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TERBUTALINE IMPAIRS THE DEVELOPMENT OF PERIPHERAL NORADRENERGIC PROJECTIONS: IMPLICATIONS FOR AUTISM SPECTRUM DISORDERS AND PHARMACOTHERAPY OF PRETERM LABOR

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Abstract

Terbutaline, a β_2-adrenoceptor agonist, is used off-label for long-term management of preterm labor; such use is associated with increased risk of neurodevelopmental disorders, including autism spectrum disorders. We explored the mechanisms underlying terbutaline's effects on development of peripheral sympathetic projections in developing rats. Terbutaline administration on postnatal days 2–5 led to immediate and persistent deficiencies in cardiac norepinephrine levels, with greater effects in males than in females. The liver showed a lesser effect; we reasoned that the tissue differences could represent participation of retrograde trophic signaling from the postsynaptic site to the developing neuronal projection, since hepatic β_2-adrenoceptors decline in the perinatal period. Accordingly, when we gave terbutaline earlier, on gestational days 17–20, we saw the same deficiencies in hepatic norepinephrine that had been seen in the heart with the later administration paradigm. Administration of isoproterenol, which stimulates both β_1- and β_2-subtypes, also had trophic effects that differed in direction and critical period from those elicited by terbutaline; methoxamine, which stimulates α_1-adrenoceptors, was without effect. Thus, terbutaline, operating through trophic interactions with β_2-adrenoceptors, impairs development of noradrenergic projections in a manner similar to that previously reported for its effects on the same neurotransmitter systems in the immature cerebellum. Our results point to the likelihood of autonomic dysfunction in individuals exposed prenatally to terbutaline; in light of the connection between terbutaline and autism, these results could also contribute to autonomic dysregulation seen in children with this disorder.

Keywords

Autism; β-Adrenergic agonists; Norepinephrine; Preterm delivery; Sympathetic nervous system; Terbutaline

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INTRODUCTION

It is abundantly clear that “classic” neurotransmitters, such as norepinephrine, serotonin and acetylcholine, act as morphogens to direct the assembly of the mammalian brain (Bruel-Jungerman et al., 2011; Lauder, 1985; Whitaker-Azmitia, 1991), a function that likely evolved from their roles in invertebrate embryogenesis (Buznikov et al., 1996, 2007). Although less well studied, similar processes operate for development of peripheral innervation; in the sympathetic nervous system, for example, the development of cholinergic neurotransmission at the ganglion dictates the differentiation, neurotransmitter subtype and outgrowth of postganglionic neurons (Azevedo and Osswald, 1986; Black, 1980; Black et al., 1976). These types of neurotrophic relationships underlie the vulnerability of the immature nervous system to “misprogramming” resulting from exposure to neuroactive chemicals, leading ultimately to neurodevelopmental disorders (Grandjean and Landrigan, 2006; Lauder, 1985; Whitaker-Azmitia, 1991).

Considerable attention has been paid to the role of illicit drugs, and pesticides or other environmental neurotoxicants, but the potential impact of therapeutic agents has been less well explored. Recent attention has turned to the effects of β-adrenergic agonists and their use in the management of preterm labor, asthma and fetal heart block (Cheslack-Postava et al., 2007; Connors, 2008; Connors et al., 2005; Hadders-Algra et al., 1986; Kilburn et al., 2009; Pitzer et al., 2001; Robinson et al., 2001; Witter et al., 2009). Clinical and epidemiological studies show an increased risk of learning disabilities and autism spectrum disorders (ASD) resulting from use of these agents in pregnancy (Connors, 2008; Connors et al., 2005; Croen et al., 2011; Hadders-Algra et al., 1986; Kilburn et al., 2009; Pitzer et al., 2001; Robinson et al., 2001; Witter et al., 2009), and animal models indicate that such exposures lead to structural, neurochemical and behavioral damage (Feenstra, 1992; Garofolo et al., 2003; Rhodes et al., 2004; Slotkin et al., 2003; Witter et al., 2009; Zerrate et al., 2007). In part, this occurs because developing cells do not display β-adrenoceptor desensitization in response to excess stimulation, and instead actually show *enhanced* responsiveness (Slotkin et al., 2003; Slotkin and Seidler, 2006). Accordingly, receptor overstimulation leads to a positive feedback that further augments the cellular response to continued or subsequent stimulation, ultimately culminating in altered cell differentiation or even cell death (Connors, 2008; Fu et al., 2004; Yan et al., 2000). Such effects are particularly important because terbutaline, a β_2-selective agonist, is still used “off label” for maintenance control of preterm labor (Goldenberg, 2002), notwithstanding its ineffectiveness for that purpose (Thornton, 2005), and despite the fact that the U.S. Food and Drug Administration specifically warns against its use (U.S. Food and Drug Administration, 2011). Prolonged terbutaline administration in the second to third trimester is associated with significantly increased incidence of ASD (Connors, 2008; Connors et al., 2005; Kilburn et al., 2009; Witter et al., 2009; Zerrate et al., 2007), a risk that is likely to be even greater in individuals with β-adrenoceptor polymorphisms that impair desensitization (Cheslack-Postava et al., 2007; Connors et al., 2005).

In our previous work, terbutaline administered to newborn rats on postnatal (PN) days 2–5, neurodevelopmentally equivalent to late second trimester human development, evoked structural, functional and behavioral anomalies (Garofolo et al., 2003; Rhodes et al., 2004; Slotkin et al., 1989, 1990; Zerrate et al., 2007). Some of the most notable changes were in the cerebellum and further, the structural alterations and pattern of neuroinflammation resembled those seen in postmortem samples of children and adults with ASD (Connors, 2008; Rhodes et al., 2004; Zerrate et al., 2007). At the neurotransmitter level, terbutaline appears to disrupt noradrenergic circuits in particular, reducing cerebellar synaptogenesis for this transmitter (Slotkin et al., 1989), while at the same enhancing the expression of both α_1- and α_2-adrenoceptors (Kreider et al., 2004; Slotkin et al., 1990); again, this likely reflects

overstimulation that preempts the normal neurotrophic role of norepinephrine (Sanders et al., 2011). The question remains as to whether the effects of terbutaline reflect a specific role involving cerebellar noradrenergic projections (i.e. regional specification), or whether there is more widespread vulnerability of noradrenergic neurons that depends instead on a specific stage of neuronal differentiation. In the newborn rat, the window for the peak of cerebellar development corresponds also to the period in which peripheral noradrenergic projections develop (Rodier, 1988; Slotkin, 1986). This presents us with the opportunity to distinguish between the two possible mechanisms. If it is specifically the cerebellum that is targeted, then peripheral noradrenergic projections will not be affected similarly, but if it is a critical period of neurodifferentiation that is responsible for the defects, then the effects will be similar for sympathetic neuronal development. In the current study, we evaluated the effects of terbutaline (β_2-agonist) on development of cardiac and hepatic noradrenergic innervation in contrast to the effects of isoproterenol (β_1- and β_2-agonist) and methoxamine (α_1-agonist) during different developmental periods. We found evidence that β_2-adrenoceptor stimulation during a critical developmental period leads to deficiencies in peripheral sympathetic noradrenergic innervation, providing a link to observations of autonomic dysfunction reported in ASD (Anderson et al., 2012; Fan et al., 2009; Witter et al., 2009).

METHODS

Animal treatments

All procedures utilized tissues that were archived from earlier studies and maintained frozen at −45° C, so that no additional animals were actually used for this study. Details of animal husbandry, institutional approvals, maternal and litter characteristics, and growth curves, have all been presented in earlier work from the original animal cohorts (Garofolo et al., 2003; Kreider et al., 2004; Slotkin et al., 1996; Thai et al., 1996). Timed-pregnant Sprague-Dawley rats were housed individually and given free access to food and water. For studies of gestational terbutaline treatment, dams received daily subcutaneous injections of 10 mg/kg terbutaline sulfate (Sigma Chemical Co., St. Louis, MO) by s.c. injection on gestational days (GD) 17–20, whereas controls received equivalent volumes (1 ml/kg) of isotonic saline vehicle. Postnatal treatments were conducted similarly with daily s.c. injections to the pups on PN2–5, PN21–24 (i.e. immediately after weaning) or for four consecutive days in adulthood (males only, body weight 250–300 g); treatment groups comprised 10 mg/kg terbutaline sulfate, 1.25 mg/kg *l*-isoproterenol HCl (Sigma) or 10 mg/kg methoxamine (Sigma), each with corresponding saline control groups. The PN21–24 and adult group were included to show that the effects seen with prenatal or early postnatal exposures were developmental, that is, they occur only with treatment in a critical window. Tissue samples were obtained 24 hr after the last injection, and additionally on PN60 for the cohort given terbutaline given on PN2–5. All groups comprised no more than one male and one female from a given litter.

The doses used in this study were selected so as to produce prolonged stimulation of the corresponding adrenergic receptors in heart and liver (β_2 for terbutaline, β_1 and β_2 for isoproterenol, α_1 for methoxamine), as evidenced by effects on tissue growth, receptor concentrations and receptor-mediated signal transduction (Garofolo et al., 2003; Kreider et al., 2004; Slotkin et al., 1996; Thai et al., 1996). It would be inappropriate to match the dose in newborn rats to terbutaline given to pregnant women because terbutaline is metabolized much more quickly in rats (Tegner et al., 1984); the drug is given to humans by continuous infusion or repeated oral dosing so as to maintain round-the-clock receptor stimulation (Lam et al., 2001), and we selected doses that, given once daily, achieve the same biologic effect in rats. For terbutaline, the treatment produces prolonged adenylyl cyclase activation, β_2-receptor downregulation (Auman et al., 2001a, b), metabolic activation (Kudlacz et al., 1989; Morris and Slotkin, 1985), and brain neuroinflammation and structural changes

resembling findings in ASD (Rhodes et al., 2004; Zerrate et al., 2007). The isoproterenol treatment elicits sustained, maximal elevation of heart rate (Hou et al., 1989b; Hou and Slotkin, 1989; Seidler and Slotkin, 1979) and metabolic activation in both heart (Bareis and Slotkin, 1978; Bartolome et al., 1977) and liver (Bartolome et al., 1985; Slotkin et al., 1986). The methoxamine regimen produces stimulation sufficient to downregulate α_1-receptors (Thai et al., 1996).

Assays and data analysis

Tissues were thawed on ice and deproteinized by homogenization in 0.1 N perchloric acid containing 3,4-dihydroxybenzylamine (Sigma) as an internal standard. Homogenates were sedimented at 26,000 × g for 10 minutes, the supernatant solutions were decanted, and norepinephrine was then trace-enriched by alumina adsorption, separated by reverse-phase high performance liquid chromatography and quantitated by electrochemical detection (Seidler and Slotkin, 1981); values were corrected for recovery of the internal standard. Preliminary studies verified that measured norepinephrine levels were stable even after prolonged tissue storage at −45° C.

Data are presented as means and standard errors, with treatment differences established by ANOVA utilizing the factors of treatment, tissue, sex and age. Post-hoc tests for individual treatment effects were established with Fisher's Protected Least Significant Difference Test. Significance was assumed at $p < 0.05$. Because each treatment paradigm involved a separate cohort of animals, treatment comparisons were made only to the matched control group from the same cohort.

RESULTS

As found in our earlier studies with these treatments, terbutaline given on GD17–20 had no effect on the number of fetuses and produced little or no change fetal body weight, heart weight of liver weight (Auman et al., 2001a; Slotkin et al., 2001), nor were any changes seen for the postnatal terbutaline regimens (Auman et al., 2001b; Slotkin et al., 2001). At all ages tested, neither isoproterenol nor methoxamine had any significant effects on body weights (Thai et al., 1996); liver weights were within 5% of normal, and were unchanged relative to body weight (Thai et al., 1996). However, isoproterenol produced significant cardiac hypertrophy when given on PN21–24 or in adulthood, but not earlier (Giannuzzi et al., 1995).

Terbutaline administration on PN2–5 elicited significant deficits in peripheral norepinephrine levels that were sex- and tissue-selective (Fig. 1). In males, cardiac norepinephrine showed significant decrements on PN6, persisting into young adulthood (PN60). In the liver, there was no initial deficit but values were subnormal on PN60; although this effect was individually nonsignificant, it was also indistinguishable from the significant deficit seen in the heart at the same age, and a comparison of treatment effects across the two tissues showed a significant main effect of terbutaline ($p < 0.03$) without a treatment × tissue interaction.

In our earlier work with terbutaline, we found that hepatic β-adrenoceptor downregulation was much larger than for the heart with either gestational or postnatal treatment (Auman et al., 2001a, b), reflecting the predominance of the β_2-subtype in the liver as compared to the β_1-subtype in the heart; also, unlike the heart, hepatic β_2-receptors decline sharply after birth (McMillian et al., 1983). Accordingly, we explored whether the lack of immediate effect (PN6) on liver norepinephrine levels reflected an earlier critical period. Terbutaline given prenatally on GD17–20 elicited a large decrement in liver norepinephrine concentrations measured on GD21 (Fig. 2A), whereas the same treatment given postnatally on PN2–5 did

not (Fig. 2B). Since terbutaline is a β_2-selective agonist, we then investigated whether the relative insensitivity in the postnatal period was shared by the response to isoproterenol, which stimulates both the β_1- and β_2-subtypes; isoproterenol given on PN2–5 elicited a significant increase in hepatic norepinephrine (Fig. 2B). This effect also showed a critical period, since similar treatment on PN21–24 failed to show an increase and actually produced a significant decrease (Fig. 2C); when given in adulthood, there was no effect of isoproterenol (Fig. 2D). We also examined the consequences of α_1-adrenergic receptor stimulation with methoxamine over the same developmental period in which the response to isoproterenol changed and disappeared. Methoxamine given on PN21–24 (Fig. 2C) or in adulthood (Fig. 2D) did not cause statistically significant changes in norepinephrine levels.

DISCUSSION

Our results show that terbutaline impairs peripheral sympathetic neuronal development when exposure occurs during the same critical period in which it targets development of cerebellar noradrenergic projections, and further, that these effects are not shared by agonists that operate through adrenergic receptor subtypes other than the β_2-adrenoceptor. The findings support a specific trophic role for the β_2-adrenoceptor in neuronal development involving not only the central nervous system but also peripheral sympathetic projections.

Administration of terbutaline on PN2–5 elicited immediate and long-lasting deficits in cardiac norepinephrine levels, with the effect restricted to males. This parallels our earlier work showing greater adverse cerebellar effects of the same treatment in males (Rhodes et al., 2004). Notably, terbutaline given during this period also produces persistent β-adrenoceptor downregulation (Slotkin et al., 2005), which would further augment the functional consequences of deficient presynaptic norepinephrine levels. Interestingly, though, the effects were less notable in the liver, despite the fact that there are no substantial neurochemical disparities between these noradrenergic projections and those that innervate the heart; indeed, both pathways develop with a virtually identical time course (Slotkin et al., 1995). What is different, though, is the postsynaptic receptor population in the two tissues. The neonatal heart possesses high concentrations of β_1-receptors and α_1-receptors which are maintained into adulthood (Slotkin et al., 1995). In the liver, however, the predominant β-receptor subtype is the β_2-adrenoceptor, which is extremely high in the fetus and newborn whereas α_1-receptors are low (McMillian et al., 1983; Slotkin et al., 1995). Hepatic β_2-adrenoceptors then decline and are replaced by the α_1-subtype, which then assumes the same metabolic function (gluconeogenesis) previously controlled by β_2-receptors (Exton, 1979; Katz et al., 1985; McMillian et al., 1983). If postsynaptic receptor populations are responsible for the difference in terbutaline's effects on cardiac vs. hepatic noradrenergic projections, then it would imply that there is a retrograde signal from the end-organ that contributes to the development of these neurons. There is prior evidence for retrograde control of sympathetic neuronal development based on studies of end-organ removal (Dibner et al., 1977), but to our knowledge no one has looked at whether specific neurotransmitter receptors could trigger retrograde trophic signaling. Accordingly we focused on treatments targeting different receptor populations and different critical periods for their effects on hepatic norepinephrine.

Since hepatic β_2-adrenoceptors decline sharply in the perinatal period (McMillian et al., 1983), we reasoned that terbutaline treatment earlier than PN2–5 might elicit a response more akin to that seen in the heart. This prediction was verified: when we administered terbutaline on GD17–20, we saw deficits in hepatic norepinephrine that paralleled the effect that had been seen in the heart with the later administration paradigm. We then explored whether other adrenergic receptor subtypes exerted similar trophic effects on hepatic noradrenergic projections. Administration of isoproterenol, which targets both β_1- and β_2-

adrenoceptors, produced upregulation when given on PN2–5, the opposite effect from that obtained with terbutaline in the heart with the same regimen, or in the liver with the earlier regimen. It is thus evident that the β_1-adrenoceptor also exerts trophic control over development of hepatic noradrenergic projections but in a direction opposite to that of the β_2-receptor. There is ample precedent for these divergent trophic actions, since the two subtypes can have opposite effects on signaling pathways mediating apoptosis (Chesley et al., 2000; Shizukuda and Buttrick, 2002; Zaugg et al., 2000). The β_1-dependent trophic component likewise displayed a critical period, since isoproterenol administration on PN21–25 decreased norepinephrine instead of increasing it; administration in adulthood had no impact, reinforcing the concept that these are indeed *developmental* effects. Finally, we examined the effects of methoxamine (α_1-adrenoceptor agonist), administered during the period in which hepatic α_1-adrenoceptors spike (McMillian et al., 1983) or in adulthood. There was no effect, reinforcing the unique trophic roles of β-adrenoceptors as distinct from the α_1-subtype. It should be noted that the methoxamine regimen was sufficient to cause persistent overstimulation of the α_1-receptors, as evidenced by downregulation of this receptor subtype (Thai et al., 1996), so the lack of effect on presynaptic norepinephrine did not reflect administration of a subeffective dose.

In the present study, we pursued the duration of the synaptic defects elicited by terbutaline in only one model (treatment on PN2–5) and found that the effect lasted into young adulthood. However, persistence is not required to produce lasting defects in sympathetic function. The perinatal stage is a critical period in which presynaptic stimulation of postsynaptic targets is required for proper development of end-organ function, so that early deficiencies result in permanently subnormal responses (Hou et al., 1989a, b; Hou and Slotkin, 1989; Navarro et al., 1991; Slotkin et al., 2003). Accordingly, later-emerging changes in receptor expression and tissue function are apparent after terbutaline exposure on GD17–20 or PN2–5 but not with exposure on PN11–14 (Slotkin et al., 2005).

In summary, our results point to a specific trophic role for β-adrenoceptors modulating the development of peripheral noradrenergic projections, likely through retrograde signaling via postsynaptic receptors located in the target tissues. This role is not shared by the α_1-subtype. The results are of clinical relevance for two specific reasons. First, terbutaline is widely used in the long-term management of preterm labor, so that tens of thousands of newborns are exposed to this treatment each year in the U.S. alone (Goldenberg, 2002). Although there has been considerable work on increased risk of neurodevelopmental disorders resulting from such exposure (Cheslack-Postava et al., 2007; Connors, 2008; Connors et al., 2005; Hadders-Algra et al., 1986; Kilburn et al., 2009; Pitzer et al., 2001; Robinson et al., 2001; Witter et al., 2009), our results point to the likelihood of autonomic consequences as well, including cardiovascular and metabolic dysfunction. The second point is the potential relationship to ASD. Prolonged prenatal terbutaline exposure increases the risk of ASD, especially in association with β_2-adrenoceptor polymorphisms that enhance responsiveness (Cheslack-Postava et al., 2007; Connors, 2008; Connors et al., 2005; Witter et al., 2009), and some of our morphological findings for cerebellar development parallel those in ASD (Rhodes et al., 2004; Vargas et al., 2004; Zerrate et al., 2007). ASD is also associated with autonomic dysfunction (Anderson et al., 2012; Fan et al., 2009; Witter et al., 2009) and there is a subset of ASD patients who show specific defects in sympathetic activation (Hirstein et al., 2001). The present work shows that terbutaline exposure during the relevant developmental period for its use in preterm labor, impairs development of peripheral sympathetic projections, with the same sex selectivity found for the incidence of ASD (male > female). Our earlier work detailed adverse effects of terbutaline on expression of postsynaptic adrenergic and cholinergic receptors in these same target tissues (Auman et al., 2001a, b; Slotkin et al., 2005), properties that would amplify presynaptic defects; and indeed, we identified parallel changes in both cardiac and hepatic function in response to

sympathetic and parasympathetic stimuli (Auman et al., 2001a; Hou and Slotkin, 1989; Navarro et al., 1991; Slotkin et al., 2005). The present results are therefore relevant for outcomes of inappropriate terbutaline use in preterm labor, specifically pointing to the need to examine whether exposed children show autonomic dysfunction, as well as providing a contributory component to autonomic manifestations in ASD (Anderson et al., 2012; Fan et al., 2009; Hirstein et al., 2001; Witter et al., 2009).

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Abbreviations

ANOVA	analysis of variance
ASD	Autism Spectrum Disorders
GD	gestational day
PN	postnatal day

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Highlights

- Prenatal terbutaline exposure is associated with increased incidence of autism
- Developing rats given terbutaline showed deficient peripheral noradrenergic development
- Vulnerability depended on the concentration of β_2-adrenoceptors in the target organs
- Effects were not shared by stimulants acting at β_1- or α_1-adrenoceptors
- Impaired noradrenergic development could contribute to autonomic dysfunction in autism or in offspring of women given terbutaline for preterm labor

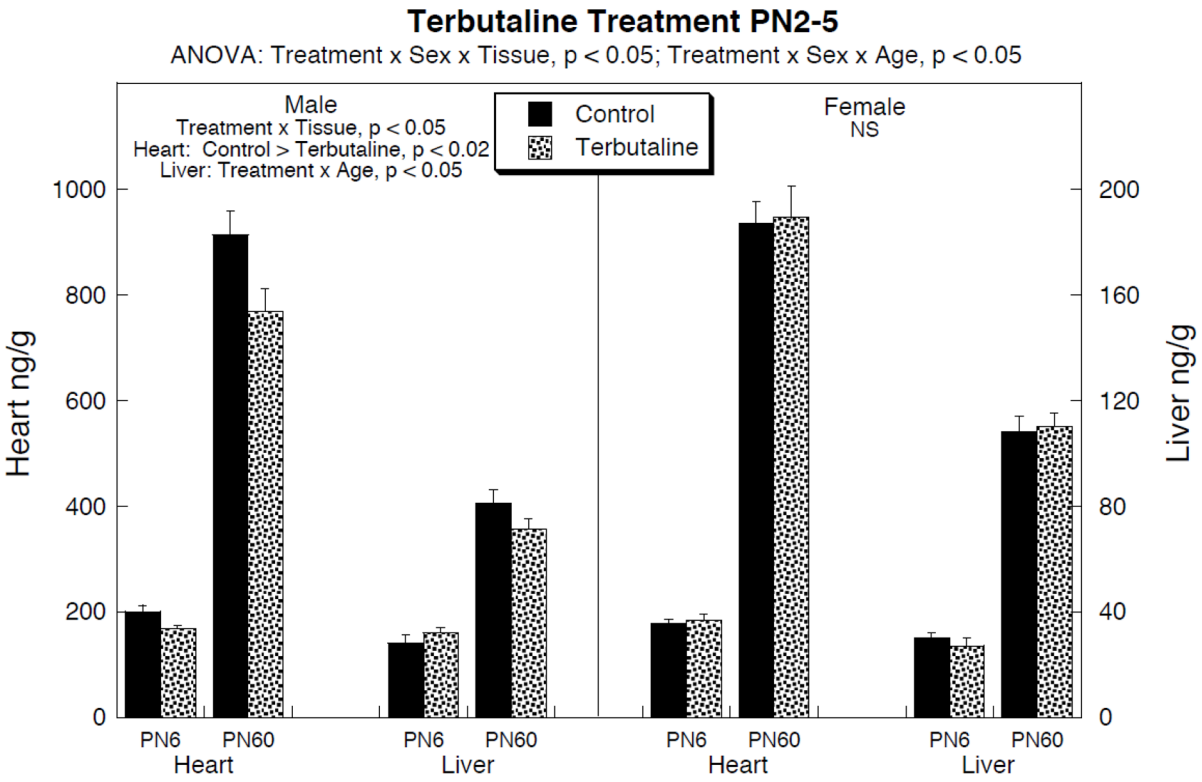

Figure 1.

Effects of terbutaline given on PN2–5 on norepinephrine levels in heart and liver (note different scales). Data represent means and standard errors obtained from 8–10 animals in each group for each age and sex. ANOVA appears at the top of the panel; lower-order tests for males and females were carried out because of the treatment interaction with sex, and these appear within the panel. For males, values were separated by tissue because of the treatment × tissue interaction. For the heart, there was no interaction of treatment × age, so only main treatment effects are reported, whereas tests at each age were carried out for the liver (significant treatment × age interaction) but were not significant; however, the decrement in both the heart and liver on PN60 were significant taken together ($p < 0.03$ for the main treatment effect, no interaction of treatment × tissue). No lower order tests were carried out for females because of the absence of significance for the overall ANOVA. NS = not significant.

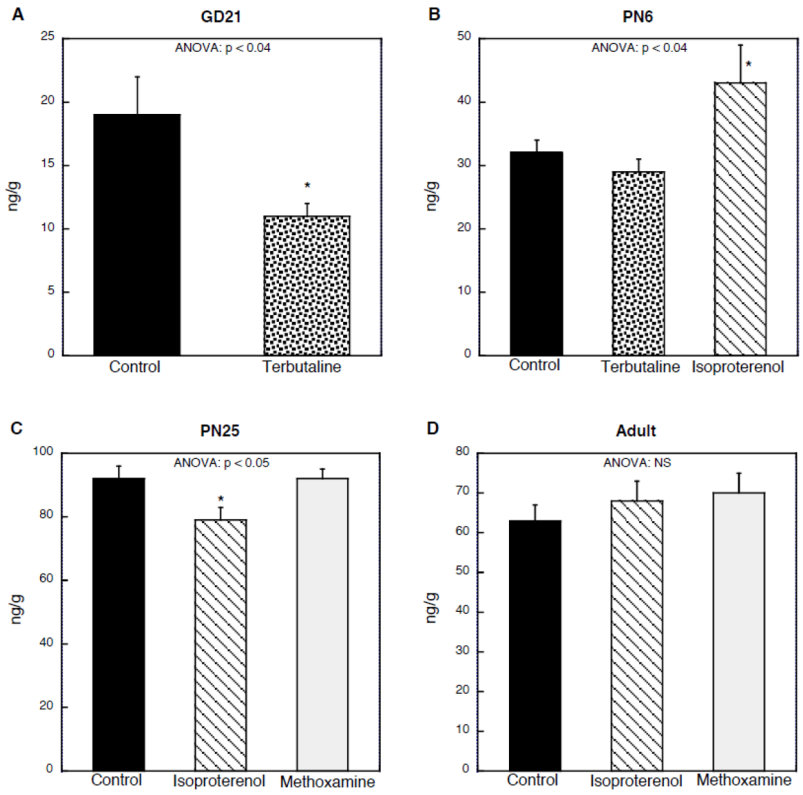

Figure 2.

Effects of adrenergic agonists on liver norepinephrine levels. The indicated agents were given for four days preceding the age at which evaluations were carried out, shown at the top of each panel: (A) terbutaline given on GD17–20, evaluated on GD21 (n=14); (B) terbutaline or isoproterenol given on PN2–5, evaluated on PN6 (control n=23, terbutaline n=17, isoproterenol n=6; (C) isoproterenol or methoxamine given on PN21–24, evaluated on PN25 (control n=11, isoproterenol n=12, methoxamine n=17); (D) isoproterenol or methoxamine given for four days in adulthood, evaluated on the fifth day (control n=12, isoproterenol n=17, methoxamine n=8). Values are shown without separation by sex: (A) sex was not determined in the fetuses; (B) and (C) there was no interaction of treatment × sex; (D) subjects were males only. ANOVA appears at the top of each panel and asterisks denote individual groups that differ from the corresponding control. Note different scales for each panel. NS = not significant.