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Sex Differences in Long-Term Consequences of Prenatal Diazepam Exposure: Possible Underlying Mechanisms

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KELLOGG, C. K. *Sex differences in long-term consequences of prenatal diazepam exposure: Possible underlying mechanisms*. PHARMACOL BIOCHEM BEHAV **64**(4) 673–680, 1999.—Prenatal exposure to diazepam, a benzodiazepine (BZD) compound, leads to pronounced effects on responses to stressors in exposed animals when they reach adulthood. Many of the responses are sex specific. The mechanisms mediating the effects of the exposure on the organism have not been elucidated; however, the time course for the appearance of altered function following in utero drug exposure indicates that the exposure interfered with neural organization of mechanisms mediating responses to stressors. The article discusses possible mechanisms that relate to sites of action of the drug in the developing brain: the $GABA_A$ receptor, and the mitochondrial BZD receptor. The mechanisms mediating the sex-specific impact of diazepam on the developing brain appear to be complex and interactive. © 1999 Elsevier Science Inc.

$GABA_A$ receptor Trophic factor Mitochondrial benzodiazepine receptor Steroid synthesis Neurosteroids

PRENATAL exposure of experimental animals to benzodiazepine (BZD) compounds has clearly been associated with long-term consequences in the exposed progeny (27,62): specifically, 1) integrated responses (i.e., neural, hormonal, behavioral, and immune responses) to environmental stressors are altered; 2) the consequences of the exposure are developmentally delayed, with the major effects emerging after the onset of puberty, long after any persisting drug has been cleared from the organism (66); and 3) many of the consequences are sex specific (see below). The mechanisms whereby these compounds induce the consequences remain elusive, but consideration of the particular consequences of the exposure, that is, the effects on responses to stimuli that threaten individual survival, as well as consideration of the sites of interaction of these drugs in the brain can provide a testable hypotheses.

BZDs are anxiolytic and anticonvulsive compounds that exert their predominant pharmacologic effects via binding to a specific site on the $GABA_A$ receptor. This site is referred to as the central-type BZD receptor, and occupancy of this site by BZDs facilitates the action of GABA on the chloride channel (63,73). This positive modulatory effect of the BZDs has been observed in synaptoneurosomal preparations of rat fetal cortex from gestation day 20 (28). Many of the consequences of prenatal exposure to the BZD compound, diazepam (DZ), are related to interaction of the drug with the $GABA_A$ receptor because the effects are prevented by concurrent exposure to DZ and a central-type BZD receptor antagonist (67,68). However, several BZDs can also bind to a site on the mitochondrial outer membrane (2). This site, referred to as the mitochondrial BZD receptor, has been associated with steroid biosynthesis in several organs (19,50). Furthermore, prenatal DZ exposure in the rat has been linked to later altered binding to this site (60), and mitochondrial function is altered by the exposure (46,47). The problem that will be addressed in this article is to consider how BZD action at these different sites during early development could alter neural organization leading to altered stressor-induced responses in a sex-specific manner.

SEX-SPECIFIC EFFECTS OF DEVELOPMENTAL DZ EXPOSURE

Several studies have demonstrated that early DZ exposure affects sex-specific behaviors. The earliest report that developmental exposure to DZ induced sex-related consequences indicated that the sex-specific nature of the exposure could depend upon the time of exposure (17). Prenatal exposure to DZ (10 mg/kg to the dam) over the second week of gestation in the rat had no effect on the acquisition or retention of a simultaneous choice discrimination task in animals tested as

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adults; however, exposure over the third week of gestation affected performance on this task most clearly in male rats, and postnatal exposure (days 3 through 18 to the dam) resulted in substantial deficits, with the effect now most pronounced in females. Guillamon et al. (20) analyzed two sex-specific behaviors—open-field exploration, and continuously reinforced lever-pressing responses—in adult animals that had been exposed to DZ prenatally (2.5 mg/kg to the dam) or postnatally from birth to day 16 (2.5 mg/kg to the pups). They observed no sex-related effects of the prenatal exposure on either behavior. The effects of postnatal DZ exposure on open-field exploration also were not sex related, whereas the exposure significantly affected lever pressing only in males, reducing the response rate to that normally observed in females. Because the typical sex-specific nature of open-field exploration and free-operant behavior appear to be organized under the action of sex steroids over the same developmental period (10,72), the sex-selective nature of postnatal DZ exposure on one behavior but not the other was suggested to relate to the particular sex steroid involved in the organization of the different behaviors (20). More recent work from the laboratory has shown that early postnatal exposure to DZ (1.0 or 2.5 mg/kg to the pups from birth to day 16) facilitates maternal behavior (clearly a sex-related behavior) in virgin female rats tested as adults (14). Furthermore, early postnatal DZ exposure induced maternal behaviors in adult males, whereas early postnatal exposure to the $GABA_A$ antagonist, picrotoxin (0.5 mg/kg), disrupted maternal behavior in females (65). Early developmental manipulation of $GABA_A$ receptors does appear capable of inducing gender-specific effects.

Whereas the studies cited above showed that developmental DZ exposure led to sex-specific effects by mainly affecting behavior in one sex but not the other, we have observed that early DZ exposure can lead to a reversal of typical sex-specific responses. The nature of the testing environment (novel vs. familiar) markedly affects the amount of social interaction displayed between two adult male rats (strangers to each other): social interaction is significantly reduced between males tested in the novel environment compared to interaction in a familiar environment (16,55). Reduced social interaction observed between males in a novel environment has been considered to represent a response to an anxiogenic situation (16). The nature of the environment has no impact on social interaction between two adult female rats (55). However, following prenatal exposure to DZ (1.0 or 2.5 mg/kg to the dam over the last week of gestation), the amount of social interaction in adult male rats was not related to the nature of the environment (31). In contrast, adult female rats prenatally exposed to the higher dose of DZ were responsive to the nature of the environment (Fig. 1). The early exposure to the higher dose of DZ thus reversed the sex-specific nature of environment-related social interaction.

These results suggest that perhaps the presence of DZ during in utero brain development in rats may have interfered with the organizational effects of specific gonadal steroids. It has been generally accepted that steroid hormones influence neural development by binding to cytoplasmic steroid receptors, and the hormone–receptor complex is then translocated to the nucleus where the complex binds to specific DNA sequences in regulatory regions of target genes, thereby modulating gene expression (25). Testosterone produced in fetal male testes has been considered the critical hormone involved in sexual differentiation. This hormone can interact with androgen receptors, but following aromatization of testosterone to estrogen, the estrogen formed can interact with estrogen

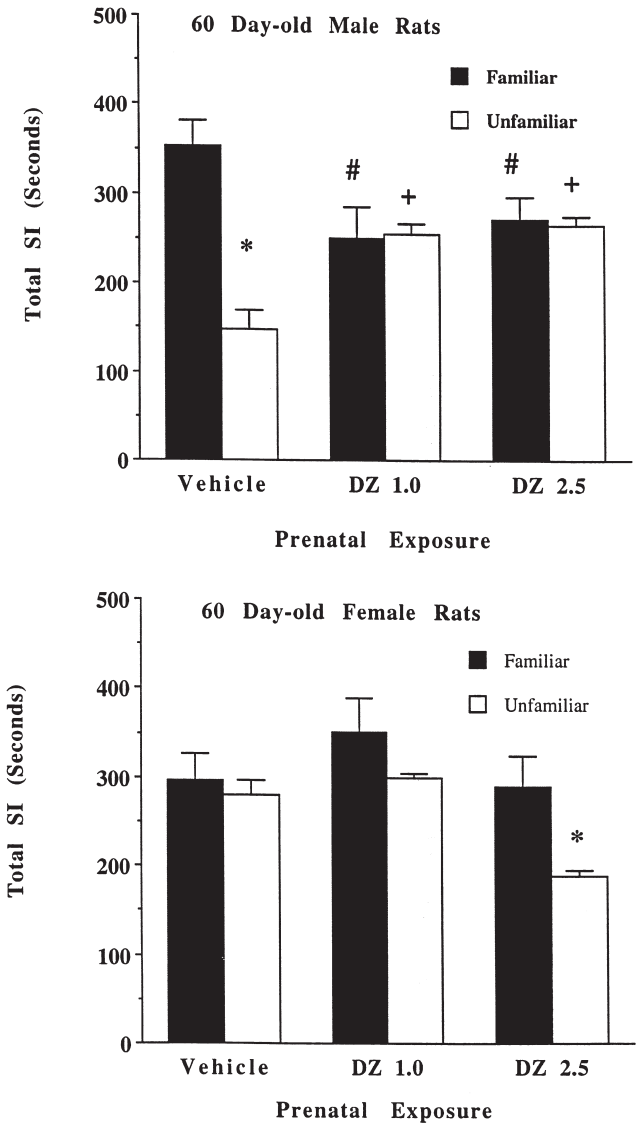

FIG. 1. Total social interaction (in seconds) in young adult male and female rats as a function of environmental challenge (confronting a stranger in a familiar vs. an unfamiliar environment) and prenatal DZ exposure. Animals were exposed in utero to DZ (1.0 or 2.5 mg/kg to pregnant dam) or vehicle (40% propylene glycol, 10% ethanol) over gestation days 14 through 20. All animals were weaned at postnatal day 28 and housed by sex and litter until day 55 when they were individually housed. All rats were prehandled daily for 5 days before testing. On the 2 days prior to testing, rats to be tested in the familiar environment were transported to the test room and individually exposed to the test chamber. Animals to be tested in an unfamiliar environment were transported to the test room, but remained in their home cage. On the test day, two rats (unacquainted) of the same sex and familiarization experience were placed in the chamber together for 7.5 min, where their behavior was videotaped for later scoring. * Indicates significant difference between environments for respective group; # indicates significant difference from familiar environment, vehicle group; + indicates significant difference from unfamiliar environment, vehicle group. From Kellogg et al. (31).

receptors. The presence of testosterone induces maleness, whereas its absence has been associated with femaleness (45). If the in utero presence of DZ interfered with these classic organizational actions of sex steroids, one would predict that early DZ exposure would affect only one sex: by drug interference with the action of androgens, early DZ exposure could disrupt male-typical development, or by mimicking the action(s) of androgens, DZ could induce male-like behaviors in females. However, in addition to genomic actions of sex steroids, reduced metabolites of the sex steroids, which can interact with membrane receptors (42,51), have also been proposed to play a role in sexual differentiation (7,64). It is becoming apparent that the role of gonadal steroids in brain sexual differentiation is not as simplistic as it is often made out to be, and must be redefined (53). Likewise, the mechanisms via which early DZ exposure disrupts sex-specific stressor-responsive behaviors in adult animals are undoubtedly complex and interactive.

DEVELOPMENTAL DZ EXPOSURE: LACK OF EFFECT ON MATURE GONADAL FUNCTION

Many of the behaviors shown to be altered by prenatal DZ exposure normally emerge with the onset of puberty. Sex-specific maternal behavior clearly emerges with mature reproductive function in females (44). Likewise, the mature sex-specific profile of environment-related social interaction emerges over adolescent development. The nature of the environment did not influence social interaction between two male rats tested at a late juvenile age (28 days) but exerted a marked influence on social interaction in animals tested just after the onset of puberty [35 days; (54)]. Thus, in males, the impact of the novel environment on social interaction emerges with the onset of reproductive function. Furthermore, while gonadal hormones are essential for the emergence of adult male-typical social interaction, they are not required for maintenance of the behavior in adult male rats (55). In contrast to males, the nature of the environment profoundly affected social interaction in females tested at a late juvenile age, whereas the environment had no effect on social interaction between two adult females (15). The impact of different environments on female social interaction, therefore, dissipates over adolescent development.

The fact that early DZ exposure affects stressor-responsive behaviors that mature after the onset of puberty in a sex-specific manner raises the question of whether the impact of the early exposure may in part be related to an effect on gonadal function, leading to deficient pubertal production of sex steroids. As indicated above, DZ can bind to mitochondrial BZD receptors in various organs, and thereby influence steroidogenesis. However, there does not appear to be any long-term effect upon gonadal steroidogenesis following developmental exposure to DZ. Segovia et al. (65) measured plasma levels of testosterone, estrogen, and progesterone in adult animals exposed to $GABA_A$ receptor ligands over early postnatal development, and observed no effects of early treatment, even though the exposures markedly altered maternal behavior. This observation is consistent with observations made in our laboratory showing no effect of prenatal exposure to DZ on male sexual behavior or on female reproduction and fecundity (unpublished). The observation that in utero DZ exposure affected stressor responses that emerge after the onset of puberty but did not affect reproductive behaviors that also emerge after puberty suggests differential in utero organization of the different behaviors. The sex-specific effects of developmental DZ exposure on adolescent organization of social interaction could relate to an effect of the early exposure on specific neural targets of gonadal hormones. The effects of the early drug exposure would then become apparent with the onset of puberty and the accompanying increased production of sex steroids.

$GABA_A$ RECEPTOR FUNCTION AND DEVELOPMENTAL DZ EXPOSURE

Because BZD receptors bind to a site on $GABA_A$ receptors, the early DZ exposure might be expected to affect $GABA_A$ receptor maturation and/or function. Moreover, $GABA_A$ receptor function in certain brain regions normally changes with adolescent development: therefore, this complex could be a target of pubertal hormonal changes. Considerable evidence indicates that $GABA_A$ receptors are targets of steroid hormones. In particular, 5α-reduced metabolites of deoxycorticosterone, progesterone, and testosterone have been shown to act as positive modulators of $GABA_A$ receptors (42,51).

$GABA_A$ receptors are pentameric structures, and at least 16 subunits have been cloned (52,63). The distribution of the different subunits varies not only from region to region in the brain, but may also vary from cell to cell within a region (52,79). The composition of pentameric $GABA_A$ receptors in mature brains then may differ likewise. Developmentally, the expression of mRNAs for different subunits follows a prolonged time course, with mature levels of predominant subunits reached by late juvenile ages (58).

Sex differences have been reported in $GABA_A$ receptor function in naive adult animals (78). Prenatal exposure to DZ does alter function of the $GABA_A$ receptor in adult animals, and some of the reported effects are sex related. The sensitivity of cortical $GABA_A$ receptors to GABA was altered in adult males exposed to DZ in utero, thereby masking any modulatory effect of acute DZ (added in vitro) on $GABA_A$ receptors (31). The early exposure did not lead to a similar effect in females. The sensitivity of $GABA_A$ receptors to GABA does not normally differ between adult males and females; thus, in this case, the early exposure did not affect a normal sex-specific response; however, the effect was assessed in animals in a basal, rather than a challenged, state. Sex differences may become apparent when animals are challenged.

Because $GABA_A$ receptors are multimeric, the function of the receptor can be influenced by allosteric interactions among different binding sites around the complex. Chloride facilitates binding to the BZD site, and exposure to environmental stressors enhances this facilitation (21). Furthermore, the impact of the chloride ion on the BZD site has been observed to be sex specific: BZD binding was considerably more sensitive to chloride in control males than in females under basal conditions (33). Additionally, whereas environmental restraint enhanced the sensitivity to chloride in males, it reduced the sensitivity in females (33). Prenatal DZ exposure enhanced the potency of chloride on BZD binding in both males and females (33), but in males, the impact of the exposure interacted significantly with environmental condition (presence or absence of restraint). Schlumpf et al. (61) have observed that prenatal exposure to DZ enhanced the hypothermic response to DZ administered acutely to adults, an effect consistent with the enhanced sensitivity to GABA: both compounds are positive modulators at the complex. However, prenatal exposure to DZ also enhanced the sensitivity to the $GABA_A$ antagonist, bicuculline, a negative modulator of the complex (8).

The effect of early DZ exposure on the responsiveness of $GABA_A$ receptors to certain ligands as well as to environmental stimuli, however, raises the question of whether the early exposure may have altered composition of $GABA_A$ receptors, because the functional responsiveness of $GABA_A$ receptors has been shown to be linked to subunit composition (75). There have been reports of a transient decrease in mRNA levels of the α_1 subunit during early postnatal development and of an increase in mRNA levels for the same subunit in specified cortical regions in the adult following prenatal exposure to DZ (62). However, we observed no effect of prenatal DZ exposure on mRNA levels (measured using ribonuclease protection assays) of α_1, α_2, β_1, β_2, or the short or long variants of the γ_2 subunit in cortical, hypothalamic, or brainstem homogenate preparations minimal effect in adult animals and in fetuses (in preparation). The lack of an effect of prenatal DZ exposure on mRNA levels of predominant $GABA_A$ subunits is consistent with the lack of an effect of the early exposure on binding to either the BZD or GABA recognition sites on the receptor complex, which suggests no major effect on the proteins involved in ligand binding (26,62). Furthermore, the early exposure also did not affect displacement of BZD binding by Cl 218–872 (26). In addition to binding to receptors composed of some of the subunits analyzed in our study, this drug also can bind with high affinity to $GABA_A$ receptors composed of subunits (such as α_5, β_3, and γ_3) not evaluated in our study (41). Therefore, although effects on mRNA for some subunits cannot be ruled out, there does not seem to be overwhelming evidence that early developmental exposure to DZ affects the composition of $GABA_A$ receptors to a degree that could explain the marked altered responsiveness of these receptors to pharmacologic agents, endogenous substances, or environmental stimuli.

The responsiveness of cortical $GABA_A$ receptors to environmental stressors emerges over adolescent development in drug-naive animals. Although environmental stimuli differentially affected chloride-facilitated BZD binding in adult male rats, the complex was not responsive to any environmental stimuli in male rats at 28 days (57). Furthermore, while forced swimming enhanced maximal GABA-stimulated chloride uptake in synaptoneurosomal preparations in control adult male rats, males tested at an early adolescent age (35 days) were unresponsive to this stressor (33). Additionally, prenatal exposure to DZ prevented the changes in $GABA_A$ receptor responsiveness from taking place over adolescence (33). The expression of predominant $GABA_A$ subunits in several regions is adult-like in male rats by a late juvenile age (58). Changes in composition of $GABA_A$ receptors, therefore, probably do not account for the marked pubertal-related changes in receptor responsiveness to environmental stressors.

However, neither changes in receptor function that normally take place over adolescent development nor the impact of prenatal DZ exposure on adolescent-related changes in receptor function need involve direct changes in the complex. Conceivably, the $GABA_A$ receptor in the cerebral cortex could be part of neural circuitry that is recruited over adolescence under the action of gonadal steroids, and developmental exposure to DZ may interfere with recruitment of that circuitry. Consistent with this hypothesis, while acute DZ treatment modulates environment-specific social interaction in adult males, the differential response to the novel environment cannot be modulated by acute DZ treatment at 35 days of age (56). Because $GABA_A$ receptor function can be modulated in vitro by DZ from late gestational ages (28), the failure of DZ to modify this particular behavior until young adulthood may indicate that the neural circuitry mediating this response is recruited over adolescence. Cells containing $GABA_A$ receptors may become part of this circuitry late in development. We have recently observed that environmental stressors activate areas in adult male rat brain that are not activated in males at 28 days of age (32). Therefore, the gender-specific nature of many of the consequences of prenatal DZ exposure, including the effect on $GABA_A$ receptor function, may relate to an impact of the exposure on gender-specific neural organization that takes place during the time of exposure, that is, in utero.

POTENTIAL TARGETS OF DZ ACTION DURING IN UTERO BRAIN DEVELOPMENT

An impact of early DZ exposure on neural organization is clearly supported by the time course for the appearance of the consequences of prenatal DZ exposure: the most marked effects emerge late in postnatal development, long after the drug has been cleared from the organism. Possible mechanisms regulating neural growth and organization that relate to DZ action at both $GABA_A$ receptors and mitochondrial BZD receptors should be considered as potential targets mediating the delayed effects. There is growing acceptance that GABA exerts a trophic influence on developing brains (4), and because DZ can modulate $GABA_A$ receptors from fetal ages in the rat (28), the presence of DZ in fetal brains could affect the trophic influences of GABA. It also has become apparent over recent years that GABA can exert depolarizing influences on developing neurons (6,11), in contrast to its predominant hyperpolarizing action in the mature brain. The depolarizing action of GABA on developing neurons promotes the influx of calcium into cells (6,40), and intracellular calcium exerts diverse influences on neurons (43). Thus, GABA can exert a major influence on neural excitability during brain development, and control over ionic currents is a critical factor in regulating neural differentiation (71). Clearly, modulation of GABA receptors can influence neuronal growth. The introduction of a neuroactive steroid into hippocampal cultures prepared from 18-day rat embryos decreased the area and length of neurites, and the modulation of chloride channels was considered as the initial step in the mechanism underlying this retraction of neurites (9). We have observed that exposure of fetal cortical cultures to DZ (10^{-5} M) also reduced neurite growth (unpublished observations).

Diazepam action at $GABA_A$ receptors in utero could affect neural differentiation and growth, with sex-specific consequences, by affecting the expression of other trophic factors. In hippocampal neurons cultured for 5 days, stimulation of GABA receptors activated voltage-gated calcium channels and induced c-*fos* immunoreactivity, an index of neural activation (6). Furthermore, this same stimulation enhanced levels of BDNF (brain-derived neurotrophic factor) mRNA. In contrast, activation of GABA receptors in neurons cultured for 3 weeks did not induce any evidence of neural activation, an observation consistent with the fact that the depolarizing effect of GABA on neurons dissipates with development. BDNF is a member of the neurotrophin family of neurotrophic factors (3), factors that shape the structure of the nervous system, are critical to the survival of neurons and play a role in differentiation as well (69). Alteration of BDNF expression during early development could have consequences throughout development. BDNF-deficient mice (those that survived more than a day or two after birth) showed few structural abnormalities of the brain, and only cranial and spi-

nal sensory neurons were substantially reduced in number (24). Levels of GABA appeared to be normal in the cerebral cortex and hippocampus, and the number of GABAergic neurons appeared normal. However, the BDNF-deficient mice showed markedly reduced neuropeptide and calcium binding protein expression in the cortex, hippocampus, and striatum. Select neuropeptides and calcium binding proteins normally are expressed in specific GABAergic neurons. Furthermore, the specific nature of calcium binding proteins in cortical GABAergic cells switches during normal postnatal development (1), and expression of specific neuropeptides in cortical neurons has been demonstrated to take place over late postnatal development (48). BDNF regulates the expression of neuropeptides in developing and adult brains (13,49). Clearly, if the ability of cells to produce BDNF is affected by early DZ exposure, this could affect the differentiation of specific cells throughout development.

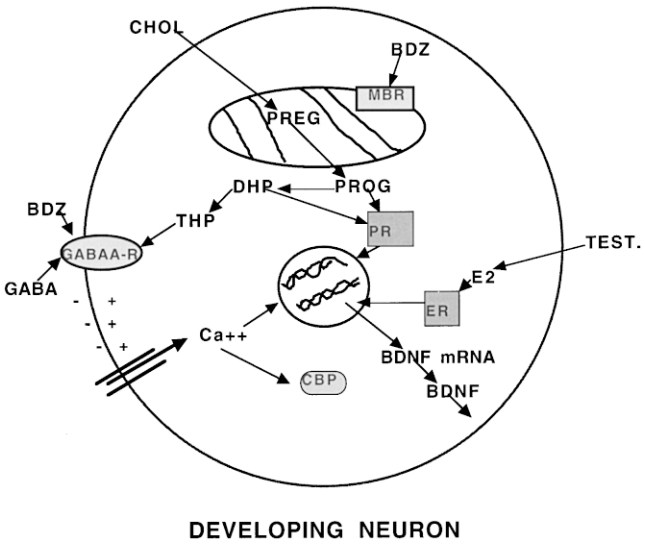

FIG. 2. Proposed mechanisms of action of DZ on developing neurons. Not all the mechanisms described will necessarily be present in each neuron. DZ can interact with the $GABA_A$ receptor (73) where it could alter the depolarizing action of GABA (11) on developing neurons. GABA stimulation in culture has been associated with increased calcium (Ca^{++}) flux and increased BDNF mRNA levels (6). Calcium can influence gene transcription via several routes (43). Sexual dimorphism from this route of interaction could arise from: 1) the sexually dimorphic distribution of specific calcium binding proteins [CBP; (39)] or, 2) the sex-specific presence of testosterone (TEST), which can be aromatized to estradiol [E_2; (22)]. The E_2 formed, by binding to the estrogen receptor (ER), can influence BDNF mRNA transcription via action at purported regulatory sties on the BDNF gene (70). Diazepam also binds to the mitochondrial BZD receptor (MBR). Simulation of MBRs affect de novo steroid synthesis in the brain (35), a process that may be taking place in neurons during early development (12,74). An influence on steroid synthesis could influence cellular levels of 5α-reduced steroids such as DHP and THP. This could alter cellular function via action at different sites: DHP can bind to progesterone receptors (PR) and influence gene transcription (59). A sexually dimorphic distribution of PR has been described (76). THP is a positive modulator of the $GABA_A$ receptor (42,51). Thus, there are several interactive mechanisms whereby in utero exposure to DZ could alter neural organization leading to sex-specific altered adaptive responses in the exposed organisms as young adults. Other abbreviations: CHOL = cholesterol; PREG = pregnenolone, PROG = progesterone.

Observations from our laboratory indicate that prenatal exposure to DZ during late gestation exerts sex-specific effects on BDNF mRNA levels during late fetal and early postnatal development (30). The sex-specific nature of the effect of in utero DZ exposure on BDNF mRNA could result from an interaction of cellular responses to drug action with the trophic actions of specific sex steroids. Putative estrogen response elements have been identified on BDNF genes (70), and the aromatization of testosterone to estrogen is a major factor in sexual differentiation (22,45). Conceivably then, an impact of GABA modulation on BDNF expression could interact with the transcription action of estrogen in male brains. Furthermore, in specific brain areas calcium-binding proteins are sexually dimorphic: the levels of calbindin-D28k are reportedly higher in the medial basal hypothalamus of male compared to female rats during late fetal development (39). Such a sexually dimorphic distribution could regulate the impact of GABA-mediated calcium influx. DZ modulation of $GABA_A$ receptor function, however, is only one mechanism via which the drug could modify neural organization.

As indicated above, DZ also can bind to the mitochondrial BZD receptor, a site that has been associated with regulation of steroidogenesis in several organs (19,50). Furthermore, the brain has the capability for de novo synthesis of steroids from cholesterol (5), and recent evidence indicates this ability in developing brains. The P450scc enzyme essential for the conversion of cholesterol to pregnenolone (the precursor of progesterone and dihydroepiandrosterone) has been identified in central and peripheral nervous systems during embryogenesis (12). A recent study indicated that this enzyme appeared in Purkinje cells immediately after differentiation (74). Additionally, the mRNA for the 5α-reductase enzyme responsible for the conversion of steroids such as progesterone and testosterone to 5α-reduced metabolites has been observed in germinal zones and differentiating fields during fetal development in the rat (37). Considered together, these observations suggest that a synchronized formation of precursor hormones and 5α-reduced metabolites may take place in developing neurons, rather than in glia as is typical in adult brain (18). Drugs acting at the mitochondrial BZD receptor influence de novo neurosteroidogenesis (35), apparently by facilitating the intramitochondrial flux of cholesterol (36). The presence of DZ then during fetal development could affect brain steroidogenesis (rather than gonadal steroidogenesis), which in turn, could affect levels of 5α-reduced steroids, potent modulators of $GABA_A$ receptor function.

Although there has been ample evidence gathered demonstrating the distribution and high activity of 5α-reductase in specific brain regions during fetal development (38), information on endogenous levels of potential 5α-reduced steroids has been lacking. To fill this gap, we recently measured levels of 5α-reduced progestins and androgens in fetal brains during late gestation (29). The results indicated that progesterone is rapidly converted to its 5α-reduced metabolites throughout the last week of gestation, whereas the conversion of testosterone is very low. The levels of progesterone were considerably lower in fetal than in adult brains, whereas the levels of the reduced metabolites were similar. Thus, the conversion of progesterone to 5α-reduced metabolites in the brain appears to decrease between fetal and adult ages. 5α-Reduced metabolites of progesterone, therefore, may play a major role in fetal neural organization. The immediate product of the 5α-reduction of progesterone is DHP (5α-pregnan-3-20-dione), which is then converted to 3α,5α-THP by the 3α-hydroxysteroidoxidoreductase enzyme. Although 3α,5α-THP modu-

lates the $GABA_A$ receptor (42,51), DHP has been shown in cell lines to bind to the progesterone receptor and regulate progesterone receptor binding to DNA (59). Such an action of DHP in vivo could have sexually dimorphic consequences during development, because recent evidence identified progesterone receptors in specific regions of the hypothalamus in males but not females during fetal and early postnatal development(76).

There are multiple mechanisms, therefore, whereby DZ present in the brain during late fetal development in the rat could influence neural growth and differentiation with sexually dimorphic consequences. A schematic diagram illustrating possible mechanisms is shown in Fig. 2. The different sites of action need not function exclusively of each other: affecting steroidogenesis via action at mitochondrial BZD receptors could affect $GABA_A$ receptor function by altering levels of specific 5α-reduced steroids. Altered $GABA_A$ function (either directly by DZ action or indirectly via DZ action at the mitochondrial site) could, in turn, influence intracellular calcium levels. Clearly, the mechanisms potentially engaged by DZ could vary in different cell groups. Cells expressing both $GABA_A$ membrane receptors and intracellular progesterone receptors, for example, have the potential for a different impact of DZ than cells expressing only one of those receptors. Sexually dimorphic distribution of potential targets of DZ action on fetal brain has been described in several hypothalamic nuclei (39,76). The hypothalamus has been shown to be a primary target of prenatal DZ exposure (67,68), and manipulation of various transmitter systems in this region has been shown to affect cortical $GABA_A$ receptor responsiveness to environmental stressors in naive adult animals (23,34). The initial impact of DZ in utero that leads to altered integrated responses to environmental stressors then may be on specific cells in the hypothalamus, a region long known to be important in homeostasis, and a region showing considerable sexual dimorphism. The observation that prenatal exposure to DZ affects mature stressor responsiveness but does not affect mature reproductive behaviors suggests that the impact of the drug is on developing mechanisms and systems specifically related to organization of mature stressor responsiveness.

SUMMARY

Although it has been known for some time that early developmental exposure to centrally acting drugs such as BZDs affects later neural and behavioral function, mechanisms that could mediate the effects of the exposure have remained elusive. However, the growing understanding of the trophic role of GABA in normal brain development and the rapidly emerging picture of neurotrophin action on the brain supports earlier hypotheses that manipulation of $GABA_A$ receptors during development could have long-lasting consequences. Although the initial results that demonstrated dose-related effects of prenatal exposure to DZ on brain function at a time period long after the drug had been cleared from the organism (67) were startling in the context of classic pharmacology, developmental insults are now considered etiological factors in behavioral disorders, such as schizophrenia, that emerge in late adolescence, young adulthood (77). The time course of appearance of the consequences of prenatal DZ exposure and the nature of the consequences suggest that understanding how BZDs influence the brain during early development will aid in understanding how insults to the developing brain in general can affect later neural organization and function.

REFERENCES

1. Alcantara, S.; de Lecea, L.; del Rio, J. A.; Ferrer, I.; Soriano, E.: Transient colocalization of parvalbumin and calbindin D28k in the postnatal cerebral cortex: Evidence for a phenotypic shift in developing nonpyramidal neurons. Eur. J. Neurosci. 8:1329–1339; 1996.
2. Anholt, R. R. H.; Petersen, P. L.; DeSouza, E. B.; Snyder, S. H.: The peripheral benzodiazepine receptor: Localization to the mitochondria outer membrane. J. Biol. Chem. 261:576–583; 1986.
3. Barde, Y.-A.: The nerve growth factor family. Prog. Growth Factor Res. 2:237–248; 1990.
4. Barker, J. L.; Lauder, J. M., eds: Perspectives on developmental neurobiology: GABA as a trophic signal, vol. 5. Maylasia: Gordon & Breach Science Publishers; 1998:101–322.
5. Baulieu, E.-E.; Robel, P.: Neurosteroids: A new brain function? J. Steroid Biochem. Mol. Biol. 37:395–403; 1990.
6. Berninger, B.; Marty, S.; Zafra, F.; Berzaghi, M. P.; Thoenen, H.: GABAergic stimulation switches from enhancing to repressing BDNF expression in rat hippocampal neurons during maturation in vitro. Development 121:2327–2335; 1991.
7. Beyer, C.; Gonzalez-Mariscal, G.: Functional implications of progesterone metabolism: Effects on psychosexual development, brain sexual differentiation, and perception. In: Archer, T.; Hansen, S., eds. Behavioral biology: Neuroendocrine axis. Hillsdale, NJ: LEA; 1991:151–165.
8. Bitran, D.; Primus, R. J.; Kellogg, C. K.: Gestational exposure to diazepam increases seizure sensitivity to convulsants that act at the GABA-benzodiazepine receptor complex and facilitates bicuculline antagonism of GABA-gated chloride channel. Eur. J. Pharmacol. 196:223–231; 1991.
9. Brinton, R. D.: The neurosteroid 3α-hydroxy-5α-pregnan-20-one induces cytoarchitectural regression in cultured fetal hippocampal neurons. J. Neurosci. 14:2763–2774; 1996.
10. Cales, J. M.; Guillamon, A.: Sex differences and the early postnatal gonadal steroids effect in the free-operant behaviour of the rat. Neurosci. Lett. Suppl. 26:249; 1986.
11. Cherubini, E.; Martina, M.; Sciancalepore, M.; Strata, F.: GABA excites immature CA3 pyramidal cells through bicuculline-sensitive and -insitive chloride-dependent receptors. Perspect. Dev. Neurobiol. 5:289–304; 1998.
12. Compagnone, N. A.; Bulfone, A.; Rubenstein, J. L. F.; Mellon, S. H.: Expression of the steroidogenic enzyme P450scc in the central and peripheral nervous systems during rodent embryogenesis. Endocrinology 136:2689–2696; 1995.
13. Croll, S. D.; Wiegand, S. J.; Anderson, D. K.; Lindsay, R. M.; Nawa, H.: Regulation of neuropeptides in adult rat forebrain by the neurotrophins BDNF and NGF. Eur. J. Neurosci. 6:1343–1353; 1994.
14. del Cerro, M. C. R.; Izquierdo, M. A. P.; Perez-Laso, C.; Rodriquez-Zafra, M.; Guillamon, A.; Segovia, S.: Early postnatal diazepam exposure facilitates maternal behavior in virgin female rats. Brain Res. Bull. 38:143–148; 1995.
15. Etzel, B. A.: Adult female-specific environment-related social interaction: Role of reproductive hormones during different periods of development. Thesis submitted to the University of Rochester in partial fulfillment of the requirements for the degree Doctor of Philosophy; 1998.
16. File, S. E.: How good is social interaction as a test of anxiety? Anim. Models Psychiatr. Disord. 1:151–166; 1988.
17. Frieder, B.; Epstein, S.; Grimm, V. E.: The effects of exposure to diazepam during various stages of gestation or during lactation on the development and behavior or rat pups. Psychopharmacology (Berlin) 83:51–55; 1984.
18. Garcia-Segura, L. M.; Chowen, J. A.; Naftolin, F.: Endocrine glia: Roles of glial cells in the brain actions of steroid and thyroid hormones and in the regulation of hormone secretion. Front. Neuroendocrinol. 17:180–211; 1996.

19. Gavish, M.; Bar-Ami, S.; Weizman, R.: The endocrine system and mitochondrial benzodiazepine receptors. Mol. Cell. Endocrinol. 88:1–13; 1992.
20. Guillamon, A.; Cales, J. M.; Rodriguez-Zafra, M.; Perez-Laso, C.; Caminero, A.; Izquierdo, M. A. P.; Segovia, S.: Effects of perinatal diazepam administration on two sexually dimorphic nonreproductive behaviors. Brain Res. Bull. 25:913–916; 1990.
21. Havoundjian, H.; Paul, S. M.; Skolnick, P.: Acute, stres-induced changes in the benzodiazepine/gamma-aminobutyric acid receptor complex are confined to the chloride ionophore. J. Pharmacol. Exp. Ther. 237:787–793; 1986.
22. Hutchison, J. B.; Beyer, C.; Hutchison, R. E.; Wozniak, A.: Sex differences in the regulation of embryonic brain aromatase. J. Steroid Biochem. Mol. Biol. 61:315–322; 1997.
23. Inglefield, F. R.; Kellogg, C. K.: Hypothalamic $GABA_A$ receptor blockade modulates cerebral cortical systems sensitive to acute stressors. Psychopharmacology (Berlin) 116:339–345; 1994.
24. Jones, K. R.; Farinas, I.; Backus, C.; Reichardt, L. F.: Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. Cell 76:989–999; 1994.
25. Kawata, M.: Roles of steroid hormones and their receptors in structural organization in the nervous system. Neurosci. Res. 24:1–46; 1995.
26. Kellogg, C. K.: Benzodiazepines: Influence on the developing brain. Prog. Brain Res. 73:207–228; 1988.
27. Kellogg, C. K.: Early developmental modulation of $GABA_A$ receptor function: Influence on adaptive responses. Perspect. Dev. Neurobiol. 4:219–234; 1998.
28. Kellogg, C. K.; Pleger, G. L.: GABA-stimulated chloride uptake and enhancement by diazepam in synaptoneurosomes from rat brain during prenatal and postnatal development. Dev. Brain Res. 49:87–95; 1989.
29. Kellogg, C. K.; Frye, C. A.: Endogenous levels of 5 alpha-reduced progestins and androgens in fetal vs. adult brains, Dev. Brain Res. 115:17–24; 1999.
30. Kellogg, C. K.; Pleger, G. L.: Altered BDNF mRNA during development may underlie delayed responses to developmental insults. Soc. Neurosci. Abstr. 25:486, no. 191–10, 1999.
31. Kellogg, C. K.; Primus, R. J.; Bitran, D.: Sexually dimorphic influence of prenatal exposure to diazepam on behavioral responses to environmental challenge and on γ-aminobutyric acid (GABA)-stimulated chloride uptake in the brain. J. Pharmacol. Exp. Ther. 256:259–265; 1991.
32. Kellogg, C. K.; Awatramani, G. B.; Piekut, D. T.: Adolescent development alters stressor-induced Fos immunoreactivity in rat brain. Neuroscience 83:681–689; 1998.
33. Kellogg, C. K.; Taylor, M. K.; Rodriguez-Zafra, M.; Pleger, G. L.: Altered stressor-induced changes in $GABA_A$ receptor function in the cerebral cortex of adult rats exposed in utero to diazepam. Pharmacol. Biochem. Behav. 44:267–273; 1993.
34. Kellogg, C. K.; Inglefield, J. R.; Taylor, M. K.; Pleger, G. L.: Importance of hypothalamic function to stressor-induced responsiveness of the $GABA_A$ receptor in the cerebral cortex: A non-corticosterone influence. Brain Res. 609:244–252; 1993.
35. Korneyev, A.; Pan, B. S.; Polo, A.; Romeo, E.; Guidotti, A.; Costa, E.: Stimulation of brain pregnenolone synthesis by mitochondrial diazepam binding inhibitor receptor ligands in vivo. J. Neurochem. 61:1515–1524; 1993.
36. Krueger, K. E.; Papadopoulos, V.: Peripheral-type benzodiazepine receptors mediate translocation of cholesterol from outer to inner mitochondrial membranes in adrenocortical cells. J. Biol. Chem 265:15015–15022; 1990.
37. Lauber, M. E.; Lichtensteiger, W.: Ontogeny of 5α-reductase (type I) messenger ribonucleic acid expression in rat brain: Early presence in germinal zones. Endocrinology 137:2718–2730; 1996.
38. Lephart, E. D.: Expression of neural 5α-reductase messenger ribonucleic acid: Comparison to 5α-reductase activity during prenatal development in the rat. Endocrinology 127:1121–1128; 1990.
39. Lephart, E. D.: Dimorphic expression of calbindin-D_{28k} in the medial basal hypothalamus from perinatal male and female rats. Dev. Brain Res. 96:281–284; 1996.
40. Lin, M.-H.; Takahashi, M. P.; Takahashi, Y.; Tsumoto, T.: Intracellular calcium increase induced by GABA in visual cortex of fetal and neonatal rats and its disappearance with development. Neurosci. Res. 20:85–94; 1994.
41. Luddens, H.; Korpi, E. R.; Seeburg, P. H.: $GABA_A$/benzodiazepine receptor heterogeneity: Neurophysiological implications. Neuropharmacology 34:245–254; 1995.
42. Najewska, M. D.: Neurosteroids: Endogenous bimodal modulators of the $GABA_A$ receptor. Mechanism of action and physiological significance. Prog. Neurobiol. 38:379–395; 1992.
43. Mattson, M. P.: Calcium as sculptor and destroyer of neural circuitry. Exp. Gerontol. 27:29–49; 1992.
44. Mayer, A. D.; Freeman, N. C.; Rosenblatt, J. S.: Ontogeny of maternal behavior in the laboratory rat: Factors underlying changes in responsiveness from 30–90 days. Dev. Psychobiol. 12:425–439; 1979.
45. McEwen, B. S.: Gonadal steroid influences on brain development and sexual differentiation. In: Greep, R., ed. Reproductive physiology. Baltimore: University Park 1983;99–145.
46. Miranda, R. C.; Wagner, J. P.; Kellogg, C. K.: Early developmental exposure to benzodiazepine ligands alters brain levels of thiobarbituric acid-reactive products in young adult rats. Neurochem. Res. 14:1119–1127; 1989.
47. Miranda, R.; Ceckler, T.; Guillet, R.; Kellogg, C. K.: Early developmental exposure to benzodiazepine ligands alters brain ^{31}P NMR spectra in young adults rats. Brain Res. 506:85–92; 1990.
48. Naus, C. C. G.; Miller, F. D.; Morrison, J. H.; Bloom, F. E.: Immunohistochemical and *in situ* hybridization analysis of the development of the rat somatostatin-contained neocortical neuronal system. J. Comp. Neurol. 269:448–463; 1988.
49. Nawa, H.; Pelleymounter, M. A.; Carnahan, J.: Intraventricular administration of BDNF increases neuropeptide expression in newborn rat brain. J. Neurosci. 14:3751–3765; 1994.
50. Papadopoulos, V.; Mukhin, A. G.; Costa, E.; Krueger, K. E.: The peripheral-type benzodiazepine receptor is functionally linked to Leydig cell steroidogenesis. J. Biol. Chem. 265:3772–3779; 1990.
51. Paul, S. M.; Purdy, R. H.: Neuroactive steroids. FASEB J. 6:51–63; 1992.
52. Persohn, E.; Malherbe, P.; Richards, G.: Comparative molecular neuroanatomy of cloned $GABA_A$ receptor subunits in the rat CNS. J. Comp. Neuroanat. 326:193–216; 1992.
53. Pilgrim, C.; Hutchison, J. B.: Developmental regulation of sex differences in the brain: Can the role of gonadal steroids be redefined? Neuroscience 60:843–855; 1994.
54. Primus, R. J.; Kellogg, C. K.: Pubertal-related changes influence the development of environment-related social interaction in the male rat. Dev. Psychobiol. 22:633–643; 1989.
55. Primus, R. J.; Kellogg, C. K.: Gonadal hormones during puberty organize environment-related social interaction in the male rat. Horm. Behav. 24:311–323; 1990.
56. Primus, R. J.; Kellogg, C. K.: Developmental influence of gonadal function on the anxiolytic effect of diazepam on environment-related social interaction in the male rat. Behav. Pharmacol. 1:437–446; 1990.
57. Primus, R. J.; Kellogg, C. K.: Gonadal status and pubertal age influence the responsiveness of the benzodiazepine/GABA receptor complex to environmental challenge in male rats. Brain Res. 561:299–306; 1991.
58. Roberts, A. A.; Kellogg, C. K.: Synchronous postnatal increase in α1 and γ2L $GABA_A$ receptor mRNAs and high affinity zolpidem binding across three regions of rat brain. Dev. Brain Res, (in press).
59. Rupprecht, R.; Reul, J. m. H. M.; Trapp, T.; van Steensel, B.; Wetzel, C.; Damm, K.; Zieglgansberger, W.; Holsboer, F.: Progesterone receptor-mediated effects of neuroactive steroids. Neuron 11:523–530; 1993.
60. Schlumpf, M.; Parmar, R.; Lichtensteiger, W.: Prenatal diazepam induced persisting downregulation of peripheral (ω3) benzodiazepine receptors on rat splenic macrophages. Life Sci. 52:927–934; 1993.
61. Schlumpf, M.; Ramseier, H. R.; Abriel, H.; Youmbi, M.; Baumann, J.; Lichtensteiger, W.: Diazepam effects on the fetus. Neurotoxicology 10:501–516; 1989.

62. Schlumpf, M.; Parmar, R.; Butikofer, E. E.; Inderbitzin, S.; Salili, A. R.; Schreiber, A. A.; Ramseier, H. R.; van Loveren, H.; Lichtensteiger, W.: Delayed developmental neuro- and immunotoxicity of benzodiazepines. Arch. Toxicol., Suppl. 17:261–287; 1994.
63. Schwartz, R. D.: The $GABA_A$ receptor-gated ion channel: Biochemical and pharmacological studies of structure and function. Biochem. Pharmacol. 37:3369–3375; 1988.
64. Segovia, S.; Guillamon, A.: Searching for sex differences in the vomeronasal pathway. Horm. Behav. 30:618–626; 1996.
65. Segovia, S.; Cruz, M.; del Cerro, R.; Ortega, E.; Perez-Laso, C.; Rodriguez-Zafra, M.; Angeles, M.; Isquierdo, P.; Guillamon, A.: Role of $GABA_A$ receptors in the organization of brain and behavioural sex differences. Neuroreport 7:2553–2557; 1996.
66. Simmons, R. D.; Kellogg, C. K.; Miller, R. K.: Prenatal diazepam: Distribution and metabolism in perinatal rats. Teratology 28:181–188; 1983.
67. Simmons, R. D.; Kellogg, C. K.; Miller, R. K.: Prenatal diazepam exposure in rats: Long-lasting, receptor-mediated effects on hypothalamic norepinephrine-containing neurons. Brain Res. 293:73–83; 1984.
68. Simmons, R. D.; Miller, R. D.; Kellogg, C. K.: Prenatal exposure to diazepam alters central and peripheral responses to stress in adult rat offspring. Brain Res. 307:39–46; 1984.
69. Snider, W. D.; Johnson, E. M.: Neurotrophic molecules. Annu. Neurol. 26:489–506; 1989.
70. Sohrabji, F.; Miranda, R.; Toran-Allerand, D.: Identification of a putative estrogen response element in the gene encoding BDNF. Proc. Natl. Acad. Sci. 92:11110–11114; 1995.
71. Spitzer, N. C.: A developmental handshake: Neuronal control of ionic currents and their control of neuronal differentiation. J. Neurobiol. 22:659–673; 1991.
72. Stewart, J.; Cygan, D.: Ovarian hormones act early in development to feminize adult open-field behavior in the rat. Horm. Behav. 14:20–32; 1980.
73. Tallman, J. F.; Gallaher, D. W.: The GABAergic system: A locus of benzodiazepine actions. Annu. Rev. Neurosci. 8:21–44; 1985.
74. Ukena, K.; Usui, M.; Kohchi, C.; Tsutsui, K.: Cytochrome P450 side-chain cleavage enzyme in the cerebellar purkinje neuron and its neonatal change in rats. Endocrinology 139:137–147; 1998.
75. Verdoon, T. A.; Draguhn, A.; Ymer, S.; Seeburg, P. H.; Salmann, B.: Functional properties of recombinant rat $GABA_A$ receptors depend upon subunit composition. Neuron 4:919–928; 1990.
76. Wagner, C. K.; Nakayama, A. Y.; DeVries, G. J.: Potential role of maternal progesterone in the sexual differentiation of the brain. Endocrinology 139:3658–3661; 1998.
77. Weinberger, D. R.: Schizophrenia as a neurodevelopmental disorder: A review of the concept. In: Hirsch, S. R.; Weinberger, D. R., eds. Schizophrenia. London: Blackwood; 1995.
78. Wilson, M. A.: GABA physiology: Modulation by benzodiazepines and hormones. Crit. Rev. Neurobiol. 10:1–38; 1996.
79. Wisdon, W.; Laurie, D. J.; Monyer, H.; Seeburg, P. H.: The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J. Neurosci. 12:1040–1062; 1992.