTP nick-end labeling; UTP, uridine triphosphate; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2.

Key words: corticosterone, 11β-HSD2 knockout mice, growth, developmental landmarks, BrdU.

Glucocorticoids have profound effects on brain development both pre- and postnatally. Elevated glucocorticoid levels inhibit neuronal proliferation, differentiation and migration as well as dendritic arborization (de Kloet et al., 1988; Baud, 2004). Maintenance of low glucocorticoid exposure during critical periods of brain development is essential. Postnatally, this is ensured, in part, by the stress hyporesponsive period, during which the hypothalamic–pituitary–adrenal (HPA) axis is relatively unresponsive (Walker, 1986; Schmidt et al., 2003). However, the stress hyporesponsive period is not a complete protection as corticosteroid binding globulin levels are also very low, so 'free' steroids are not minimized. Moreover, the HPA axis is still responsive to some stressors (Viau et al., 1996; Dent et al., 2000). Additional mechanisms restraining glucocorticoid action are uncertain.

11β-Hydroxysteroid dehydrogenase type 2 (11β-HSD2) catalyzes the rapid inactivation of corticosterone (cortisol in humans) to inert 11-dehydrocorticosterone (cortisone). In the distal nephron, 11β-HSD2 protects mineralocorticoid receptors (MR) from activation by corticosterone (Edwards et al., 1988). Its deficiency leads to apparent mineralocorticoid excess in which glucocorticoids illicitly activate MR causing sodium retention, hypertension and hypokalemia. 11β-HSD2 is also expressed in the placenta (Benediktsson et al., 1997) where it protects the developing fetus from excess maternal glucocorticoid exposure (Benediktsson et al., 1997).

Although 11β-HSD2 is barely expressed in the adult brain, the enzyme is highly expressed in the developing CNS, until the end of mid-gestation in rats, mice and humans (Brown et al., 1996; Diaz et al., 1998). Thereafter expression becomes more restricted as each brain area ceases to proliferate and differentiates. After birth, high 11β-HSD2 expression occurs only in the proliferating, external granule layer (EGL) of the cerebellum and in several differentiating and migrating nuclei of the thalamus (Roland et al., 1995; Robson et al., 1998). Cerebellar development is particularly sensitive to high levels of glucocorticoids produced by either exogenous administration or in response to the stress induced by maternal separation (Bohn and Lauder, 1978; Mirescu et al., 2004). Furthermore, elevating maternal corticosterone levels in the lactating rat is anticipated to saturate the 11β-HSD2 barrier and indeed results in profound lifelong effects on brain biochemistry and behavior (Meerlo et al., 2001).

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In this paper we test the hypothesis that 11β-HSD2 expression in the early postnatal brain acts as a further level of protection for the still developing cerebellum. To achieve this we investigated neonatal cerebellar development in mice homozygous for targeted disruption of the 11β-HSD2 gene, 11β-HSD2$^{-/-}$ mice (Kotelevtsev et al., 1999), back-crossed onto the C57BL/6J strain for 10 generations (Paterson, Bailey, Hadoke, Brownstein, Bellamy, Fleming, Seckl, Mullins, unpublished). Furthermore, we investigated whether exogenous corticosterone administered to the neonate mimicked effects observed in mice lacking the 11β-HSD2 protective barrier and whether the 11β-HSD2 null mice were more sensitive to this treatment.

EXPERIMENTAL PROCEDURES

Animals

Male and female 11β-HSD2$^{-/-}$ mice (congenic—10 generations—on the C57BL/6J background) were housed in pairs in breeding cages with bedding for nest building. The resulting offspring were compared with offspring from similarly housed C57Bl/6J control mice. The light/dark cycle was kept constant with lights on from 07:00 h to 19:00 h. Animals were given standard chow and water *ad libitum*, and all studies were carried out to the highest standards of humane care in strict accordance with the UK Animals (Scientific Procedures) Act, 1986, and international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering. All pups were weighed on day of birth and at time of kill. One or two pups were taken from each litter and decapitated on postnatal days (P) 7, 14 and 21 (preweaning) and at P28 (postweaning).

Corticosterone treatment. To assess sensitivity to exogenous physiological glucocorticoids and determine if exogenous glucocorticoids mimic or exacerbate the phenotype observed in 11β-HSD2$^{-/-}$ mice, some litters of 11β-HSD2$^{-/-}$ and control pups were treated with corticosterone (50 ng/g i.p.) daily between P4 and 13. Pups were weighed daily at time of injection and two mice per litter were killed at P14 or P21.

Cell proliferation studies. BrdU (40 μg/g i.p.) was injected 24 h prior to kill at P7, 14 and 21.

In situ hybridization histochemistry

In situ hybridization histochemistry to determine 11β-HSD2 mRNA expression patterns in the cerebellum of C57BL/6 mice was carried out according to the method described in Brown et al. (1996). Briefly, a 666–base pair mouse 11β-HSD2 complementary DNA (cDNA) fragment corresponding to bases +393–1059, was amplified from a 2.74λ61BS clone containing 1.6 kilobase mouse 11β-HSD2 cDNA clone, with unique primers incorporating T3/T7 polymerase binding sites at their 5′ ends. The polymerase chain reaction fragment was purified on Chromaspin columns (Clontech, Palo Alto, CA, USA) and used as a template for mouse ^{35}S-labeling of 11β-HSD2 antisense and sense riboprobes. Sagittal 20 μm cryostat sections were thaw mounted onto 3-amino propyl silane coated slides. Tissue sections fixed, prehybridized, hybridized (3×10^6 cpm/section ^{35}S-uridine triphosphate (UTP)-labeled probe at 50 °C for 12–14 h) and washed as described previously (Holmes et al., 1997).

Morphometric and immunocytochemical analysis of cerebella

Brains from decapitated neonates were quickly dissected and immersion fixed in 4 ml neutral-buffered formalin (Sigma, Poole, Dorset, UK) for 24 h. Brains were then washed in 0.1 M phosphate buffer and stored at 8 °C in 70% ethanol until wax-embedded. Samples were wax-embedded in a standard protocol, 6 μm sections from the midsagittal region of the cerebellum were cut using a microtome, collected on electrostatically charged microscope slides (Superfrost, VWR Int., Lutterworth, Leicestershire, UK) and stored at RT prior to staining or immunocytochemical analysis.

Morphometric analyses. Sections at the midline of the cerebellum were stained with hematoxylin and eosin. Total area of the section (total cerebellar area), area of the molecular layer (ML) and area of the granule layer were measured using computer-assisted image analysis (MCID, Research Imaging, St. Catherines, Ontario, Canada). Mean measurements were calculated from three mid-sagittal sections for each brain.

Immunocytochemistry for cell proliferation (bromodeoxyuridine, BrdU) and glial projections (glial fibrillary acidic protein, GFAP) and TUNEL (terminal-deoxynucleotidyl transferase dUTP nick-end labeling) staining for assessment of apoptosis measurement were carried out on adjacent mid-sagittal sections of the cerebellum. Primary antibodies for BrdU and GFAP were monoclonal antibodies raised in mice (Sigma). Immunohistochemistry was carried out using a *mouse-on-mouse peroxidase* kit (Vector Laboratories, Peterborough, UK) and processed in a Sequenza (Thermo Shandon, Runcorn, Cheshire, UK). In brief, slides were heated to 50 °C for 15 min in an oven, deparaffinized in xylene for 5 min, then rehydrated through decreasing ethanols to be washed in tap water. Slides were then placed in an antigen unmasking solution (Vector Laboratories), microwaved (850 W) for 15 min, cooled under running water for 20 min, washed in PBS, treated with 0.01% Triton for 5 min, endogenous peroxidase activity was inactivated by treatment with 3% hydrogen peroxide for 5 min, blocking serum added for 1 h prior to incubation with the primary antibody for 30 min. Sections were then incubated with biotinylated secondary antibody for 10 min and finally with an avidin-bound biotinylated peroxidase complex for 5 min. Visualization of the antibody complex was possible with diaminobenzidine (DAB) staining. Control slides were processed without primary antibody and, in the case of BrdU, sections from mice not having a BrdU injection. These slides revealed no non-specific staining (data not shown).

TUNEL analysis was carried out using a commercially available kit (DeadEnd Colorimetric System; Promega, Southampton, UK), according to the manufacturer's protocol. In brief, sections were dewaxed in xylene and rehydrated in graded ethanols before antigen retrieval was performed with proteinase K and endogenous peroxidase quenched with hydrogen peroxide. Sections were then incubated with terminal-deoxynucleotidyl transferase (TdT) and biotinylated nucleotide. Nuclear DNA containing incorporated biotinylated nucleotides was then labeled by avidin bound horseradish-peroxidase using DAB as the chromogen. Sections were counterstained in hemotoxylin before mounting. Positive controls were created by pre-treating tissue with DNAse 1, while negative controls omitted the TdT enzyme step.

Quantification of the immunostained sections was carried out in three midsagittal sections from each cerebellum by counting labeled and non-labeled cells in each cerebellar layer in lobule 2 (indicated by a box in Fig. 3a). This was carried out using a color capture, computer driven image analysis package (KS Imaging, Imaging Associates, Bicester, UK). For hippocampal cell proliferation assessment, BrdU-labeled cells were counted in three coronal sections (~Bregma −2.06 mm) in the hilus and dentate gyrus for each brain.

Assessment of neurodevelopment

Negative geotaxis. At P7 pups were removed from their mothers and placed on an inclined plane of approx 30° with their head facing downwards. The number of mice which completed a reflex turn to face up the slope within a 1 min period was recorded.