

Neuroinflammation and Behavioral Abnormalities after Neonatal

Terbutaline Treatment in Rats: Implications for Autism*

M.C. Zerrate, M. Pletnikov, S.L. Connors, D.L. Vargas, F.J. Seidler, A.W.

Zimmerman, T.A. Slotkin, C.A. Pardo

Department of Neurology (MCZ, DLV, AWZ, CAP), Neuroimmunopathology Laboratory (MCZ, DLV, CAP), Pathology (CAP) and Department of Psychiatry and Behavioral Sciences (MP, AWZ), Johns Hopkins University School of Medicine, Baltimore, Maryland 21287; Kennedy Krieger Institute (AWZ, SLC), Baltimore, Maryland 21205; Department of Pharmacology and Cancer Biology (FJS, TAS), and Psychiatry and Behavioral Sciences (TAS), Duke University Medical Center, Durham, NC.

Neuroinflammation in Terbutaline-exposed Rat Model of Autism

Corresponding Author:

Carlos A. Pardo, MD

Department of Neurology

Pathology 627, 600 North Wolfe Street

Baltimore, Maryland 21287

Telephone: 410-614-4548

Fax: 410-502-6736

e-mail: cpardo@jhmi.edu

Number of text pages: 33

Number of tables: 1

Number of figures: 4

Number of references: 42

Abstract words: 234

Introduction words: 592

Discussion words: 1399

Abbreviations: β 2AR, β 2-adrenoceptor; Iba-1, ionized calcium binding adapter molecule 1

Section: Inflammation, Immunopharmacology, and Asthma

Abstract

Autism is a neurodevelopmental disorder presenting before 3 years of age with deficits in communication and social skills, and repetitive behaviors. In addition to genetic influences, recent studies suggest that prenatal drug or chemical exposures are risk factors for autism. Terbutaline, a β 2-adrenoceptor agonist used to arrest preterm labor, has been associated with increased concordance for autism in dizygotic twins. We studied the effects of terbutaline on microglial activation in different brain regions and behavioral outcomes in developing rats. Newborn rats were given terbutaline (10 mg/kg) daily on postnatal days (PN) 2-5 or PN 11-14, and examined 24 hr after the last dose and at PN 30. Immunohistochemical studies showed that administration of terbutaline on PN 2-5 produced a robust increase in microglial activation on PN 30 in the cerebral cortex as well as in cerebellar and cerebrocortical white matter. None of these effects occurred in animals given terbutaline on PN 11-14. In behavioral tests, animals treated with terbutaline on PN 2-5 showed consistent patterns of hyper-reactivity to novelty and aversive stimuli when assessed in a novel open field, as well as in the acoustic startle response test. Our findings indicate that β 2-adrenoceptor overstimulation during an early critical period results in microglial activation associated with innate neuroinflammatory pathways and behavioral abnormalities similar to those described in autism. This study provides a useful animal model for understanding the neuropathological processes underlying autism spectrum disorders.

Introduction

Autism is a human neurodevelopmental disorder of early childhood onset, defined by impairments in social interaction, communication skills, and stereotyped, repetitive behaviors (Lord et al., 2000). Despite efforts to clarify contributing factors, the etiology and pathophysiology of this clinically heterogeneous group of disorders remain poorly understood. Both genetic as well as gestational factors may interact and contribute to the development of autism. Animal models of autism are needed in order to understand its causes and to study pathogenic mechanisms, as well as to prevent and treat it. Several potential rodent models of autism have been developed, and include prenatal valproate exposure (Schneider and Przewlocki, 2005), neonatal serotonin depletion (Hohmann et al., 2000), the *engrailed 2* transcription factor (Cheh et al., 2006), prenatal influenza infection (Shi et al., 2003), and neonatal Borna Disease Virus (BDV) infection (Hornig et al., 1999; Pletnikov et al., 2002), among others. Each model targets specific characteristics of behavior or neuroanatomy found in autism. Prenatal drug or chemical exposures have been suggested as potential contributors to the etiology of autism (Szpir, 2006a, 2006b) including standard therapeutic interventions used in perinatal or pediatric medicine (Rhodes et al., 2004; Connors et al., 2005; Szpir, 2006b). Interestingly, one of these is terbutaline, a β 2-adrenoceptor (β 2AR) agonist developed for asthma and frequently used for preterm labor as a tocolytic agent (Lam et al., 1998). Terbutaline crosses the placenta (Bergman et al., 1984) and, by overstimulating

β 2ARs in the fetal brain, can disrupt the replication and differentiation of developing neurons (Slotkin et al., 2001). Studies of offspring of women treated with terbutaline during pregnancy showed impaired school performance, cognitive dysfunction and an increased incidence of psychiatric disorders (Hadders-Algra et al., 1986; Feenstra, 1992; Pitzer et al., 2001). Notably, the use of terbutaline in pregnancy has also been linked to increased concordance for autism in dizygotic twins (Connors et al., 2005). Earlier work showed that in the developing rat, terbutaline treatment alters neural cell replication and differentiation, synaptogenesis and expression of synaptic proteins involved in neurotransmission, culminating in biochemical and structural damage to the cerebellum, hippocampus, and somatosensory cortex (Slotkin et al., 2003; Rhodes et al., 2004). Indeed, some of the anatomical features of this model resemble those reported in postmortem examinations of corresponding brain regions in autistic individuals (Kemper and Bauman, 1998; Bailey et al., 1998). Terbutaline treated rats also show modified cell signaling that may result in an imbalance of autonomic cardiovascular responses (Slotkin et al., 2005), similar to those that have been documented in autistic children (Ming et al., 2005).

Neuroglial cells, such as microglia and astroglia, are enriched in β 2ARs (Mantyh et al., 1995; Tanaka et al., 2002), which similarly function to control replication and differentiation of these cells (Hodges-Savola et al., 1996). Neuroinflammation, mostly associated with innate immunity, characterized by activation of microglia and astroglia as well as increased cytokines and chemokines, has recently been documented in postmortem studies of autism

brains (Vargas et al., 2005). At present, whether neuroglial activation is destructive, reparative, or reflects a retained fetal pattern, is unknown. It is also unclear whether neuroinflammation in autism correlates with behaviors seen in this disorder. In the current study, we have examined the processes of neuroinflammation after terbutaline treatment of neonatal rats in an effort to correlate β 2AR overstimulation in the developing brain with neuroinflammation, as well as with subsequent behavioral abnormalities. Since earlier results showed increases in astroglial markers (Rhodes et al., 2004), we concentrated on studies of the microglial responses to determine if astrogliosis was part of a general neuroinflammatory reaction associated with neuronal dysfunction.

Methods

Animals and Treatments.

All experiments were carried out in accordance with the declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Timed pregnant Sprague-Dawley rats (Charles River, Raleigh, NC) were shipped by climate-controlled truck (transit time, 1 hr) on the second day of gestation, prior to implantation of the embryos in the uterine wall; thus, shipping occurred without any communication between maternal and fetal circulation. Fetal abnormalities at this early stage of development always result in failure to implant in the uterine wall, or in spontaneous abortion shortly thereafter. After arrival, the pregnant rats were housed in breeding cages with a 12-h light/dark cycle and free access to food and water. To obviate the initial, pretreatment differences among litters, pups from all litters were randomized on the day after birth and redistributed to the dams with litter sizes of 10 pups to ensure standardized nutrition and maternal care. For morphological studies pups were then given daily subcutaneous injections of 10 mg/kg terbutaline hemisulfate (Sigma-Aldrich, St. Louis, MO) or equivalent volumes of saline (1 ml/kg) on either postnatal day (PN) 2- 5 or PN 11- 14. For tocolytic therapy in humans, doses typically lie in the range of 0.5 mg/kg/day, but can also be as high as 1-2 mg/kg/day (Lam et al., 1998). In light of the fact that terbutaline has a much shorter half-life in the rat (Tegner et al., 1984) we used a proportionally higher dose (10 mg/kg) that likely

lies at the upper end of potential human exposures but is also of shorter duration than that often used in terbutaline maintenance therapy (Lam et al., 1998). Within a given litter, all pups received the same treatment (saline or terbutaline) to avoid the possibility that the dams might discriminate between control and treated pups. These regimens elicit robust β AR stimulation in the neonate while retaining selectivity toward β 2ARs (Slotkin et al., 2001; Slotkin et al., 2003). Because the rat is an altricial species, these treatment periods (PN 2-5 and PN 11-14) span the equivalent of the second to third trimester of human brain development (Rodier, 1988). This is the period during human pregnancy when mothers were treated with terbutaline in a study of twins with autism (Connors et al., 2005) and in which other mechanisms potentially interfering with β 2AR signaling may be operative in the development of autism (Beverdors et al., 2005).

Experiments for morphological and immunohistochemical analysis were conducted using 20-30 animals in each treatment group for each regimen, divided approximately equally between males and females and between each of the two age points, with no more than one male and one female taken from the same litter. Twenty-four hours after the last terbutaline injection (PN 6 or PN 15 for the two regimens), half of the animals were euthanized by perfusion and brains prepared for morphological studies. The rest of the animals were weaned on PN 21 and euthanized on PN 30. The animals used for morphologic examination were part of the same cohort examined in an earlier study (Rhodes et al., 2004).

A separate cohort of animals was used for behavioral analysis. Timed-

pregnant Fischer344 rats (Harlan, IN) were obtained one week before term and were housed as already described. At birth, litters were randomized and culled to eight pups per litter, since this strain has fewer pups than the Sprague-Dawley rat. Offspring were treated with terbutaline or saline using the PN 2-5 regimen and were evaluated in adolescence, using 4 males and 4 females from each treatment group, with no more than one male and one female derived from each litter.

Morphological Studies and immunocytochemistry

Animals were anesthetized with euthasol (1.25 ml/kg) and perfused through the heart with saline containing 0.1% heparin followed by 4% paraformaldehyde in Tris buffer. The brains were removed, postfixed in 4% paraformaldehyde for 24 hours, and transferred to 70% ethanol. Fixed tissue was paraffin processed, and cut into 8 μ m sections for immunohistochemical studies. Fifty-five brains from terbutaline exposed rats and 52 from control rats were processed for morphological and immunocytochemical studies.

Immunohistochemistry to identify the patterns of microglial activation was performed using the avidin-biotin-peroxidase complex method as described previously (Vargas, et al., 2005). A polyclonal antibody that recognizes the ionized calcium binding adapter molecule 1 (Ito et al., 1998; Ito et al., 2001), anti-Iba1 (Wako, Richmond, VA), was used at a dilution of 1:250 for immunostaining of the microglia population. Paraffin sections (8 μ m) mounted on slides were deparaffinized and treated with an antigen retrieval method that included boiling

the sections in distilled water in a microwave oven for 15 minutes. Following this pre-treatment the slides were treated with a blocking solution (5% normal goat serum in phosphate-buffered saline containing 0.4% Triton X-100) for 1 hour at room temperature. Biotinylated goat anti-rabbit secondary antibody was used at 1:200 (Vector Laboratories, Burlingame, CA). Sections were incubated with streptavidin-peroxidase complex, developed with diaminobenzidine (DAB) and counterstained with cresyl violet.

The magnitude of microglial (Iba1) immunostaining was quantified by the fractional area of immunoreactivity method (Gundersen et al., 1988) using a light microscope interfaced with a Stereo Investigator System (MicroBrightfield, Inc., Williston, VT). The cerebral cortex, subcortical white matter, the cerebellar granular cell layer and cerebellar white matter were outlined for quantitative studies. The fractional area (FA) of immunoreactivity for microglia (Iba-1) in those regions was used as the outcome measure for microglial reaction. A group of 30 points was systematically placed in random positions, at 20 μ m intervals, within the boundary of each region. The sum of the points falling over structures of interest (e.g., microglia) was divided by the total number of grid points sampled, to estimate the fraction of the area of the region occupied by a particular type of cell. This method measures the percentage of the area of interest that is immunoreactive for a specific antibody (FA of immunoreactivity). One individual, who was blinded to the diagnostic groups, performed the counting procedure.

Behavioral Analysis

Open field test:

Horizontal and vertical locomotor activities of the control and treated groups were recorded over a period of 30 minutes using the automated activity chambers (Accuscan Instruments, Columbus, OH) at PN 35. The size of the test arena was 15"x15"x12". Tests were run at 9-11 AM and 4-6 PM for all rats in a pseudorandom manner during the light phase of the dark-light cycle. The test chambers were cleaned after each animal. After testing the animal was returned to its home cage.

Acoustic startle response (ASR) and pre-pulse inhibition (PPI) of the acoustic startle response:

The ASR and PPI of the ASR were measured in the control and experimental rats one week after the open field test, i.e., at PN 42. Two identical startle chambers (SDI, San Diego, CA) were used for measuring startle reactivity and plasticity. Each rat was placed in a Plexiglas cylinder (9 cm in diameter) within each chamber. A loudspeaker mounted 24 cm above the cylinder provided the broadband background noise and acoustic stimuli. Presentations of the acoustic stimuli were controlled by the SR-LAB[®] software (SDI) and interface system, which also rectified, digitized, and recorded responses from the accelerometer. Sound levels were measured inside the startle cabinets by means of the digital sound level meter (Realistic, Tandy, Fort Worth, TX). The accelerometer sensitivities within each startle chamber were calibrated regularly and were found to remain constant over the test period.

For testing, a rat was placed in a plastic enclosure inside the startle

chamber and left there for five minutes of the acclimation period with the background noise of 70dB. Immediately afterwards, the test session was started and it consisted of the pseudorandom presentations of the following trials, six presentations for each type of trial: no pulse (P0); only pulse of 120dB; and the five types of prepulse-pulse combinations – P74-120; P78-120; P82-120; P86-120, and P90-120 -- where the first number indicates the intensity of the prepulse and the second number is the intensity of the pulse. The prepulse-to-pulse interval was held constant at 40 msec. The duration of the prepulse and pulse stimuli was kept at 50 msec. The maximum voltages within 50-ms reading windows, starting at stimulus onset, were used as the measures of startle amplitudes. For each animal, PPI was assessed by comparing mean startle amplitudes between the pulse only trial and each of the prepulse-pulse combinations.

Statistical Analysis

Data are presented as means and standard errors. To avoid type 1 statistical errors that might arise from repeated testing of different attributes of the same data sets, treatment differences were first established by a global, multivariate ANOVA incorporating all factors: treatment (control or terbutaline), regimen (PN 2-5 or PN 11-14), sex, brain region (morphology), age (morphology), and session (behavior) or pulse intensity (behavior). Data in the multivariate tests were log-transformed because of the heterogeneous variance inherent in comparisons across different ages and regions (morphology), or different sessions or pulse intensities (behavior). Lower-order tests were then conducted

as justified by the interaction of treatment with the other variables and, where appropriate, individual values in the terbutaline group were compared to the corresponding controls using Fisher's Protected Least Significant Difference. In the absence of interactions of treatment with other variables, only main treatment effects are reported without separate testing of individual values. Significance was assumed at $p < 0.05$ for main effects of treatment; however, for interactions at $p < 0.1$, we also examined whether lower-order main effects were detectable after subdivision of the interactive variables. To enable us to present disparate values across ages and regions on the same axis, some data are given as the percent change from corresponding control values; however, statistical analyses were performed only on the original data. For reference, the corresponding control values are shown in Table 1, combined across the two treatment cohorts (controls for the PN 2-5 regimen and PN 11-14 regimen) but the comparisons between terbutaline and control groups were conducted only on the appropriately matched control cohort.

Results

Microglial Reactions after Terbutaline Exposure

For assessment of microglia reactions (FA of Iba1 immunoreactivity), the global ANOVA indicated overall treatment effects that interacted with age, regimen (PN 2-5 vs. PN 11-14) and brain region (Figure 1). We subdivided the analysis into the two different treatment regimens and for these measures, there were significant effects with terbutaline given either on PN 2-5 or PN 11-14; however, because the treatment effects were not interactive with sex, data were combined for males and females. For the early treatment regimen, we found interactions of treatment with age and region; the effects were more notable at the PN 30 age point than at PN 6. Individually significant differences were found for cerebellar white matter on PN 30, cerebrocortical white matter on both PN 6 and PN 30 and cerebral cortex on PN 30. For the later treatment regimen, there was only a weak interaction of treatment with age and region, reflecting a significant difference for cerebral cortex on PN 15. As shown in Figure 1, the net effect of PN 2-5 terbutaline administration was to produce initial deficits on PN 6 followed by a robust increase in microglial activation on PN 30 (Figure 2) across three of the four regions, effects that were not seen when terbutaline treatment was shifted to PN 11-14. The magnitude of microglial increase at PN 30 parallels cytomorphological changes as prominent cytoplasm and ramified processes were observed in the microglial population of cortical and cerebellar regions (Figure 2), changes consistent with microglial activation (Graeber and Streit,

1990).

Behavior

In keeping with an earlier report on animals at PN 30 (Rhodes et al., 2004), terbutaline administration in the cohort used for behavioral analysis did not affect body weight of the rats assessed later in adolescence (data not shown). Figure 3 demonstrates the data for the open field test. Neonatal injections of terbutaline on PN 2-5 had significant effects on novelty-induced locomotor activity in rats, producing a consistent pattern of hyperactivity (main effect of treatment, $p < 0.002$ across measures of both horizontal and vertical activity). For horizontal activity (total distance covered, Figure 3A), three-factor ANOVA (treatment, sex, session) confirmed a significant main effect of terbutaline; we subdivided the results by sex in light of sex differences in other parameters of open field behavior (see below) and found that the effect was statistically robust in females but not significant in males, although the lack of a treatment \times sex interaction and the small number of subjects indicates the need for some caution in ascribing sex selectivity to the effect on distance covered. We also examined whether terbutaline treatment altered the spatial preference for locomotor activity, comparing distance covered along the margins of the field vs. distance covered in the center (data not shown). Again, for both these parameters there was significant hyperactivity caused by terbutaline treatment with some indication of sex selectivity ($p < 0.04$ for the main effect of terbutaline along the margins; $p < 0.03$ for treatment \times sex \times session for the center). However, terbutaline did not

cause a significant shift in the proportion of time spent in the two spatial zones. For vertical activity (Figure 3B), we again found a robust main effect of terbutaline without any sex interaction; here, the effects were clearly present in both males and females.

Terbutaline administration on PN 2-5 also had significant effects on startle responses indicative of hyperactivity, and for these measures, the effect was clearly preferential for females. Three-factor ANOVA for the peak amplitude of the ASR (Figure 4A) indicated a main effect of the treatment as well as a significant sex interaction. Lower-order ANOVAs for male and female rats demonstrated a significant effect in females but not males. Similarly, latency to the peak effect (Figure 4B) showed a main effect of terbutaline that was significant in both males and females. PPI was generally unaffected by terbutaline treatment, so that the decline in startle responses with increasing pre-pulse intensity was seen equally in control and terbutaline-exposed animals (no significant interaction of treatment \times PPI intensity); accordingly, the treatment effects described above were apparent irrespective of changes in pre-pulse intensity used to modulate the startle response.

Discussion

In earlier studies, architectural and biochemical anomalies in the cerebellum, somatosensory cortex and hippocampus were identified after terbutaline exposure on PN 2-5, whereas few effects were seen when the exposure window was shifted to PN 11-14 (Rhodes et al., 2004). In the current work we similarly identified both innate neuroinflammatory responses as represented by microglial activation and behavioral anomalies centered around the same critical period, which corresponds to the second to third trimester of human brain development (Rodier, 1988). Not only is this the period in which terbutaline is most often used to arrest preterm labor (Lam et al., 1998), but it is also the period in which other mechanisms related to overstimulation of the β 2AR, such as stress, may contribute to the development of autism (Beverdorf et al., 2005). As detailed below, the changes seen in previous work and in the current study parallel those documented in autism, thus reinforcing a mechanistic connection between autism and β 2AR overstimulation (Connors, et al., 2005), as well as providing evidence that the terbutaline model may prove useful in pursuing both etiology and treatment paradigms.

Robust reactions were observed in microglial activation with the early administration of terbutaline (PN 2-5). An initial deficit in microglial activation in cerebrocortical white matter at PN 6 was followed at PN 30 by strongly increased activation in cerebellar white matter, cerebrocortical white matter, and cortex. Just as was found from earlier work on cerebellar and cerebrocortical

architecture (Rhodes et al., 2004), there was no corresponding effect when the treatment occurred later (PN 11-14), outside the window of the corresponding period in which terbutaline exposure is likely to occur in human fetal development. Equally important, our findings at PN 30 resemble the neuroinflammatory changes seen as innate immune responses, documented in examinations of postmortem brain tissue in patients with autism (Vargas et al., 2005), where marked microglial and astroglial activation was found in the cerebral cortex, white matter and cerebellum. Interestingly, previous immunoblot studies of brain tissues in this model had also shown small but significant elevations in GFAP on PN 6, and elevated numbers of glial cells in different areas of the brain (Rhodes et al., 2004). A similar finding of astrogliosis in postmortem brain samples of autistic children (Vargas et al., 2005) has been reported. Here, our findings for PN 30 suggest that the higher glial numbers seen earlier may entail effects on microglia as well as astroglia.

Neuroglia are not only the first line of defense in response to CNS injury, but are also essential for normal neurodevelopment. Microglia and astroglia play key roles in cortical modeling, neuroaxonal guidance and synaptic plasticity; they are required for neuronal activity and axonal functioning (Aschner et al., 1999). Hence, activation of microglial responses may represent a mechanism that contributes to the ultimate disruption of cerebellar and cerebrocortical architecture noted for terbutaline treatment (Rhodes et al., 2004), or the corresponding changes in autism (Vargas et al., 2005). Indeed, given the high concentration of β 2ARs on glial cells and the key role played by this receptor in

their proliferation and differentiation (Hodges-Savola et al., 1996; Tanaka et al., 2002), it is not only possible, but rather likely, that overstimulation of glial β 2ARs by terbutaline provides the driving force for the subsequent morphologic and behavioral abnormalities we observed. By extension then, microglial activation might contribute directly to the etiology and neuropathology of autism.

Two aspects of our findings have especially important implications for autism: the time course for appearance of microglial activation and the nature of the neuroanatomical lesions. We found a delayed onset between terbutaline administration and the appearance of neuroinflammation, as microglial activation was not apparent on PN 6 but was robust by PN 30. This delay is in keeping with the later emergence of a neurobehavioral disorder that nevertheless originates from an insult during pregnancy. The time course thus supports recent views that chemical and physiologic insults during pregnancy may have long-lasting effects for brain structure and function (Stanwood and Levitt, 2004), and may contribute to the increase in the incidence of autism spectrum disorders as well as other neurodevelopmental disabilities (Szpir, 2006a). Equally important, if microglial activation proves to be part of the pathogenesis of autism, then our model suggests there may be a window of opportunity when therapeutic interventions might be started. This would be similar to the situation in adrenoleukodystrophy in which treatment is effective if begun prior to the onset of neuroinflammation (Moser, 2006). The second factor is that our findings bear a distinct relationship to the neuroanatomical features of autism. Neuroanatomical abnormalities have been well documented in autistic patients. Excessive growth in brain size during

the first two years of life in children with autism has been shown by Courchesne and colleagues (Courchesne, 2004) and significant increases in subcortical white matter, mostly in the frontal cortex, has been demonstrated by magnetic resonance imaging studies (Herbert, 2005). Our findings for terbutaline show corresponding regional effects, and again, microglial activation may provide the mechanistic link. Microglia promote production of myelin by oligodendrocytes in vitro (Hamilton and Rome, 1994), which in turn may contribute to the increased white matter found in the autism brain. In addition, direct stimulation of β 2ARs on developing oligodendrocytes by itself stimulates expression of myelin basic protein (Sato-Bigbee et al., 1999).

Encouraged by the neuropathological findings, we conducted a preliminary behavioral analysis of rats treated with terbutaline on PN 2-5. A consistent pattern of hyper-reactivity was found in the open field tests and in the acoustic startle response. The behavioral findings in our study correlate with those from other animal models of autism (Andres, 2002), and are analogous to the clinical presentation of many autistic patients who display auditory hypersensitivity as well as impulsive, hyperactive and irritable features. Although the small size of the cohort studied here made statistical detection of sex differences problematic, we did observe tendencies toward greater effects in female rats, which corresponds to their greater loss of pyramidal neurons in the cerebral cortex and thinning of the hippocampal layers (Rhodes et al., 2004). At the same time, the loss of cerebellar Purkinje cells tended to be greater in males (Rhodes et al., 2004), so a more detailed behavioral battery may detect further

sex differences after terbutaline treatment. Most obviously, future development of this animal model should include assessment of possible social deficits (the most disabling feature of autism spectrum disorders). The observed hyper-reactivity in the terbutaline-treated rats could result from a loss of cerebellar Purkinje cells and pyramidal neurons in the somatosensory cortex, and/or thinning of hippocampal layers. Similar behavioral outcomes have been described in rats after neonatal lesions of the hippocampus or cerebellum (Altman, 1987).

However, because our studies were conducted in different laboratories and institutions, the rat strain used for morphology was different from that used for the behavioral studies, which may influence the relationship we have drawn between the two sets of findings. Nevertheless, the fact that parallel anomalies were found in the two strains also supports the generalizability of our conclusions, since the results span both an outbred (Sprague-Dawley) and inbred (Fischer 344) strain. If there is a direct relationship between the morphological abnormalities and behavioral outcomes, then we would predict that the PN 11-14 treatment regimen would not elicit the same effects on open field and acoustic startle performance. Future work should define whether this is the case.

Our data indicate that β 2AR overstimulation during an early developmental period in the rat results not only in neuroinflammation similar to that found in autism, but also in behavioral abnormalities seen frequently in children with this disorder. Thus, overstimulation of the β 2AR by terbutaline at PN 2-5 can provide a useful animal model for autism, to facilitate understanding of neuropathology in this disorder and to develop potential future treatments, as

well as providing a mechanistic underpinning for our observations of autism in dizygotic twins exposed to this agent prenatally (Connors et al., 2005). Of course, it is doubtful that any one animal model can recapitulate all the aspects of autism, but individually they can characterize specific contributory mechanisms or outcomes. Our model, like others, provides important clues to the neuropathology and corresponding behavioral anomalies of a few key features of autism, but nevertheless also points toward mechanisms that can be exploited both for understanding the pathogenesis of autism and for designing interventions that may prevent or offset the effects of neurodevelopmental processes that culminate in this disorder.

References

- Altman J (1987) Morphological and behavioral markers of environmentally induced retardation of brain development: an animal model. *Environ Health Perspect* **74**:153-168.
- Andres C (2002) Molecular genetics and animal models in autistic disorder. *Brain Res Bull* **57**:109-119.
- Aschner M, Allen JW, Kimelberg HK, LoPachin RM and Streit WJ (1999) Glial cells in neurotoxicity development. *Annu Rev Pharmacol Toxicol* **39**:151-173.
- Bailey A, Luthert P, Dean A, Harding B, Janota I, Montgomery M, Rutter M and Lantos P (1998) A clinicopathological study of autism. *Brain* **121 (Pt 5)**:889-905.
- Bergman B, Bokstrom H, Borga O, Enk L, Hedner T and Wangberg B (1984) Transfer of terbutaline across the human placenta in late pregnancy. *Eur J Respir Dis Suppl* **134**:81-86.
- Beversdorf DQ, Manning SE, Hillier A, Anderson SL, Nordgren RE, Walters SE, Nagaraja HN, Cooley WC, Gaelic SE and Bauman ML (2005) Timing of prenatal stressors and autism. *J Autism Dev Disord* **35**:471-478.

Cheh MA, Millonig JH, Roselli LM, Ming X, Jacobsen E, Kamdar S and Wagner GC (2006) En2 knockout mice display neurobehavioral and neurochemical alterations relevant to autism spectrum disorder. *Brain Res* **1116**:166-176.

Connors SL, Crowell DE, Eberhart CG, Copeland J, Newschaffer CJ, Spence SJ and Zimmerman AW (2005) beta2-adrenergic receptor activation and genetic polymorphisms in autism: data from dizygotic twins. *J Child Neurol* **20**:876-884.

Courchesne E (2004) Brain development in autism: early overgrowth followed by premature arrest of growth. *Ment Retard Dev Disabil Res Rev* **10**:106-111.

Feenstra MG (1992) Functional neuroteratology of drugs acting on adrenergic receptors. *Neurotoxicology* **13**:55-63.

Graeber MB and Streit WJ (1990) Microglia: immune network in the CNS. *Brain Pathol* **1**:2-5.

Gundersen HJ, Bendtsen TF, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sorensen FB, Vesterby A et al (1988) Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. *APMIS* **96**:379-394.

Hadders-Algra M, Touwen BC and Huisjes HJ (1986) Long-term follow-up of children prenatally exposed to ritodrine. *Br J Obstet Gynaecol* **93**:156-161.

Hamilton SP and Rome LH (1994) Stimulation of in vitro myelin synthesis by microglia. *Glia* **11**:326-335.

Herbert MR (2005) Large brains in autism: the challenge of pervasive abnormality. *Neuroscientist* **11**:417-440.

Hodges-Savola C, Rogers SD, Ghilardi JR, Timm DR and Mantyh PW (1996) Beta-adrenergic receptors regulate astrogliosis and cell proliferation in the central nervous system in vivo. *Glia* **17**:52-62.

Hohmann CF, Richardson C, Pitts E and Berger-Sweeney J (2000) Neonatal 5,7-DHT lesions cause sex-specific changes in mouse cortical morphogenesis. *Neural Plast* **7**:213-232.

Hornig M, Weissenbock H, Horscroft N and Lipkin WI (1999) An infection-based model of neurodevelopmental damage. *Proc Natl Acad Sci U S A* **96**:12102-12107.

Ito D, Imai Y, Ohsawa K, Nakajima K, Fukuuchi Y, Kohsaka S (1998) Microglia-specific localisation of a novel calcium binding protein, Iba1. *Brain Res Mol Brain Res* **57**:1-9.

Ito D, Tanaka K, Suzuki S, Dembo T, Fukuuchi Y (2001) Enhanced expression of Iba1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain. *Stroke* **32**:1208-1215.

Kemper TL and Bauman M (1998) Neuropathology of infantile autism. *J Neuropathol Exp Neurol* **57**:645-652.

Lam F, Elliott J, Jones JS, Katz M, Knuppel RA, Morrison J, Newman R, Phelan J and Willcourt R (1998) Clinical issues surrounding the use of terbutaline sulfate for preterm labor. *Obstet Gynecol Surv* **53**:S85-S95.

Lord C, Cook EH, Leventhal BL and Amaral DG (2000) Autism spectrum disorders. *Neuron* **28**:355-363.

Mantyh PW, Rogers SD, Allen CJ, Catton MD, Ghilardi JR, Levin LA, Maggio JE and Vigna SR (1995) Beta 2-adrenergic receptors are expressed by glia in vivo in the normal and injured central nervous system in the rat, rabbit, and human. *J Neurosci* **15**:152-164.

Ming X, Julu PO, Brimacombe M, Connor S and Daniels ML (2005) Reduced cardiac parasympathetic activity in children with autism. *Brain Dev* **27**:509-516.

Moser HW (2006) Therapy of X-linked adrenoleukodystrophy. *NeuroRx* **3**:246-253.

Pitzer M, Schmidt MH, Esser G and Laucht M (2001) Child development after maternal tocolysis with beta-sympathomimetic drugs. *Child Psychiatry Hum Dev* **31**:165-182.

Pletnikov MV, Moran TH and Carbone KM (2002) Borna disease virus infection of the neonatal rat: developmental brain injury model of autism spectrum disorders. *Front Biosci* **7**:d593-d607.

Rhodes MC, Seidler FJ, bdel-Rahman A, Tate CA, Nyska A, Rincavage HL and Slotkin TA (2004) Terbutaline is a developmental neurotoxicant: effects on neuroproteins and morphology in cerebellum, hippocampus, and somatosensory cortex. *J Pharmacol Exp Ther* **308**:529-537.

Rodier PM (1988) Structural--functional relationships in experimentally induced brain damage. *Prog Brain Res* **73**:335-348.

Sato-Bigbee C, Pal S and Chu AK (1999) Different neuroligands and signal transduction pathways stimulate CREB phosphorylation at specific developmental stages along oligodendrocyte differentiation. *J Neurochem* **72**:139-147.

Schneider T and Przewlocki R (2005) Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. *Neuropsychopharmacology* **30**:80-89.

Shi L, Fatemi SH, Sidwell RW and Patterson PH (2003) Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. *J Neurosci* **23**:297-302.

Slotkin TA, Auman JT and Seidler FJ (2003) Ontogenesis of beta-adrenoceptor signaling: implications for perinatal physiology and for fetal effects of tocolytic drugs. *J Pharmacol Exp Ther* **306**:1-7.

Slotkin TA, Tate CA, Cousins MM and Seidler FJ (2001) Beta-adrenoceptor signaling in the developing brain: sensitization or desensitization in response to terbutaline. *Brain Res Dev Brain Res* **131**:113-125.

Slotkin TA, Tate CA, Cousins MM and Seidler FJ (2005) Imbalances emerge in cardiac autonomic cell signaling after neonatal exposure to terbutaline or chlorpyrifos, alone or in combination. *Brain Res Dev Brain Res* **160**:219-230.

Stanwood GD and Levitt P (2004) Drug exposure early in life: functional repercussions of changing neuropharmacology during sensitive periods of brain development. *Curr Opin Pharmacol* **4**:65-71.

Szpir M (2006a) New thinking on neurodevelopment. *Environ Health Perspect* **114**:A100-A107.

Szpir M (2006b) Tracing the origins of autism: a spectrum of new studies.

Environ Health Perspect **114**:A412-A418.

Tanaka KF, Kashima H, Suzuki H, Ono K and Sawada M (2002) Existence of functional beta1- and beta2-adrenergic receptors on microglia. *J Neurosci Res* **70**:232-237.

Tegner K, Nilsson HT, Persson CG, Persson K and Ryrfeldt A (1984) Elimination pathways of terbutaline. *Eur J Respir Dis Suppl* **134**:93-100.

Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW and Pardo CA (2005) Neuroglial activation and neuroinflammation in the brain of patients with autism. *Ann Neurol* **57**:67-81.

FOOTNOTES:

*This work was supported by the Fund for Autism Research established by Mr. Peter Emch, Cure Autism Now and The Fetal Physiology Foundation. Dr. Pardo is supported by a NIH-NIDA K08-DA16160. The authors declare that they have no conflicts of interest. Theodore Slotkin, Frederic Seidler and Andrew Zimmerman have provided expert witness testimony on behalf of government agencies, corporations and/or individuals.

For Reprints:

Carlos A. Pardo, MD

Department of Neurology

Pathology 627, 600 North Wolfe Street

Baltimore, Maryland 21287

e-mail: cpardo@jhmi.edu

LEGENDS FOR FIGURES

Figure 1. Microglial responses in rats given terbutaline either on PN 2-5 or PN 11-14, assessed 24 hr after the last dose of terbutaline and on PN 30. Data represent means and standard errors, presented as the percentage change from control values (Table 1). ANOVA across both treatment regimens, both sexes and all regions appears at the top, with lower-order tests for each regimen within the panel. Values are shown combined for males and females because of the absence of a treatment \times sex interaction. Because of the treatment interactions with age and region, lower-order tests were conducted for each individual value and asterisks denote those for which the terbutaline group differs from the corresponding control.

Figure 2. Microglial reactions in cerebellum and cerebral cortex in animals given terbutaline on PN 2-5 and evaluated at PN 30 as demonstrated by immunostaining with anti-Iba-1 antibodies. Marked differences in microglial responses were seen in the cerebellum (A-D) and cerebral cortex (E-F) when control tissues (A, E) were compared with terbutaline-exposed rats (B, F). The morphological features of microglia are consistent with a pattern of activation (C & D) as seen in microglia localized in the molecular (C) and white matter (D) layer of cerebellum in terbutaline-exposed rats.

Figure 3 Open field activity in animals given terbutaline on PN 2-5. Data

represent means and standard errors and ANOVA across all sessions is shown at the top of each panel; lower-order evaluations for each sex appear within the panels but tests of individual sessions were not conducted because of the absence of a treatment \times session interaction.

Figure 4. Effects of PN 2-5 terbutaline treatment on acoustic startle responses: (A) startle amplitude, (B) latency to peak amplitude. Data represent means and standard errors and ANOVA across all sessions is shown at the top of each panel; lower-order evaluations for each sex appear within the panels but tests of individual sessions were not conducted because of the absence of a treatment \times session interaction.

TABLE 1. Control Values for Microglial Quantification (fractional area)

Age	PN6		PN15		PN30	
	Male	Female	Male	Female	Male	Female
Cerebellum White matter	0.21±0.03	0.23±0.06	0.41±0.03	0.41±0.05	0.26±0.03	0.24±0.03
Cerebellum Granular Cell Layer	0.10±0.01	0.10±0.01	0.14±0.01	0.13±0.02	0.14±0.02	0.15±0.02
Brain White matter	0.101 ±0.008	0.103 ±0.013	0.31±0.02	0.24±0.02	0.21±0.03	0.20±0.03
Cerebral cortex	0.083 ±0.009	0.093 ±0.004	0.19±0.03	0.24±0.02	0.10±0.01	0.11±0.01

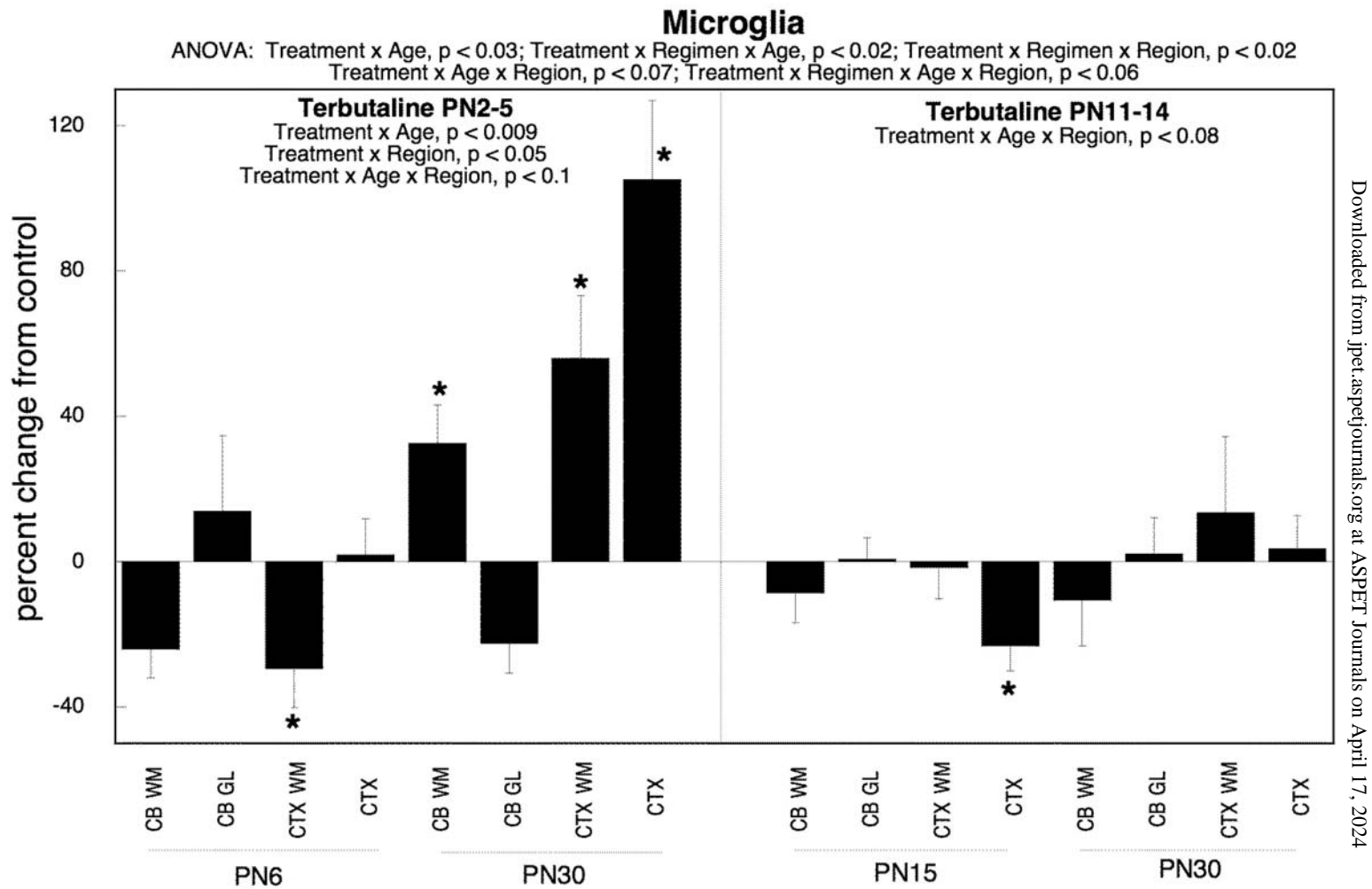


Figure 1

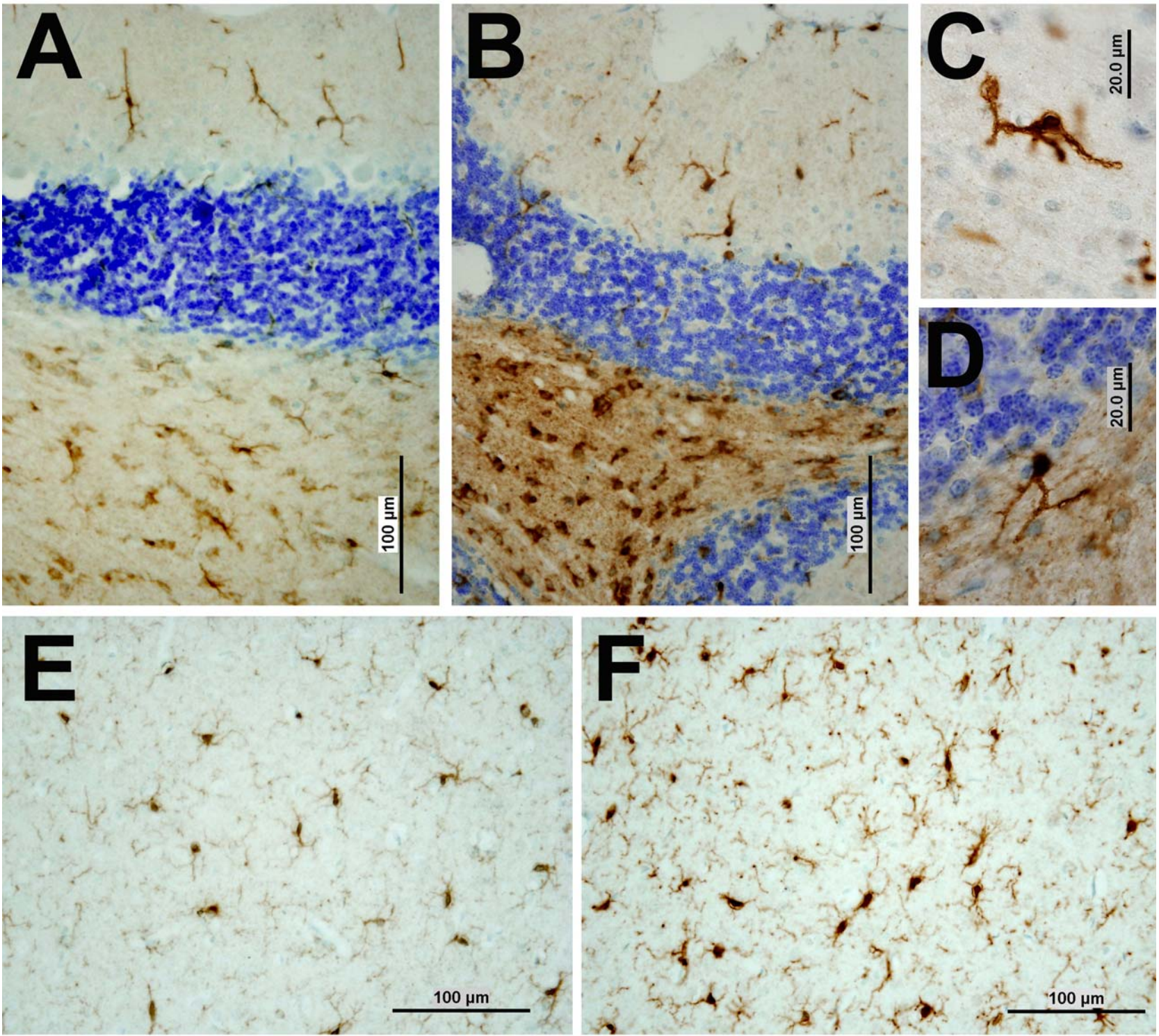


Figure 2

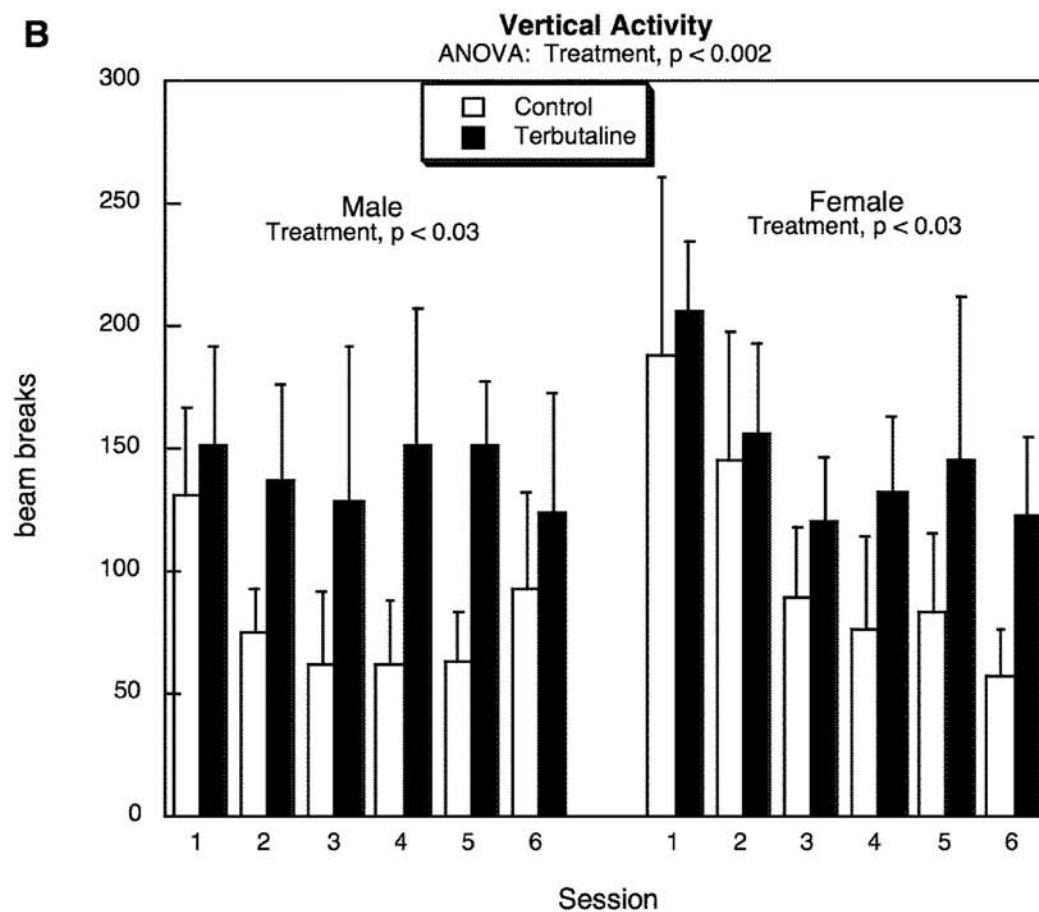
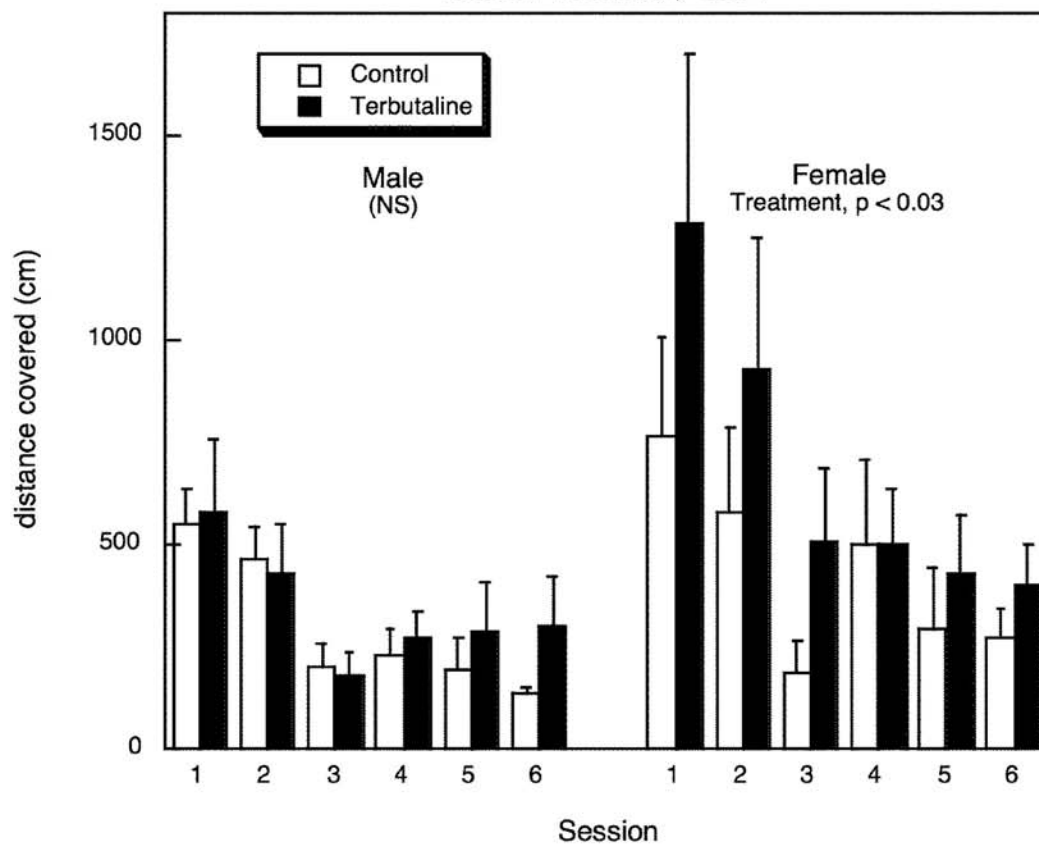


Figure 3

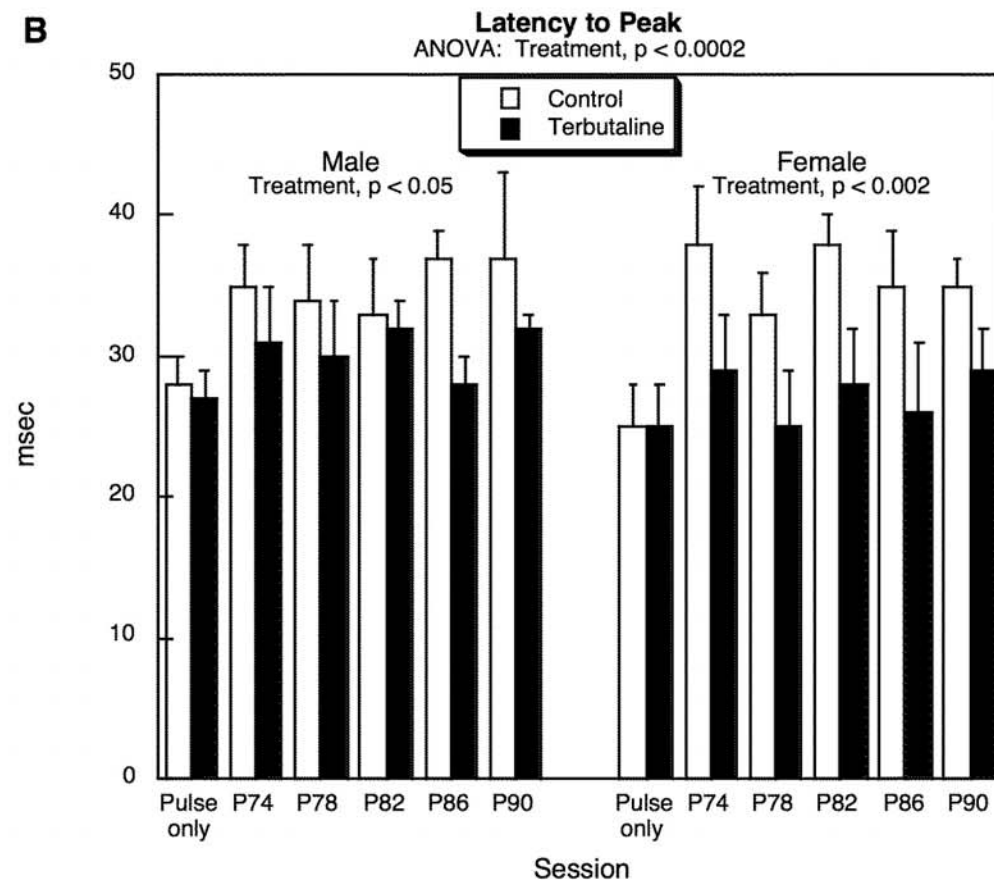
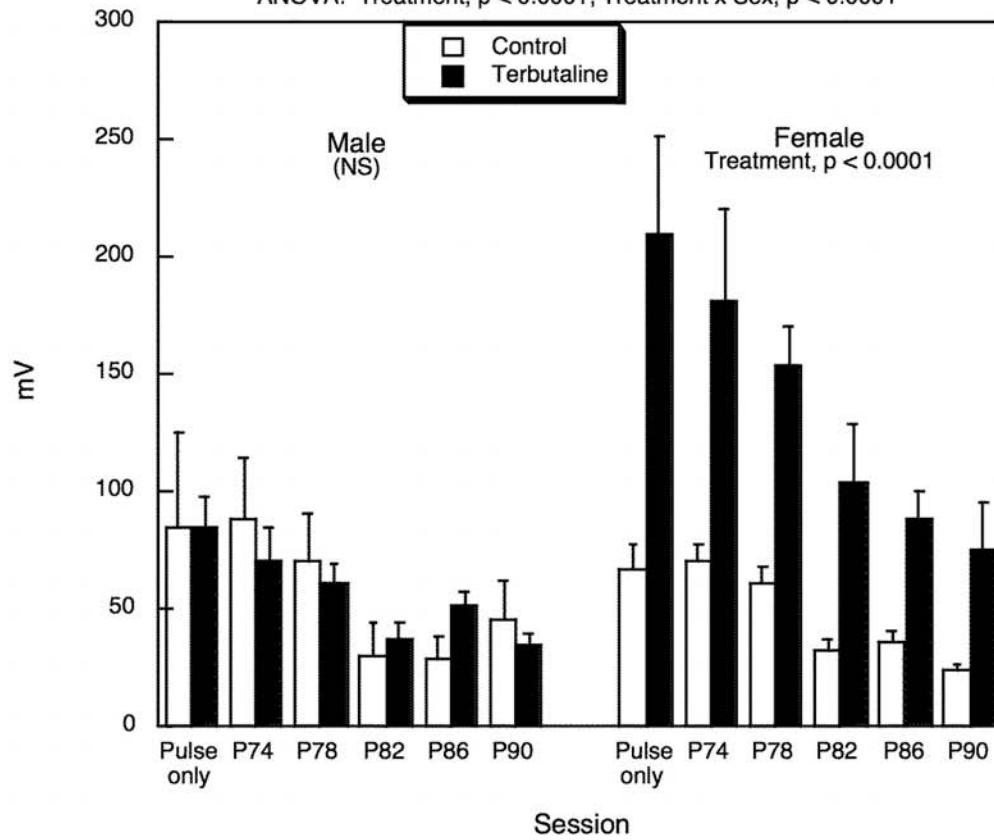
ANOVA: Treatment, $p < 0.0001$; Treatment \times Sex, $p < 0.0001$ 

Figure 4