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Research Report

Region-, age-, and sex-specific effects of fetal diazepam exposure on the postnatal development of neurosteroids

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ABSTRACT

Fetal exposure to diazepam (DZ), a positive modulator of $GABA_A$ receptors and an agonist at mitochondrial benzodiazine receptors, induces long-term neural and behavioral effects. This study evaluated whether the early manipulation influenced the normal development of brain levels of neurosteroids or altered steroid action at $GABA_A$ receptors. Pregnant dams were injected over gestation days 14 through 20 with DZ (2.5 mg/kg) or the vehicle. Male and female offspring were analyzed at five postnatal ages. The levels of progesterone (P), dihydroprogesterone (DHP), 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP), testosterone (T), dihydrotestosterone, and 5α-androstan-3α,17β diol were measured in the cerebral cortex and diencephalon. The results indicated that development of brain steroid levels and the impact of fetal DZ exposure were region- and sex-specific. Age-related changes in brain steroids did not mirror associated changes in circulating P and T. Age regulated the levels of all 3 progestins in the cerebral cortex, and fetal DZ exposure interacted with the development of P and DHP. The development of 3α,5α-THP in the cortex was markedly influenced by sex, with levels in males decreasing over postnatal development whereas they increased over postpubertal development in females. An adolescent surge in T levels was observed in male cortex and fetal DZ exposure prevented that surge. Steroid levels in the diencephalon were altered by age mainly in females, and DZ exposure had little effect in this region. The data support region-specific regulation of brain steroid synthesis. Only in the cerebral cortex are relevant mechanisms readily modifiable by fetal DZ exposure. However, neither sex nor fetal DZ exposure altered the response of $GABA_A$ receptors in adult cortex to neurosteroid.

1. Introduction

Experimental studies have demonstrated that perturbation of the developing brain can lead to lasting consequences, and that the effects of early perturbations often do not become apparent until much later in the life of the organism (Zagon and Slotkin, 1992). Furthermore, several clinical neurobehavioral disorders that are expressed in adulthood have come to be regarded as neurodevelopmental disorders (Keshavan and Murray, 1997). Basic research designed to define principles of neurodevelopment and the effects of interference in brain development can help elucidate understanding of developmental psychopathology.

We have examined the effects of late fetal exposure of rats to the benzodiazepine (BZD) diazepam (DZ) in order to evaluate how an early developmental manipulation could influence

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brain organization and lead to later altered function. DZ binds to a specific site on $GABA_A$ receptors (the central BZD binding site), and it is a positive modulator of GABA action at these receptors (Möhler et al., 2000). GABA is now thought to play an important trophic role in brain development (Barker et al., 1998), and GABA action at $GABA_A$ receptors in fetal brains results in depolarization, whereas hyperpolarization is characteristic of GABA action in mature brains (Cherubini et al., 1991). Interference in functioning of this receptor complex during fetal development should alter brain organization and could lead to later dysfunction. Indeed, we have described diverse long-term consequences of fetal DZ exposure and have defined developmental neural changes that may affect brain organization (Kellogg, 1998, 1999; Kellogg et al., 2000; Roberts et al., 2001). Many of the latent effects of fetal exposure to DZ can be prevented by co-exposure during fetal development to a BZD central site antagonist, implicating in utero action at $GABA_A$ receptors in mediation of the long-term effects (Kellogg, 1992). However, clearly, there are some latent effects that are not prevented by co-exposure to the central site antagonist. Furthermore, DZ not only binds at the BZD site on $GABA_A$ receptors, but it can also bind to a site on mitochondria referred to as the mitochondrial BZD receptor (MBR), a site that can influence the synthesis of neurosteroids from cholesterol (Plassart-Schiess and Baulieu, 2001).

Steroids can influence neuronal function either via action at intracellular receptors that translocate to the nucleus when bound by steroids and regulate gene expression or by interaction with certain cell membrane neurotransmitter receptors. Steroids exert a broad spectrum of effects on the brain (Rupprecht et al., 2001). Important steroids shown to affect $GABA_A$ receptor function include reduced metabolites of progesterone and testosterone (Bitran et al., 2000; Rosellini et al., 2001). We have reported very high levels of the progesterone metabolite, 3α,5α-tetrahydroprogesterone (3α,5α-THP), in rat fetal brain during the last week of gestation, the developmental period during which we expose animals in utero to DZ. The conversion of progesterone to its reduced metabolites appeared to be many fold greater in fetal than in adult brains (Kellogg and Frye, 1999). We have also demonstrated that neurosteroids influence function at $GABA_A$ receptors in fetal brain (Kellogg et al., 1998b). These observations led us to evaluate the hypothesis that the presence of DZ in fetal brains during late gestation could interact with the fetal action of neurosteroids. Consequences of such an interaction could include effects on development of brain neurosteroid levels or long-term effects on $GABA_A$ receptor function.

While levels of 3α,5α-THP have been reported to be developmentally regulated in the cerebral cortex (Grobin and Morrow, 2001), and fetal exposure to alcohol has been reported to alter the development of the neurosteroid, pregnenolone sulfate (Caldeira et al., 2004), few studies have addressed the development of brain steroids in a systematic way. In the present study, we measured levels of progesterone (P) and its reduced metabolites, dihydroprogesterone (DHP) and 3α,5α-THP as well as levels of testosterone (T) and its reduced metabolites, dihydrotestosterone (DHT) and 5α-androstan-3α,17β diol (3α-diol) in the cerebral cortex and diencephalon from neonatal to adult ages in both male and female rats. We selected these two regions for analysis as we have previously reported changes in neurotransmitter function in both of these regions in adult rats exposed in utero to DZ (Kellogg, 1992, 1998, 1999). Also, we have observed the effects of fetal exposure to DZ to be more pronounced in males than in females. Steroid levels were measured at postnatal ages corresponding to neonatal, early juvenile, late juvenile, adolescent and young adult periods of brain development. The results indicated that developmental regulation of brain steroid levels, as well as the consequences of fetal DZ exposure on neurosteroid development, are region- and sex-specific. We also measured 3α,5α-THP-facilitation of GABA-mediated chloride uptake in the cortex of adult male and female rats to determine whether fetal DZ exposure altered sensitivity to this neurosteroid. The results indicated that neither sex nor prenatal exposure affected facilitation by 3α,5α-THP in adult cortex.

2. Results

2.1. *Analysis of brain steroids*

2.1.1. *Cerebral cortex*

2.1.1.1. Progestins. The levels of the different progestins measured in male and female cortex at different ages are illustrated in Fig. 1. A 3-way ANOVA of progesterone levels indicated that the levels varied significantly with age ($P < 0.01$) and that age interacted significantly with treatment ($P < 0.05$). Probing this interaction with 1-way ANOVA of each treatment group indicated that in the control group (vehicle and uninjected, collapsed over sex) P levels changed significantly with age ($P < 0.03$), with the level at PD 60 significantly greater than levels at PD 7, 14, and 28. Hence, in control rats, irrespective of sex, P levels in the cortex increase after the onset of puberty (which occurs around PD 30–32). ANOVA of P levels in DZ-exposed rats also indicated that the levels varied significantly with age ($P < 0.05$), with levels at PD 7 less than those at PD 60. However, the analysis also indicated that in DZ-exposed animals, P levels at PD 14 were significantly greater than at PD 7 and PD 28. This indicates a transient surge in P levels after the first week of life in the cortex of DZ-exposed pups, irrespective of sex.

Three-way ANOVA of DHP levels in the cortex indicated that the levels varied significantly with treatment ($P < 0.0001$) and age ($P < 0.0001$) and that treatment interacted significantly with age ($P < 0.001$). Furthermore, there was a significant 3-way interaction between sex, age, and treatment ($P < 0.03$). A separate 2-way ANOVA of DHP levels in each sex indicated that DHP levels in females varied significantly only with treatment ($P < 0.0001$). DHP levels in females, collapsed across postnatal age, were higher in DZ-exposed animals than in controls. Two-way ANOVA of DHP levels in males indicated that levels varied significantly with age and treatment and that age interacted significantly with treatment ($P < 0.0001$). Probing this with 1-way ANOVA of DHP levels in each of the two treatment groups indicated that DHP levels in the vehicle (control) group did not vary with age. However, DHP levels varied significantly with age