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Award Number: W81XWH-04-1-0365

TITLE: Mechanism for Prenatal LPS-Induced DA Neuron Loss

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REPORT DATE: March 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2005	Annual (1 Mar 04 - 28 Feb 05)

4. TITLE AND SUBTITLE
Mechanism for Prenatal LPS-Induced DA Neuron Loss

5. FUNDING NUMBERS
W81XWH-04-1-0365

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates. All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

A small percentage of patients with Parkinson's disease (PD) are caused by genetic defects (familial Parkinson's disease). The etiologies of the majority of patients are still unknown. Recent advance in our laboratories suggests that prenatal exposure to bacterial toxin, lipopolysaccharide (LPS), could be an important etiology for some PD patients. A key finding is that animals exposed to LPS prenatally display fewer than normal number of dopamine (DA) neuron in the midbrain, a hall marker pathology in human patients. The mechanism for such DA neuron loss is not known. The preliminary data suggested that prenatal LPS may interfere DA neuron precursor cells (progenitor cells) migration to substantia nigra or DA neuron process outgrowth and therefore reduce the number of DA neurons in the midbrain. We proposed to use both in vivo and in vitro approaches to investigate these possibilities. A significant progress has been make in the last eleven months. Implementation of this proposal has resulted in three major findings:
1. Prenatal bacterial LPS induce loss of BrdU positive cells in the midbrain.
2. The toxicity of prenatal LPS requires removal of mitotic signal (s) to the dividing progenitor (stem) cells.
3. Prenatal LPS reduced dopamine neuron process total length that prevents dopamine neurons to reach trophic-rich striatal target tissue, a positive mechanism underlying the dopamine neuron loss in the prenatal LPS model.

14. SUBJECT TERMS
LPS, prenatal, Parkinson's disease, cytokine, dopamine, etiology

15. NUMBER OF PAGES
46

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction:

The cause of non-familial Parkinson's disease (PD) is currently unknown, although numerous environmental toxins have been implicated. To date, all studies evaluating the role of environmental toxins in PD have assumed exposure to toxin during adult life. In contrast, studies in our laboratory suggest that prenatal exposure may be just as important. We have shown that treating gravid female rats with the bacteriotoxin lipopolysaccharide (LPS) produces offspring born with fewer dopamine (DA) neurons. This DA neuron reduction persists throughout adulthood and progresses with age. These animals exhibit reductions in striatal DA, increased DA activity, increases in pro-inflammatory cytokines (that remain elevated through 16 months), increased levels of oxidized proteins, and the development of a-synuclein aggregates. We have proposed the pre-natal LPS-exposed rat as a new animal model of PD. The relevance of this model to PD is supported by the fact that LPS is known to enter the human chorioamniotic environment of the fetus in women with bacterial vaginosis (BV). BV increases pro-inflammatory cytokines, including tumor necrosis factor (TNFa) and interleukin 1 beta (IL-1ß), and induces several teratogenic effects. Unfortunately, the mechanisms responsible for the LPS-induced changes in the DA system are unknown. Preliminary *in vivo* studies using BrdU (marker incorporated into dividing cells) suggest that LPS may reduce the mitotic activity in the subventricular zone of the mesencephalon, which could lead to reduced migration of DA progenitors into the nigra. In vitro studies, using our newly developed 2-dimensional (2D) micro-island culture system which allows us to evaluate the very early stages of development of the nigro-striatal pathway, suggest that LPS may interfere with the extension of DA processes to the trophic-rich striatum. This could lead to loss of DA neurons due to inadequate trophic support. We will evaluate these two possibilities in the following specific aim.

Specific Aim: Rat fetal brains exposed to LPS at E10.5 will have reduced numbers of DA neurons in their mesencephalons due to reduced progenitor cell activity or inhibited process extension. Experimental series 1: Gravid rats will be injected (i.p.) with 10,000 EU/kg LPS or Hank's Balanced Salt Solution (HBSS) at E10.5. The animals will also receive BrdU (70 mg/kg) at E10.5 and E11.0 to label dividing cells in the midbrain. The fetuses will be harvested at E18.5 and the brains assessed for the number of TH immuno-reactive (THir)/BrdU cells, NeuNir (neuronal marker)/BrdU cells, GFAPir (astrocyte marker) and Ox-42ir cells (microglia marker) in the substantia nigra. The density of the THir staining in the striatum and medial forebrain bundle (MFB) will be used as an index of process extension. If the LPS-induced DA neuron loss is due to reduced development/conversion of progenitor cells to DA neurons, we will see reduced numbers of THir/BrdU double labeled cells. If alterations in process extension are responsible, we will see reduced striatal THir density with normal cell counts in the nigra. Experimental Series 2: Gravid female rats will be treated with BrdU as above. At E10.5, the abdomen will be reflected and LPS (800 EUs) will be injected into each amniotic sac in one uterine horn (HBSS in the other). The abdomen will be sutured closed. At E14.5 the rostral mesencephalic tegmentums (nigra) and lateral ganglionic eminences (striata) will be harvested from each uterine horn, pooled, and assessed using 2D micro-island cultures. Various combinations of the LPS exposed (affected) and unaffected nigras and striata will be co-cultured together and immunostained after 96 hrs. Evaluation of these combinations should reveal which tissue is more affected (nigra or striatum). Other pups from this treatment protocol will be taken by C-section at E21, and cross fostered until sacrificed at P5 or P15 (4 days after the 2 phases of apoptotic culling, respectively). These animals will be assessed for THir/BrdU and NeuNir/BrdU cell counts, striatal DA biochemistry (HPLC) and GSH/GSSG, striatal and

mesencephalic trophic activity, and striatal TH density and cytokine protein levels (ELISA). These studies should reveal if progenitor cells convert normally into DA neurons or alternatively, whether they die off during development due to inadequate access to trophic support, and as a result, undergo excess culling at P1 or P15.

BODY:

Overall progress:

Nearly 35% of the proposed work has been finished even though we only had 10 months to perform the proposed work before writing this report. Also, we recently moved into new facilities in the Cohn Research Pavilion that slowed the initiation of some of the studies. In addition, we have recently shown that we can produce the same DA neuron loss in mice and have successfully established breeding lines for the LPS studies. The reason for studies in mice is twofold. First, there is an economic impact (significantly reduced housing costs). Second, establishing the model in mice allows us to utilize knockout technology (see below). Although we have successfully converted the model to mice, we found that we could not perform some of the detailed analyses on sprouting in the fetal brain of the mouse (very difficult because of size), and we did not feel comfortable performing the intra-amniotic fluid injections into gravid E9.5 mice. The procedure could be performed, but we were unsure of its reliability). We therefore switched back to performing the studies in rats and currently have many of the initially planned experiments under way. Despite these problems, we feel we have made excellent progress.

Specific progress:

1. Prenatal LPS exposure reduces BrdU positive cells in the midbrain. The midbrain is where the dopamine (DA) neurons are located. These neurons are generated from the subventricular zone in the ventral midbrain inferior to the aqueduct. The precursors of DA neurons can proliferate and mature into DA neurons providing the proper signals are present (Ling et al., 1998;Potter et al., 1999). In the planned experiments, we gave gravid female rats the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) at a time when the DA neurons are just starting to develop (embryonic day 10.5 or E10.5). The animals were injected every 12 hours for two days. The BrdU was detected using a fluorescent antibody against BrdU. As our preliminary data suggested and our the current studies confirmed, there was an overt reduction in BrdU positive cells in the area ventral to aqueduct in the brains harvested from E18.5 embryos in the fetuses of dams injected with LPS vs. controls (**Figure 1**). This reduction indicates less dividing progenitor cells present in the embryos exposed to prenatal LPS injection. This reduction can be the result of several potential mechanisms: 1) LPS might have reduced mitotic signaling within the ventricular zone; 2) LPS may have increased migration into non-target areas (we are exploring this in other sections currently; 3) the progenitor cells were killed by LPS or more likely TNFα mediated cell death; 4) BrdU containing cells differentiated into neurons which were killed by the LPS-induced toxicity. One of the central issues is the phenotype of these BrdU labeled cells and double stains are under way to determine the status of the cells. We will also be performing

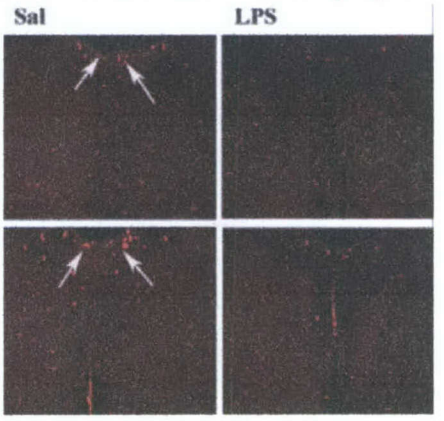

Figure 1. Prenatal LPS reduced midbrain BrdU positive cells. Left panel: E18.5 embryos exposed to saline at E10.5; right panel: E18.5 embryos exposed to LPS at E10.5. Arrows indicate the area rich in BrdU staining.

double labeling to evaluate the population of cells within the nigra to demonstrate that these cells actually migrated into the SN. As we showed in preliminary studies, if the number of BrdU labeled DA neurons is reduced in the SN, it suggests a progenitor related issue. In animals exposed to LPS prenatally we have also shown increases in numbers of microglia (Ox-6 staining) and astrocytosis (GFAP staining). There are obvious differences amongst the groups, but the cell counting is not complete yet.

2. LPS is toxic to midbrain neuroprogenitor cells when mitotic signally was removed. Following the confirmatory studies described above, we sought to determine the effects of LPS on progenitor cells. Brain neuroprogenitor cells were established from E14.5 rat embryos. These cells were maintained in the serum free medium consisting of 48.5% F12, 48.5% high glucose DMEM (Life Science/Gibco), 2% B27 supplement, 1% Pen/Strep antibiotics, and epidermal growth factor (EGF) at 20 ng/ml (Potter et al., 1999). Three weeks later, these cells were plated out in 48-well plates. LPS and several other toxins known to be toxic to DA neurons such as TNFa (McGuire et al., 2001), rotenone (Ling et al., 2004), MPP+ (Chun et al., 2001), paraquat (Brooks et al., 1999), dieldrin (Fleming et al., 1994), and L-dopa (Ling et al., 1996) were added into plates at 10μM. The supernatants were collected at various time points. Lactate dehydrogenase (LDH) release was measured to indicate toxicity (Liu et al., 2005). Vehicle HBSS and DMSO were used as negative control and H_2O was used as positive control. The data was presented as index over control value. LPS appeared not to be toxic to neuroprogenitor cells in the cultures containing EGF that prevented progenitor cell differentiation (**Figure 2**). However, the same amount of LPS was toxic when EGF was removed from media (**Figure 3**). This suggested that the progenitor cell itself is not a target for LPS toxicity, but rather, that the differentiated cell was susceptible. This also explains our prior observations in rats and mice as to why LPS does not reduce DA neurons if it is given to gravid females before embryonic day 10.5 at which time DA neuron are absent. However, it does not explain why LPS does not reduce DA neurons when it is given after E12 to a rat or after E11 in a mouse. Thus, there is a distinct "window of vulnerability" where fetuses are susceptible to the effects of LPS. In addition, the effects in rat post-

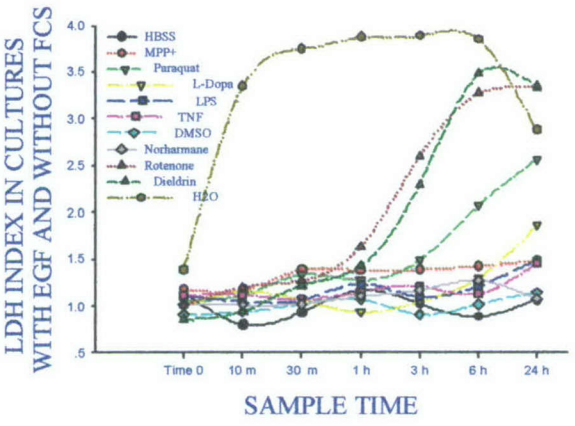

Figure 2. Neuroprogenitor cells established form E14.5 embryos were maintained in EGF containing media. These cells were plated out in naked plastic plates with the same media to prevent differentiation of the progenitor cells. All toxins were added into the wells after 72 hrs at 10μM. H_2O was used to replace media in the positive control wells. The supernatants were collected for LDH assessments at varying time points. Dieldrin, rotenone, paraquat, and l-dopa were toxic to the progenitor cells. LPS, TNFa, MPP+, and Norharmane were not toxic. This suggests that the progenitors are not susceptible to LPS or the pro-inflammatory cytokine TNFa.

differentiation (>E12.0) could well be different from those pre-differentiation (<0.10).

3. Prenatal LPS exposure reduced tyrosine hydroxylase (TH) immunoreactive process in the striatum at E18.5 embryos. Gravid females were treated and embryos were collected as described above. The brains were collected and sectioned for THir staining. The process density was estimated using stereology. The MicrobrightField "Space-ball probe" technique was used to estimate the length of THir processes (**Figure 4**). Prenatal LPS exposure significantly reduced the total length of THir process in E18 brains ($F_{1,7}$=12.801, p=0.012). This result suggests that prenatal LPS retards process outgrowth of processes from DA neurons. If the processes are affected by prenatal exposure, then a given DA neuron could be deprived of trophic support from the striatum and not fully develop or die (these studies are just starting and the THir cell counts need to be completed and the remainder of the process extensio n studies need to be counted).

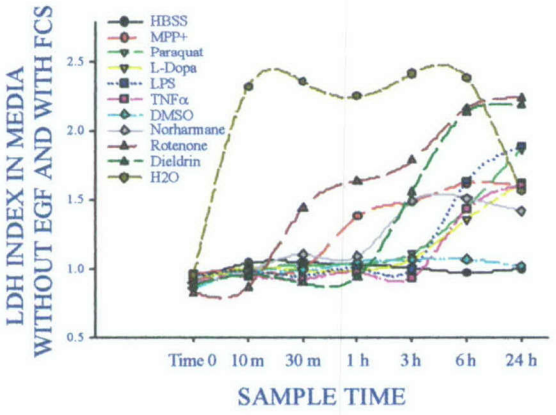

Figure 3. Mesencephalic neuroprogenitor cells were cultured in 48-well plates in media without EGF and with 10% FCS. This will initiate differentiation. All toxins were added into wells after 72 hrs at 10 μM. H_2O was used to replace media in the positive control wells. The supernatants were collected for LDH assessments at varying time points. All toxins exhibited toxicity to the cultures. This suggests that once the differentiation process started, the cells were susceptible to LPS and TNFa.

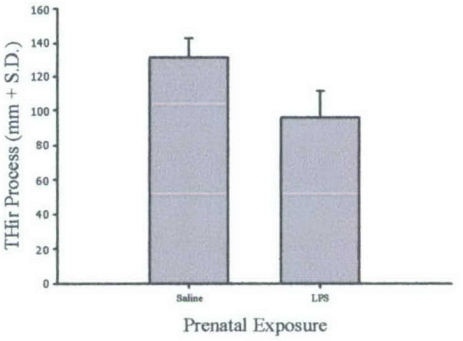

Figure 4. Prenatal LPS exposure reduced total length of THir processes in the embryos. $F_{1,7}$=12.801, P=0.012. The total length was estimated using MicrobrightField Space Balls probe.

Other Related Findings:

The fact that TNFα remains elevated for the life of the animal has always intrigued us and suggested the possibility that an epigenetic mechanism was involved. We therefore set out to determine if prenatal LPS exposure affected the methylation status of the promoter of the

TNFα gene. One of our graduate students did an excellent job in developing the bis-meta-bisulfite conjugation technique. Although we had some intriguing suggestive evidence, the results were negative even when we examined isolated microglia from these animals. Although this set of experiments does not rule out that other epigenetic mechanisms are at work, we are now convinced that the increase in TNFα seen in these animals is a post-transcriptional event.

Another possible mechanism to explain the fact that there are reduced numbers of DA neurons in these animals is that prenatal exposure somehow reduces developmental factors responsible for the DA neuron. Thus far we have looked at one month old animals, P10 animals, and are completing E18 and E14 animals at this time. Using rtPCR for Nurr1, Ptx-3, and sonic hedgehog, we have not seen alterations in these genes. Consistent with the fact that we have not seen anatomical teratologies in these animals, failing to see alterations in sonic hedge hog confirmed that finding. More importantly, since we had shown previously that these animals seemed to be born with fewer than normal DA neurons and not just a phenotypic suppression of TH, the findings of normal levels of Ptx-3 and Nurr1 are consistent with this observation. However, we need to complete the E18 and E14 studies because it is possible that LPS somehow interferes with Ptx-3 or Nurr1 just during development which then recovers. At this juncture, this hypothesis does not seem likely however.

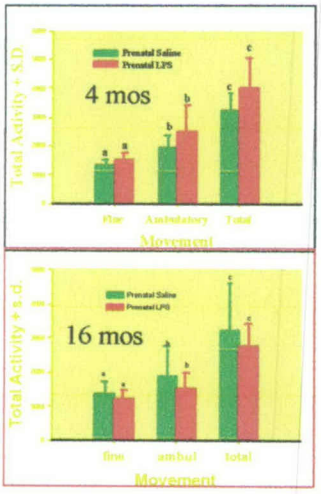

Fig. 5: Fine and total beam interuptions in animals exposed to saline or LPS prenatally.

In the course of these studies with LPS we have also observed additional findings surrounding locomotor activity and body weight. Animals are monitored (every 4 months) in a random fashion using San Diego Instrument home cage activity monitors. Early on, animals exposed to LPS prenatally are hyperactive. By 12 months they are normoactive, whereas by 16 months they are hypoactive in their home cages (**Fig. 5**). This suggests that the loss of DA neurons in the SN seen in the aged animals translates into reduced locomotor activity as would be anticipated if this is an accurate animal model of PD.

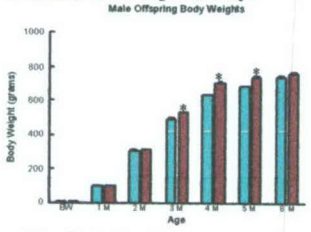

Fig. 6: Animals were weighed monthly. Males exposed to LPS are significantly heavier than saline controls at 3, 4, and 5 months.

We have also followed the body weight of these animals throughout life. Animals were weighed and body lengths and head circumference were collected at birth. They are then weighed monthly thereafter. There were no apparent differences in birth measures and the size of the litters were also equal across the prenatal saline and LPS treatment groups. However, by 4 months of age, the males were statistically heavier despite being more active (Fig 6). A similar effect was seen in females, although it was not as pronounced. This observation is particular relevant given the recent observations that patients with PD have a greater percentage of body fat than age matched controls.

Since TNFα is elevated life long in these animals we also wondered what effect this cytokine would have on the permeability of the BBB. Since TNFα is well know for its ability to reduce barrier function, we looked in aged LPS animals as well as in young animals exposed to various neurotoxins such as 6OHDA and MPTP.

The procedure involves perfusing with saline followed by heparin and then FITC labeled albumin. Examination of the sections using fluorescence reveals the vasculature filled with FITC. If the barrier is disrupted, FITC will leak into surrounding tissue. In 16 month old animals exposed to LPS prenatally, there was no evidence of leakage (**Fig 7a**). However, in the SN, there was clear evidence that focal areas of barrier disruption could be seen (**Fig 7b**). This has also been observed in rats exposed to 6OHDA and mice exposed to MPTP (data not shown). Interestingly, knockout mice (TNFα -/-) did not exhibit this effect.

Key Research Accomplishments:

The ultimate goal of this proposal is to understand the mechanism(s) through which prenatal LPS kills DA neurons. The findings collected to date seem to suggest that prenatal exposure to LPS reduces the number of BrdU labeled cells in the subventricular area of the mesencephalon where DA neurons are thought to originate. At this time, this effects could be a consequence of LPS interfering with mitotic activity or having a direct toxic effect on those cells. The in vitro data seems to suggest the later. However, the data collected also suggests that reduced process extension is also occurring. It is unlikely that this attenuation in process extension is a consequence of a direct effect of, for instance TNFα. If this were the case, then processes would have been affected in animals exposed later to LPS (e.g, at E13.5), which they were not Unless there is a unique interaction occurring here, it is more likely that the LPS administered at E10.5 is having another effect. One mechanism that we had not considered previously is that LPS may alter production of growth factors known to be critical in DA neuron development. The two logical choices at this time are GDNF and BDNF and we will examine these possibilities in the remaining period of the proposal. It is also important to note that we seem to have eliminated other possibilities such as Ptx-3, Nurr1 and Sonic hedgehog as well as an epigenetic effect on the TNFα promoter. Although these alternative mechanisms were not originally proposed, they represent viable mechanisms and their exclusion helps to elevate the possibility that effects on progenitor cells or process extension are more likely involved.

Reportable Outcomes:

Peer-reviewed article:

Paul M. Carvey, Er-Yun Chen, Jack W. Lipton, Chongwei Tong, Qin A. Chang, and Zaodung Ling. Intra-parenchymal Injection of Tumor Necrosis Factor-a and Interleukin 1-ß Produces Dopamine Neuron Loss in the Rat. J Neural Transm. 2004 Published on line Dec 7.

Ling, Z.D., Lipton, J.W., Chin, Q. Chong, C.W., Carvey, P.M. Combined Toxicity of Prenatal Bacterial Endotoxin Exposure and Postnatal 6-Hydroxydopamine in the Adult Rat Midbrain. Neuroscience. 2004;124(3):619-28.

Ling Z, Chang QA, Tong CW, Leurgans SE, Lipton JW, Carvey PM. Rotenone potentiates dopamine neuron loss in animals exposed to lipopolysaccharide prenatally. Exp Neurol. 2004 190(2):373-83.

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Conclusion:

So far, all the results from the implementation of this proposal have supported our original hypothesis outlined in the introduction. This funding, together with other funding, has helped us to understand the working mechanisms in the prenatal LPS Parkinson's disease model. These include:

1) Bacterial endotoxin lipopolysaccharide (LPS) is toxic to dopamine neurons (Gayle et al., 2002;McGuire et al., 2001).

2) LPS itself is not toxic to dopamine neurons. The presence of microglial cells and serum LPS binding protein (LBP) are required for LPS to function as a toxin neurons (Gayle et al., 2002;McGuire et al., 2001).

3) Inflammation factors induced by LPS are toxic to dopamine neurons. Such factors include: TNFa, IL-1ß, nitro oxide (NO), and other reactive oxygen species (Gayle et al., 2002).

4) Epigenetic modification of pro-inflammatory cytokine gene promoter probably is not the mechanism for prolonged pro-inflammatory cytokine elevations in brain prenatally exposed to LPS. Instead, the increase in reactive microglia in these brains is the main source of pro-inflammatory cytokines (Ling et al., 2004).

5) TNFa, which is induced by LPS in culture or in brain, kills dopamine neuron through a: Binding to TNFa receptors; b: Inducing ROS; c: Interfere process outgrowth in vivo. (McGuire et al., 2001;Gayle et al., 2002).

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Appendices:

Paul M. Carvey, Er-Yun Chen, Jack W. Lipton, Chongwei Tong, Qin A. Chang, and Zaodung Ling. Intra-parenchymal Injection of Tumor Necrosis Factor-a and Interleukin 1-ß Produces Dopamine Neuron Loss in the Rat. J Neural Transm. 2004 Dec 7.

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DOI 10.1007/s00702-004-0222-z
J Neural Transm (2004)

Journal of Neural Transmission
Printed in Austria

Intra-parenchymal injection of tumor necrosis factor-α and interleukin 1-β produces dopamine neuron loss in the rat

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Received May 10, 2004; accepted July 31, 2004
Published online December 7, 2004;

Summary. Inflammatory processes are thought to underlie the dopamine (DA) neuron loss seen in Parkinson's disease (PD). However, it is not known if the inflammation precedes that loss, or is a consequence of it. We injected tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β) into the median forebrain bundle to determine if these pro-inflammatory cytokines could induce DA neuron loss in the substantia nigra (SN) by themselves. The magnitude of the DA cell loss as well as the decreases in striatal DA, were both dose and time to sacrifice dependent. Injecting both cytokines together produced greater cell losses and DA reductions than that seen when the cytokines were injected alone. The DA neuron loss seen was more pronounced in the lateral nigra and its ventral tier and similar to that seen when other toxins are injected. These data suggest that TNFα and IL-1β can induce DA neuron loss by themselves and could produce DA neuron loss independent of other inflammatory events.

Keywords: Inflammation, cytokine, Parkinson's disease, rat, infusion.

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by loss of the dopamine (DA) producing cells in the Substantia Nigra (SN). Although a genetic basis for the disease has been identified for some, the etiology in the majority of the affected patients remains unknown. Regardless of cause, the DA neuron loss and reductions in striatal DA that characterize all patients with PD are accompanied by increases in several pathological indices (Hunot and Hirsch, 2003; Jenner, 2003). Thus, increased levels of cyclooxygenase 2 (Knott et al., 2000), nitrite (Qureshi et al., 1995), oxidized protein (Floor and Wetzel, 1998), oxidized DNA (Zhang et al., 1999), and lipid peroxidation (Yoritaka et al., 1996) as well as decreased levels of the reduced

form of glutathione (Sofic et al., 1992) and impaired mitochondrial Complex I function (Schapira et al., 1990) have all been reported. Some of these changes strongly implicate the involvement of neuroinflammation. Although the central question as to whether these changes are a consequence of DA neuron loss, are the cause of the degenerative process itself, or contribute to the progression of PD remains, there is little controversy about the need to better understand the role of inflammation in this disease process.

The brains of patients with PD also exhibit elevations in proinflammatory cytokines. Thus, Nagatsu et al. (2000) and Boka et al. (1994) have reported elevations in Tumor Necrosis Factor-alpha (TNFα) and Interleukin 1 beta (IL-1β) in the brains of patients with PD. These two cytokines in particular, are centrally involved in inflammatory processes, are a product of microglia activation which has also been noted in PD patients (McGeer and McGeer, 1998), and can support many of the inflammatory events known to be increased in PD patients. In addition to studies in patients, animal models of PD and culture systems have also demonstrated that increases in TNFα and IL-1β are associated with DA neuron loss (Castano et al., 1998; Bronstein et al., 1995; Jarskog et al., 1997; Kabiersch et al., 1998; McGuire et al., 2001; Gayle et al., 2002; Sriram et al., 2002; Ferger et al., 2004; Rousselet et al., 2002). Taken together, these data might suggest that elevations in proinflammatory cytokines lead to DA neuron loss, or at the very least, contribute to further DA neuron loss once the degenerative process has started.

Previous studies have demonstrated that injection of the bacteriotoxin lipopolysaccharide (LPS) directly into the brains of rodents leads to the loss of DA neurons (Castano et al., 1998; Herrera et al., 2000; Kim et al., 1995, 2000; Gao et al., 2002). LPS is a potent inducer of TNFα and IL-1β (Gayle et al., 2002; Paludan, 2000), and it is possible that the DA cell death seen following LPS may arise as a consequence of increased levels of either of these two cytokines. We have further shown that offspring from gravid rats exposed to LPS during development are born with fewer than normal DA neurons and have life-long increases in TNFα (Carvey et al., 2002; Ling et al., 2002, 2004). In order to ascertain whether or not elevations in TNFα and/or IL-1β contribute directly to DA cell loss independently of other LPS induced factors, we infused these two pro-inflammatory cytokines alone and in combination into the SN of young adult rats. The results show that these two pro-inflammatory cytokines can indeed produce DA neuron loss suggesting that they are not only associated with the disease process by themselves, but could actually contribute to the DA neuron loss seen in PD.

Materials and method

Animals

Male Sprague-Dawley rats (250–300 g) were purchased from Zivic-Miller (Allison Park, PA), and were allowed to acclimate to the animal facility for a period of at least 5 days before the onset of the experiments. All animals were housed in a temperature and humidity controlled environment, allowed free access to food (Agway Rat Chow; Prolab, Syracuse, NY) and water, and were kept on a 12 hour lights-on/12 hour lights-off cycle (lights on 0600). The protocols and

procedures used in these studies were approved by the Institutional Animal Care and Utilization Committee (IACUC) for Rush University Medical Center.

Cytokine concentrations

Recombinant rat cytokines were purchased from R and D System (Minneapolis, MN), and dissolved in Hank's Balanced Salt Solution (HBSS) with 1% rat serum albumin (vehicle) (Sigma-Aldrich Co.). For control injections the cytokines were heat-inactivated for 1 hr at 65°C in a water bath and dissolved in vehicle (HBSS with 1% rat serum albumin) prior to injection.

The two cytokines were injected at various concentrations alone and in combination. IL-1β was injected at 100, 200, and 10,000 pg in 3 μL vehicle. TNFα was injected at 10, 50, and 200 ng in 3 μL vehicle. 100 pg IL-1β and 10 ng TNFα concentrations were injected alone and in combination into animals that were sacrificed 14 days later. 10,000 pg IL-1β and 200 ng TNFα were injected alone and in combination and sacrificed 14 days later. 200 pg IL-1β and 50 ng TNFα were injected alone and in combination into two groups that were sacrificed 7 and 14 days later, respectively.

Cytokine injections

The rats were anesthetized with pentobarbital (40 mg/kg; Sigma-Aldrich Co.), and placed into a stereotaxic frame. A mid-cranial incision was made and the skin was reflected. A burr hole was then made on the right side of the cranium in all animals using a flexible drill (Foredom Electric Co., Bethel CT) fixed to a micro manipulator. The cytokines or inactivated cytokines were injected over five minutes into the right medial forebrain bundle (AP = − 4.3; ML = − 1.2; DV = − 7.5) using a Hamilton syringe. The needle was left in place for an additional five minutes and then slowly withdrawn. The burr hole was filled with surgical foam and skin sutured. The animals were allowed to recover in their home cages under a heating lamp for 5 hours and then returned to the animal facility. Their general health was monitored daily for the duration of the study.

Perfusion and tissue preparation

At the end of the study, the animals were anaesthetized using an overdose of pentobarbital (65 mg/kg). The chest was invaded and each animal was perfused transcardially using 300 ml ice-cold saline. During perfusion, the descending aorta was clamped to increase perfusion to the brain.

The brains were divided into rostral and caudal sections. The rostral part of the brain (4 mm from the apex) was frozen in the cold 2-methylbutane and sectioned into 1 mm slabs on a cold slate block. The right (ipsilateral to injection) and left (contralateral to the injection) striata were isolated separately. The striatal pieces ipsilateral and contralateral to the cytokine injection were immediately placed in 200 μl of ice cold antioxidant (0.4 N perchlorate, 0.05% EDTA, and 0.1% bis-metabisulfite). These pieces were then homogenized for 3 secs. using a Polytron GmbH homogenizer (setting 6; Kinematica; Luzern, Switzerland), and centrifuged (30,000 × G for 30 minutes). The total protein was measured in the pellet using a Protein Assay Kit (Bio-Rad, CA). The supernatants were assessed in duplicate for DA and homovanillic acid (HVA) using High Performance Liquid Chromatography (HPLC) as previously described (Vu et al., 2000) by comparison to 6 point standard curves run daily. The levels were expressed as ng/mg protein. The ratio of [HVA]/[DA] was also computed and served as an index of DA activity.

The caudal part of brain was immediately submerged in Zamboni's fixative (7.5% saturated picric acid, 12 mM NaH_2PO_4, 88 mM Na_2HPO_4, and 4% paraformaldehyde) for immunohistochemistry studies. After three days, Zamboni's fixative was removed and the brains were submerged through two changes of 30% sucrose. The caudal pieces were then sectioned (40 μm) on a sliding microtome and divided into six series. Sections were kept in cryoprotectant (Glycerol, 30%; Ethylene glycol, 30%; PBS 40%) until processed. Sections were immunostained for tyrosine hydroxylase (TH) as described previously (Ling et al., 2002). The endogenous peroxidase activity was eliminated with a 20-minute incubation in 0.1 M sodium periodate (Sigma Chem. Co.). Sections were then incubated at room temperature for 24 hours with a monoclonal mouse

anti-TH (1:20,000 [Immunostar, MN]; with PBS and 0.25% triton). The staining was followed by incubation with biotinylated horse anti-mouse IgG (Vector Laboratories) and avidin biotin complex (ABC) conjugated with peroxidase (Vector Laboratories). The TH immunoreactive (THir) cells were visualized with I/A solution containing nickel sulfate (2.5%, Sigma), 3,3′-diaminobenzidine (DAB, 0.05%, Sigma), and H_2O_2 (0.003%, Sigma) for a period of 5 minutes followed by 3 washes with I/A. Finally, the slides were dehydrated through graded alcohols and xylene. The slides were eventually cover-slipped (Stephens Scientific) and allowed to dry overnight.

Stereological assessment

The number of THir cell bodies in the SN area were counted by an observer blinded to treatment history using a stereological procedure described previously (Ling et al., 2002). The system used for stereological counting was an Olympus BX-50 microscope that was hard-coupled with a Prior H128 computer-controlled x-y-z motorized stage, a high sensitive Hitachi 3CCD video camera system (Hitachi, Japan), and a PC computer. THir cell counts were carried out using MicroBrightField stereological software. The SN was outlined under a low magnification (10 X) lens and approximately 10% of the outlined region was measured with a systematic, random design using dissector counting frames. Total THir neurons were estimated using the optical dissector method employing a 100 X planapo oil immersion objective with a 1.4 numerical aperture. Under the dissector principle, about 200 THir neurons were sampled by optical scanning using a uniform, systematic and random designed procedure for all measurements. Once the top of the section was in focus, the z-plane was lowered 2.5 μm (forbidden plane which was not included in the analysis). The total number of THir cells (N) within the SN was calculated using the following formula: $N = Nv \bullet Vsn$ where the Nv was the density and Vsn was the volume of the SN determined by the Cavalieri principle. THir neurons within the ventral tegmental area (medial to the accessory optic tract (Ling et al., 2004) were excluded from dissector analysis.

Statistical analysis

Two-way ANOVA was performed with treatment and side as factors. Individual group differences within a study were assessed using Tukey' *post hoc* comparison following One-way ANOVA. SN THir cell counts were compared with striatal DA levels using Pearson's correlation.

Results

Overall, the ipsilateral injections did not alter the measures in the contralateral hemisphere (Two-Way ANOVAs on all studies revealed a statistically significant effect of side). In addition, regardless of dose, cytokine, cytokine combination, or time to sacrifice, no statistically significant changes were observed in DA levels or cell counts on the side contralateral to the injection. In contrast, the injections had a profound effect on the measures on the ipsilateral side of the brain.

Striatal biochemistry

In general, injection of IL-1β and/or TNFα reduced striatal DA and increased DA activity on the side ipsilateral to the injection. In addition, this effect was dose-dependent for both cytokines. The highest combined doses of IL-1β and TNFα produced the most dramatic changes (Table 1). Thus, DA levels were reduced by both IL-1β and TNFα injection and the combined injection of both produced further striatal DA loss (Fig. 1; $F_{3,31} = 3.884$; $p < 0.05$). IL-1β (10 ng) reduced ipsilateral DA 29.9% whereas TNFα (200 ng) reduced levels 54.3%. Although the combined effect of both dosages produced a greater DA decrease (61.2%),

Table 1. Effect of 100 pg, 200 pg, and 10 ng IL-1β (low, medium, and high dose, respectively) and 10, 50 and 100 ng TNFα (low, medium, and high dose, respectively) on striatal biochemistry and tyrosine hydroxylase immunoreactive (THir) cell counts in the substantia nigra (SN) (Data that shares a common letter within group is significantly different from one another at $p < 0.05$)

Infusion		Low dose	Medium dose (MD)	MD (7 days)	High dose
			Dopamine		
Vehicle	Ipsi-	212.12 ± 22.49	207.36 ± 29.99[ab]	204.33 ± 17.19[a]	201.11 ± 20.48[ab]
	Contra-	206.73 ± 19.44	197.26 ± 25.25	215.51 ± 25.44	200.73 ± 12.19
IL-1β	Ipsi-	199.79 ± 17.49	178.76 ± 21.42	206.50 ± 29.82	140.77 ± 14.12[ab]
	Contra-	191.83 ± 25.09	194.88 ± 30.99	215.51 ± 25.44	217.13 ± 19.06
TNFα	Ipsi-	182.34 ± 14.13	154.46 ± 53.98[a]	191.24 ± 19.73	91.69 ± 15.29[a]
	Contra-	218.41 ± 30.02	204.99 ± 38.02	202.34 ± 20.95	242.46 ± 37.90
IL-1/TNF	Ipsi-	176.11 ± 16.39	130.03 ± 7.75[b]	153.41 ± 33.44[a]	77.51 ± 14.31[b]
	Contra-	192.86 ± 20.64	231.74 ± 24.26	212.44 ± 23.64	239.10 ± 51.06
			HVA		
Vehicle	Ipsi-	15.66 ± 5.01	18.74 ± 4.57	18.76 ± 4.00	18.62 ± 2.35[ab]
	Contra-	18.21 ± 3.02	17.77 ± 4.46	19.55 ± 3.54	16.78 ± 5.31
IL-1β	Ipsi-	17.31 ± 4.51	17.20 ± 4.15	17.94 ± 4.55	17.91 ± 2.84[cd]
	Contra-	19.34 ± 5.82	16.07 ± 4.80	18.05 ± 3.73	24.51 ± 6.10
TNFα	Ipsi-	20.04 ± 5.27	17.59 ± 7.86	17.02 ± 2.95	12.32 ± 1.66[ac]
	Contra-	18.73 ± 6.05	22.11 ± 7.17	19.20 ± 3.42	24.88 ± 6.42
IL-1/TNF	Ipsi-	16.42 ± 4.98	16.82 ± 4.40	15.43 ± 3.12	13.20 ± 0.83[bd]
	Contra-	17.07 ± 3.96	25.74 ± 7.81	20.23 ± 1.98	25.16 ± 5.28
			HVA/DA		
Vehicle	Ipsi-	0.075 ± 0.026	0.090 ± 0.017	0.092 ± 0.022	0.092 ± 0.007[a]
	Contra-	0.089 ± 0.022	0.091 ± 0.026	0.093 ± 0.023	0.083 ± 0.021
IL-1β	Ipsi-	0.087 ± 0.022	0.097 ± 0.024	0.088 ± 0.023	0.128 ± 0.025
	Contra-	0.099 ± 0.021	0.082 ± 0.017	0.091 ± 0.019	0.112 ± 0.018
TNFα	Ipsi-	0.109 ± 0.020	0.121 ± 0.065	0.096 ± 0.030	0.136 ± 0.024
	Contra-	0.085 ± 0.025	0.106 ± 0.016	0.094 ± 0.008	0.105 ± 0.030
IL-1/TNF	Ipsi-	0.095 ± 0.035	0.144 ± 0.040	0.104 ± 0.029	0.175 ± 0.035[a]
	Contra-	0.089 ± 0.019	0.110 ± 0.024	0.096 ± 0.009	0.138 ± 0.021
			SN THir cell counts		
Vehicle	Ipsi-	12800 ± 2590	12348 ± 784[ab]	12212 ± 1080	12468 ± 760[ac]
	Contra-	13060 ± 1731	13968 ± 4625	12727 ± 1798	12281 ± 1568
IL-1β	Ipsi-	12754 ± 740	11030 ± 1413	12920 ± 245	10720 ± 813[b]
	Contra-	11957 ± 1672	12619 ± 791	11705 ± 1333	12486 ± 972
TNFα	Ipsi-	10372 ± 1283	10235 ± 944[a]	11176 ± 1683	9620 ± 2195[c]
	Contra-	12648 ± 910	12444 ± 503	12823 ± 1354	12964 ± 1033
IL-1/TNF	Ipsi-	10144 ± 2030	9423 ± 1318[b]	10265 ± 1502	7524 ± 1011[ab]
	Contra-	12853 ± 630	12507 ± 568	12833 ± 751	12008 ± 904

this change was at best additive. The effects on DA activity ([HVA]/[DA]) were similar, although the effects were statistically not as profound (Fig. 1). Thus, the effects of IL-1β (10 ng) or TNFα (200 ng) injection on [HVA]/[DA] were significantly altered ($F_{3,31} = 6.38$; $p < 0.01$) although the combined effects of both cytokines only increased DA activity 85.1%. Again, the effects of combined cytokines on DA activity was, at best, characterized as additive.

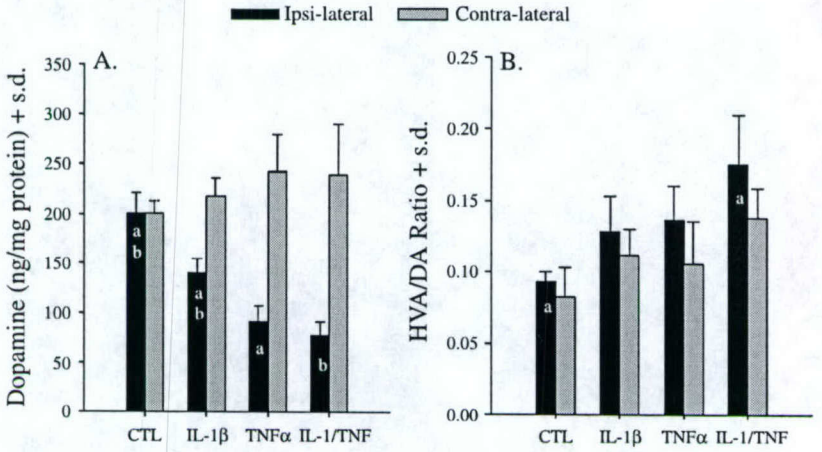

Fig. 1. The effects of IL-1β (10 ng) and TNFα (200 ng) injected into the medial forebrain bundle (MFB) alone and together on striatal DA content (left) and DA activity (right). Animals were sacrificed 14 days following injection. Bars sharing a common letter are statistically different from one another at $p < 0.05$

Substantia nigra THir cell counts

As was true for the DA biochemical results, the effects on THir cell counts were also dose-dependent (Table 1) and statistically significant (Fig. 2; $F_{3,35} = 3.206$; $p < 0.05$) although the magnitude of the effects was not as profound.

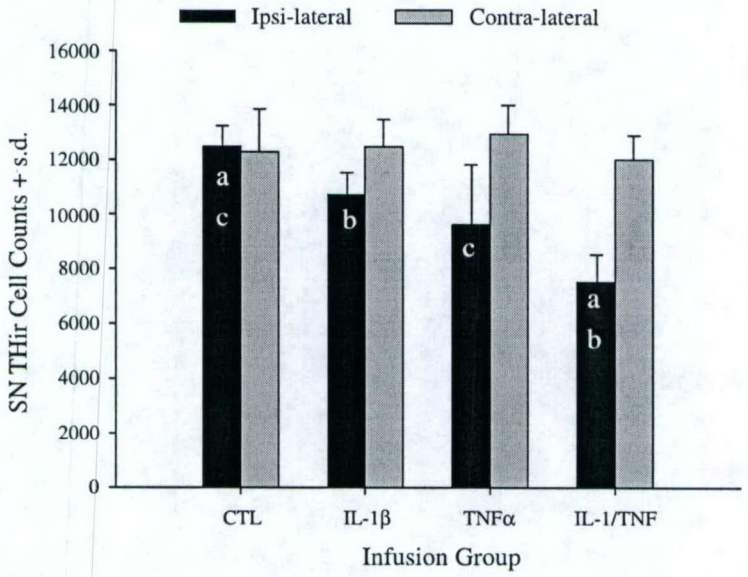

Fig. 2. The effects of IL-1β (10 ng) and TNFα (200 ng) injected into the medial forebrain bundle (MFB) alone and together on SN THir cell counts. Animals were sacrificed 14 days following injection. Bars sharing a common letter are statistically different from one another at $p < 0.05$

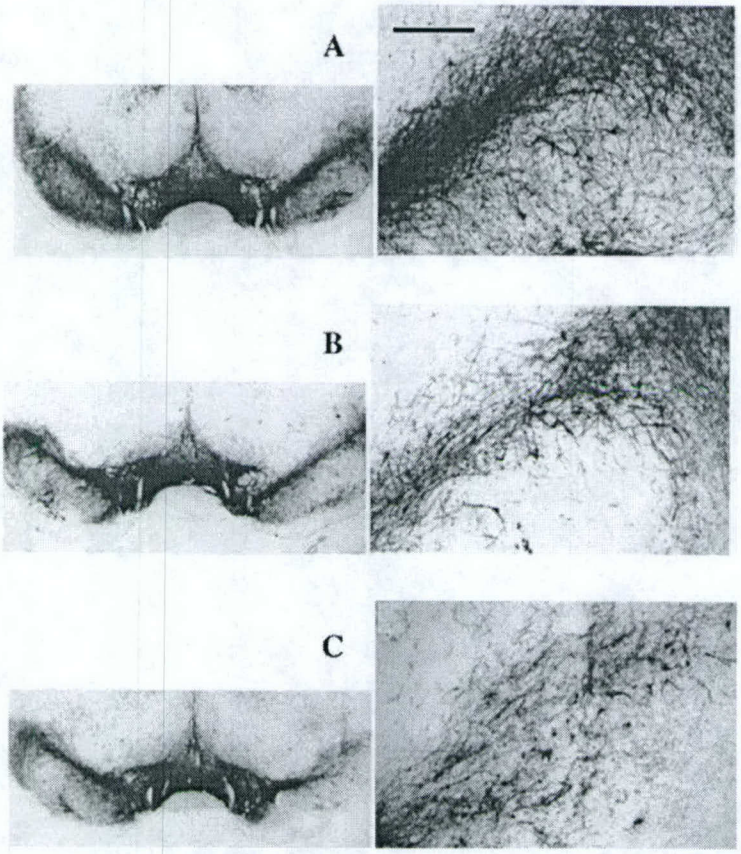

Fig. 3. Representative photomicrographs of the TH immunostaining of the mesencephalon injected with vehicle (**A**), TNFα (200 ng) (**B**), and TNFα (200 ng) plus IL-1β (10 ng) (**C**). Note that the pattern of cell loss is most pronounced in the lateral regions of the SN ipsilateral to injection and its ventral tier while sparing the VTA (right side in the left column). Within the areas of cell loss, the density of the immunoreactivity is reduced as seen in the associated higher power photomicrographs of the same sections (right column). Bar = 0.50 mm

Thus, injection of IL-1β (10 ng) produced a 12.2% decrease in THir cell counts while TNFα (200 ng) produced a 21.1% decrease. Both injections combined produced a 36.7% decrease in THir cell counts. Examination of the THir histology in the animals suggested that injection of either cytokine or combination produced greater cell loss in the lateral regions of the SN and its ventral tier (Fig. 3), but did not alter cell counts in the ventral tegmental area (VTA). The SN THir cell counts were positively correlated with the levels of DA in the striatum ($r = 0.608$, $P < 0.001$).

Effect of recovery period after injection

In one study, the animals were sacrificed 7 days following acute injection, while a second group was sacrificed after 14 days. Interestingly, neither IL-1β nor TNFα altered DA biochemistry or THir cell counts in the animals sacrificed 7

days after injection (Table 1). In contrast, both striatal DA and THir cell counts were decreased in animals sacrificed 14 days following injection suggesting that these losses were affected by the duration of recovery.

Discussion

The results from the present study revealed that IL-1β and, in particular, TNFα were both able to induce THir neuron loss, reduce striatal DA and increase DA activity in a dose- and time-to sacrifice-dependent fashion. Both pro-inflammatory cytokines had this effect and when both cytokines were combined, the effects seen were more pronounced, although best characterized as additive. Indeed, the combined effects of both agents often produced less than additive effects. In addition, the relative ability to produce a loss of THir cells was dependent upon how long after injection the animals were examined. Thus, at an intermediate dose (200 pg IL-1β or 50 ng TNFα), significant alterations were not seen after 7 days, but by 14 days, both THir cell counts and striatal DA were decreased. Taken together, these results extend previous studies and suggest that IL-1β and/or TNFα can induce DA neuron loss independent of other actions resulting from LPS exposure.

A previous study by Castano and collegues (2002) injected TNFα and/or IL-1β into the SN and analyzed the brains one week after injection. They injected only one dose of each cytokine that was 10 times lower than our highest dose of TNFα (1,000 U = ~20 ng), but 10 times lower than our highest dose of IL-1β (1,000 U = ~100 ng). In their study, they failed to demonstrate any significant changes in striatal biochemistry or DA cell counts in the SN seven days after injection, despite seeing reductions in these parameters following intranigral LPS injection. They argued that mechanisms other than IL-1β and TNFα must be responsible for the cell loss seen. The apparent differences between their results and the current findings are likely a consequence of several differences in experimental procedure. First, we also failed to demonstrate reductions in the DA indices one week after the injection and also observed that the effects were dose-dependent. Had they used different dosages or waited longer until sacrifice, they may also have seen statistically significant effects. Second, they injected the cytokines directly into the SN. This produces only focal distribution of the injectate. In contrast, we injected the cytokines into the medial forebrain bundle just rostral to the SN. Injections at this location are distributed more evenly throughout the SN by coursing back to the cell bodies along the low resistance fiber bundles coming out of the nigra (Carvey et al., 1994). Indeed, examination of photomicrographs of the SN in their manuscript revealed THir cell loss only around the needle site in contrast to the cell loss seen in our study which was more widely distributed. Finally, just because LPS was able to produce DA cell loss in their study while the cytokines did not, does not rule IL-1β and TNFα out as the factors responsible for DA cell loss. There are issues of the dose of LPS used and the resulting increases in the pro-inflammatory cytokines that must be considered. It also, however, does not rule out the possibility that indeed factors in addition to IL-1β and/or TNFα are involved in the toxicity as originally suggested in their study. Regardless, it

is clear from the present results that IL-1β and TNFα can indeed produce DA neuron loss by themselves.

The fact that IL-1β and/or TNFα produced DA cell loss after a single acute injection is remarkable, especially given the fact that significant losses required two weeks to develop. This might suggest that these pro-inflammatory cytokines either induced a cascade of events that required a prolonged period of time to kill the DA neuron, or directly killed the DA neurons, but did so slowly. A previous study demonstrated that TNFα kills DA neurons via apoptosis (McGuire et al., 2001), but this is generally a rapid process taking only 24 hours. However, even if a necrotic mechanism were involved, it is unlikely that the cell death would require more than a week. IL-1β and TNFα are known to participate in a positive, feed-forward fashion to increase the effect of one another. Thus, IL-1β increases the release of TNFα (Chao et al., 1995) while TNFα increase IL-1β (Cai et al., 2003). Anti-inflammatory cytokines are likely involved in limiting this response. However, these reciprocal feed-forward mechanisms, similarly develop rapidly (Cai et al., 2003). It is also important to appreciate that loss of DA neurons leads to increases in DA metabolism, as seen in the current study where DA activity was significantly increased. This is associated with increased production of reactive oxygen species (ROS) which, by themselves, also increase production of pro-inflammatory cytokines (Jenner, 2003). However, all of these processes would develop quickly.

It is also possible that the injected cytokines recruited the activity of another cell type. Indeed, Gao et al. (2002), demonstrated that a slow infusion of LPS into brain led to loss of DA neurons that was delayed and associated with the localized activation of microglia. Despite the infusion of LPS and the presumed increased production of pro-inflammatory cytokines such as TNFα and IL-1β produced by LPS, DA neurodegeneration was not seen in the animals for at least 4 weeks and continued even after discontinuation of the LPS infusion. Thus, the results from Gao and colleagues are not unlike those reported here. This may be significant since activated microglia are present in the PD brain where they are often seen in proximity to degenerating DA neurons (McGeer et al., 1988). Activated microglia are also often associated with degenerating DA neurons in animals treated with the DA neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; McGeer et al., 2003). It is thus possible in the present study that the injected cytokines initiated a low level inflammatory response that produced a small amount of cell loss and recruited microglia into the vicinity. This led to further inflammatory events (e.g., ROS resulting from increased DA activity) that recruited additional microglia that participated in a feed-forward process that was eventually able to produce significant cell loss. Although the exact mechanism(s) responsible for the cell loss in the current study is unknown, it is important that it (they) be determined so as to provide potential clues for the delayed and prolonged cell loss seen in patients with PD.

The pattern of DA cell loss in these animals was similar to that seen in animals exposed to other DA neuron toxins including 6-hydroxydopamine (Rodriguez et al., 2001), MPTP (Varastet et al., 1994), and prenatal LPS (Ling et al., 2004). The pattern seen in the present study is also similar to that seen in patients with PD (Hornykiewicz and Kish, 1987; Javoy-Agid et al., 1981).

Thus, cells in the lateral regions of the SN as well its ventral tier were more affected than cells in the ventral tegmental area (VTA) or more medial regions of the SN. The fact that four different toxins produce the same pattern of cell loss would suggest a common mechanism is involved or, that there is a selective vulnerability at work as originally hypothesized (Mouatt-Prigent et al., 1994).

It is likely that PD is a consequence of multiple etiological agents that are a consequence of several risk factors that involve both genetic predispositions and environmental insults. Regardless of cause, it appears that inflammatory events are involved. A central question has been, and remains; "is inflammation a consequence of the degenerative process or the cause of the DA cell loss?" The results from the present study clearly suggest that inflammation by itself can produce DA cell loss. Although it seems intuitively obvious, it demonstrates the possibility that DA cell loss does not necessarily need to be involved with the initial stages of PD pathogenesis. Thus, an initiating agent does not have to be a DA neurotoxin. Rather, it could be a non-specific toxin that produces inflammation that then happens to kill DA neurons. If true, the list of possible environmental causes and genetic risk factors would be significantly broadened.

Acknowledgements

This work was supported by a grant from the Department of Defense (17-01-1-0771), NIEHS (ES10776) and NINDS (NS0453161).

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Neuroscience 124 (2004) 619–628

COMBINED TOXICITY OF PRENATAL BACTERIAL ENDOTOXIN EXPOSURE AND POSTNATAL 6-HYDROXYDOPAMINE IN THE ADULT RAT MIDBRAIN

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Abstract—We previously reported that injection of the Gram (−) bacteriotoxin, lipopolysaccharide (LPS), into gravid females at embryonic day 10.5 led to the birth of animals with fewer than normal dopamine (DA) neurons when assessed at postnatal days (P) 10 and 21. To determine if these changes continued into adulthood, we have now assessed animals at P120. As part of the previous studies, we also observed that the pro-inflammatory cytokine tumor necrosis factor α (TNFα) was elevated in the striatum, suggesting that these animals would be more susceptible to subsequent DA neurotoxin exposure. In order to test this hypothesis, we injected (at P99) 6-hydroxydopamine (6OHDA) or saline into animals exposed to LPS or saline prenatally. The results showed that animals exposed to prenatal LPS or postnatal 6OHDA alone had 33% and 46%, respectively, fewer DA neurons than controls, while the two toxins combined produced a less than additive 62% loss. Alterations in striatal DA were similar to, and significantly correlated with (r^2=0.833) the DA cell losses. Prenatal LPS produced a 31% increase in striatal TNFα, and combined exposure with 6OHDA led to an 82% increase. We conclude that prenatal exposure to LPS produces a long-lived THir cell loss that is accompanied by an inflammatory state that leads to further DA neuron loss following subsequent neurotoxin exposure. The results suggest that individuals exposed to LPS prenatally, as might occur had their mother had bacterial vaginosis, would be at increased risk for Parkinson's disease.

Key words: Parkinson's disease, TNFα, IL-1β, dopamine, prenatal, lipopolysaccharide.

Although genetic factors account for some cases of Parkinson's disease (PD; Gasser, 1998; Polymeropoulos et al., 1997), the vast majority of PD is considered idiopathic. Several environmental factors, including chemical neurotoxins, such as rotenone, dieldrin, and paraquat have been proposed as risk factors (Brooks et al., 1999; Thiruchelvam et al., 2000; Betarbet et al., 2000; Jenner, 2001). Although these known dopamine (DA) neurotoxins have been widely discussed, their potential roles as PD risk factors are suspect because of the large dosages that would be needed. However, if exposure were to occur in an individual that already had fewer than normal DA neurons, and especially if that hypo-dopaminergic condition was associated with a pro-inflammatory state, then lower dosages of neurotoxins would be needed. Recent studies in animals from our laboratory suggest that this is possible.

We previously demonstrated that rat fetuses exposed to the Gram(−) bacteriotoxin, lipopolysaccharide (LPS) at embryonic day (E) 10.5 were born with fewer than normal DA neurons (Ling et al., 2002a,b). Assessment of tyrosine hydroxylase (TH) immunoreactive (ir) neuron counts (used as an index of DA neurons) in the substantia nigra (SN) of postnatal day (P) 10 and P21 rat pups revealed losses of 25 and 31%, respectively. Similar reductions in striatal DA and increases in DA activity ([homovanillic acid (HVA)]/[DA]) were also seen. In addition, increased levels of the pro-inflammatory cytokine tumor necrosis factor α (TNFα), were seen in the SN of these animals, indicating a pro-inflammatory state. These changes in DA and increases in TNFα are consistent with the well-established effects of LPS. Thus, LPS, acting through the CD14 and toll-like receptor-4, is a well known inducer of pro-inflammatory cytokines and has been shown to kill DA neurons both *in vitro* and *in vivo* by several investigators (Castano et al., 1998, 2002; Gayle et al., 2002). Taken together, these studies suggested that LPS and infections that increase pro-inflammatory cytokines prenatally, may be involved in the etiology of PD. We hypothesized that individuals born to mothers with bacterial vaginosis (BV), a well known inducer of LPS and pro-inflammatory cytokines in the chorioamniotic environment (Fortunato et al., 1996; Menon et al., 1995), would be at increased risk for PD. Moreover, if prenatal LPS led to a continued pro-inflammatory state, then subsequent exposure to DA neurotoxins would produce a more pronounced DA cell loss. This would then support the notion that PD is a consequence of the combined effects of multiple environmental factors with a prenatal factor representing an entirely new exposure paradigm.

We set out to assess this possibility in rats exposed to LPS prenatally followed by postnatal neurotoxin exposure. We chose the well-known DA neurotoxin, 6-hydroxydopa-

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Abbreviations: BV, bacterial vaginosis; DA, dopamine; E, embryonic day; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; EU, endotoxin unit; HBSS, Hanks' balanced salt solution; HPLC, high performance liquid chromatography; HVA, homovanillic acid; IL-1β, interleukin 1β; ir, immunoreactive; LPS, lipopolysaccharide; P, postnatal; PD, Parkinson's disease; SN, substantia nigra; TH, tyrosine hydroxylase; TNFα, tumor necrosis factor α; VTA, ventral tegmental area; 6OHDA, 6-hydroxydopamine.

0306-4522/04$30.00+0.00
doi:10.1016/j.neuroscience.2003.12.017

mine (6OHDA) as the postnatal toxin for several reasons. First, it has been shown to increase TNFα in brain, suggesting that it would further increase proinflammatory cytokine(s) in animals prenatally exposed to LPS (Mogi et al., 2000). Second, i.c.v. injection of 6OHDA kills DA cells bilaterally, which is similar to the effects of prenatal LPS (Vu et al., 2000). Third, when injected i.c.v., 6OHDA kills DA neurons in a dose-dependent manner (Carvey et al., 1994).

EXPERIMENTAL PROCEDURES

Animals

Forty-nine timed-gravid female Sprague–Dawley rats (Zivic-Miller, Allison Park, PA, USA) were delivered to Rush's animal facility at gestational day 9±12 h. Two additional male and two non-gravid female Sprague–Dawley rats were also studied. All animals were maintained in an environmentally regulated animal facility for the duration of the study (lights on 06:00–18:00 h). The protocols and procedures used in these studies were approved by the Institutional Animal Care and Utilization Committee (IACUC) of Rush University and met the guidelines of the Council on Accreditation of the Association for Assessment and Accreditation of Laboratory Animals Care (AAALAC, international). Minimal number of animals was used in the study and all survival surgeries were performed under pentobarbital anesthesia (40 mg/kg body weight) to minimize pain.

Prenatal treatment and body temperature study

LPS was purchased from Sigma Chemical Co. (St. Louis, MO, USA; L-8274; prepared from *Escherichia coli* 026:B6) and dissolved in saline (Hanks' balanced salt solution [HBSS]; Gibco-Invitrogen) at 10,000 endotoxin units (EU)/ml. At E10.5, each gravid female received a single injection (i.p.) of either 10,000 EU/kg LPS or HBSS (1 ml/kg; $n=18$ for each group). This dosage was chosen based on previous studies where it was shown to be well tolerated and did not produce fetal demise (Ling et al., 2002a). E10.5 was used because it is just prior to the birth of DA neurons at E11–E11.5 (Specht et al., 1981; Sinclair, 1999; Cohen and Sladek, 1997; Bouvier and Mytilineou, 1995) and previous studies showed it to produce the most profound DA neuron loss while earlier injections and later injections (E12.5 and greater) produced no loss at all (Ling et al., 2002b). All the animals were monitored daily for obtundation or other signs of distress, and were allowed to deliver their pups normally. The pups were weaned at P21 and housed two per cage with same-sex partners.

Increased maternal body temperature has been previously hypothesized to produce teratogenic effects in offspring (Colado et al., 1999; Albers and Sonsalla, 1995). Although the dosage of LPS used in the present study was considerably lower than those known to produce hyperthermia (Miller et al., 1997; Meszaros et al., 1991) we needed to rule out the possibility that the THir cell loss seen was simply a consequence of hyperthermia. Rectal temperature was measured in separate groups of animals treated with the same protocol (prenatal saline [$n=6$]; prenatal LPS [$n=7$]) prior to, and following i.p. LPS injection, at 30 min intervals for 5.5 h, and again at 24 h. In order to determine if the effect of LPS on body temperature was influenced by gender or gravidity, we also assessed rectal temperature in two males and two, non-gravid females.

Treatment groups, postnatal treatment, and kill protocol

One male pup from each of the 24 litters was chosen (12 LPS and 12 saline) and randomly assigned to one of two postnatal treatment groups (i.c.v. Sham Control [vehicle] or 6OHDA) creating a 2×2 design (LPS/Sham; LPS/6OHDA; Saline/Sham; and Saline/6OHDA).

At P99 the animals were subjected to the postnatal treatment protocol. One hour prior to surgery, every animal received an i.p. injection of desipramine HCl (25 mg/kg) to reduce the uptake of 6OHDA into noradrenergic terminals (Carvey et al., 1994). The animals were anesthetized with pentobarbital (40 mg/kg; i.p.) and placed in a stereotaxic frame. 5 μl of a 6OHDA solution containing 150 μg 6OHDA as the HBr salt (Research Biochemicals Inc.) in an ascorbate vehicle (0.02% ascorbate/saline) or 5 μl ascorbate vehicle was delivered into the right lateral ventricle (anterior/posterior=−3.8 mm, medial/lateral=−4.8 mm, dorsal/ventral=−6.8 mm, relative to bregma) as described previously (Carvey et al., 1994). This dose was chosen because our previous studies have demonstrated that it was likely to produce a 50% DA neuron depletion in the SN (Vu et al., 2000). The animals were allowed to recover under a heating lamp, returned to their home cages, and perfused transcardially 21 days later at P120 with ice-cold saline. The brains were quickly removed and processed for subsequent assessments as described previously (Vu et al., 2000). Briefly, the frontal part of the brain (4 mm from the apex) was isolated using a coronal knife cut, and kept for biochemistry and cytokine studies. The posterior part of brain was submerged in Zamboni's fixative (7.5% saturated picric acid, 12 mM NaH_2PO_4, 88 mM Na_2HPO_4, and 4% paraformaldehyde) for immunocytochemistry studies. Two sets of tissue punches were taken from the center of the left and right striatum, pooled and passed to high performance liquid chromatography (HPLC) assessment of DA and DA activity, or enzyme-linked immunosorbent assay (ELISA) for assessment of TNFα and interleukin 1β (IL-1β). IL-1β was assessed because it is also a pro-inflammatory cytokine, and because our previous studies revealed that it was elevated in animals exposed to LPS prenatally and killed at P10, but was not elevated in animals killed at P21 (Ling et al., 2002a,b).

DA biochemistry

DA and its metabolite HVA were assessed using HPLC with electrochemical detection in pooled (left and right) tissue punches taken from the center of each striatum as previously described (Vu et al., 2000). DA and HVA were expressed as ng/mg tissue protein (assessed using the Biorad Protein Assay Kit), and the ratio of [HVA]/[DA] was used as an index of DA activity.

ELISA for cytokine protein assessments

The pooled tissue punches from each striatum destined for cytokine assessment were submerged in 200 μl homogenate buffer (Trizma/HCl, pH 7.2, 0.1 M, sodium chloride 0.9%, EDTA 1 mM, Aprotinin 2%, leupeptin 0.5 μg/ml, pepstatin 0.7 μg/ml, phenylmethanesulfonyl fluoride 200 μM; all reagents obtained from Sigma) and homogenized as described previously (Lipton et al., 1999). To each tube, 200 μl homogenate buffer containing 0.5% NP-40 (Sigma) was then added. The tubes were shaken, centrifuged (10,000×g for 15 min), and the supernatants collected. The levels of TNFα and IL-1β were assessed in each supernatant by an ELISA run according to the manufacturer's instructions (R & D Systems, Minneapolis, MN, USA). The plates were read in a Dynatech ELISA plate reader (Dynatech Laboratories, Chanlity, VA, USA) and the concentrations of the cytokines were determined against a six-point standard curve. The protein content of each supernatant was determined using the Bio-Rad Protein Assay kit and the quantity of the cytokines was expressed as pg/mg protein.

THir and NeuNir immunocytochemistry

The number of THir and NeuNir cells (a nuclear marker expressed in most post-mitotic neurons (Mullen, 1992) were assessed using

a stereological procedure (Vu et al., 2000). The entire mesencephalon was sliced on a cryostat into consecutive 40 μm sections. Every sixth section was immunohistochemically processed for TH as described previously (Vu et al., 2000; Ma et al., 1997) using mouse anti-rat TH antibody (Immunostars, Stillwater, MN, USA; 1:20,000). The immunohistochemical procedure was continued by using a biotinylated horse anti-mouse IgG (0.5%; Vector Laboratories, Burlingame, CA, USA) for 1 h then peroxidase conjugated avidin–biotin complex (Vector Laboratories) for 1 h. The chromogen solution used to complete the reaction consisted of 0.05% 3,3′-diaminobenzidine, 0.5% nickel sulfate, and 0.003% H_2O_2 in I/A solution (10 mM imidazole/50 mM sodium acetate) to obtain a jet-black stain. The NeuN antibody (1:5000; Chemicon, Temecula, CA, USA) immunohistochemistry was performed in the same series of sections, only the chromogen was developed without nickel sulfate to generate a brown color. The sections were washed, mounted on gelatin-coated slides, dehydrated through graded alcohols, cleared in xylenes, and coverslipped with Permount.

Stereology assessments

The estimation of the total number of THir and NeuNir neurons in the SN and ventral tegmental area (VTA) was performed using the computerized optical dissector method. This method allowed for the stereological estimation of THir cells in the entire structure, independent of size, shape, orientation, tissue shrinkage or anatomical level using MicroBrightField software (Ling et al., 2002a). Since the compacta region of the SN cannot be easily distinguished from the reticulata, the two regions were combined for stereological assessment and designated SN. The VTA was identified as the region medial to the accessory optic tract and lateral to the midline. In regions where the accessory optic tract was not readily seen, the line connecting the most inferior point on the dorsal curvature between the THir cells of the SN and the VTA and the most superior point of the ventral curvature, represented the lateral boundary of the VTA (see Fig. 3).

The antibody penetration throughout the whole tissue section was assessed by dissectors using the imaging capture technique (Emborg et al., 1998; Kordower et al., 1996; Ma et al., 1999a,b). The total number (N) of SN or VTA neurons was calculated using the formula $N=N_V\ V_{SN\ or\ VTA}$, where N_V is the numerical density and $V_{SN\ or\ VTA}$ is the volume of the SN or VTA respectively, as determined by Cavalieri's principle (Cavalieri et al., 1966). The MicroBrightField software was also used to determine the total area of the SN and VTA as outlined by the blinded investigator (volume in mm^3), the density of the cells in that volume (cell counts/mm^3), and the size of the cell bodies chosen for assessment (μm^2; Ling et al., 2002a). After immunostaining and dehydration, the thickness of sections varied from 18 to 20 μm. The thickness of each section was measured and this reading was entered as a counting parameter. The upper and lower forbidden layers (4 μm) were eliminated from counting, leaving a 10–12 μm thick section as the final thickness for stereological assessment. This procedure ensures that cells layered on top of one another within a section are accurately assessed.

Statistical analysis

The various measures were assessed using two-way ANOVA. Differences between treatment groups were assessed using Tukey's post hoc analysis run following one-way ANOVA for that particular group. Pearson's two-tailed test was used to statistically correlate the cell count data with the biochemistry values. The temperature data were analyzed using a repeated measures ANOVA.

We have repeatedly shown that despite unilateral i.c.v. injection of 6OHDA, it produces equivalent bilateral effects on DA cell counts and DA biochemistry (Vu et al., 2000). Similarly, as also reported previously (Ling et al., 2002a) prenatal LPS leads to equivalent THir cell losses on both sides of the brain. As a result, all results from the left and right sides were pooled (DA biochemistry and cytokine assessments) or combined (cell counts) for statistical analysis.

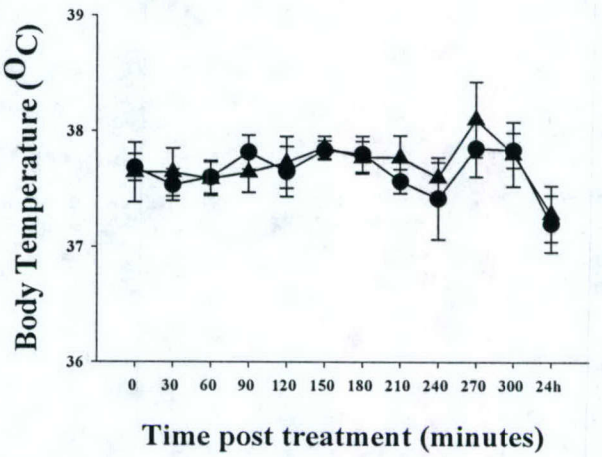

Fig. 1. Rectal body temperature in LPS- and HBSS-treated gravid rats. The rectal temperature of each group is depicted prior to (0) and following i.p. HBSS (circles) or LPS treatment (triangles) at E10.5.

RESULTS

Overall, the gravid dams tolerated the treatment protocol without difficulty. A total of 13 dams were monitored for rectal temperature fluctuations within the 24 h following injection at E10.5. No significant alterations in body temperature were noted (Fig. 1; $F_{1,12}=0.589$, $P=0.459$). Non-gravid females and males injected with 10,000 EU/kg LPS also failed to exhibit significant alterations in rectal temperature (data not shown). Only animals giving birth at approximately E21.5–22, which was 9.5–10 days after initial treatment (suggesting that the injection of LPS into the gravid females occurred at approximately E10.5) were accepted into the protocol. Neither the pup body weights nor the litter sizes in the LPS- or saline-treated groups were significantly different from one another (data not shown). In the 6OHDA lesion studies, one animal died within 24 h of i.c.v. injection of 6OHDAin the LPS/6OHDA group. One of the ELISA determinations from the Saline/Sham group had an unusually low protein content and the sample was dropped from analysis. All other values were used.

SN cell counts

The sections from the animals were immuno-stained for TH and NeuN in an effort to determine if the THir cell loss seen was a consequence of phenotypic suppression of TH or actual cell loss. If prenatal LPS or postnatal 6OHDA suppressed TH expression, but did not actually kill THir cells, then loss of THir cells would be associated with increases in NeuNir cells and the two sets of cell counts would be inversely correlated. If, on the other hand, THir cell loss reflected death of cells, there would be a reduction

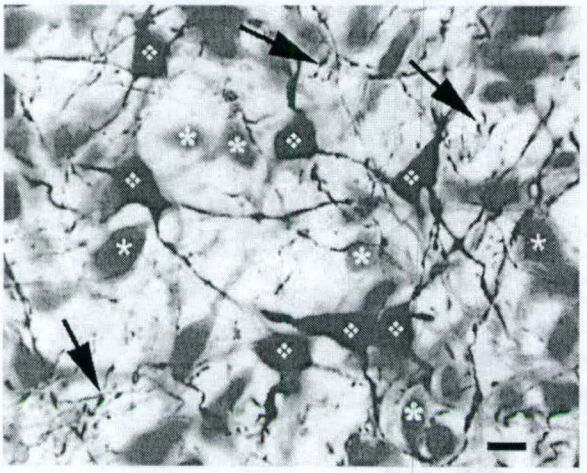

Fig. 2. Double stain of THir and NeuNir neurons in the SN compacta. This photomicrograph of the compacta region of the SN depicts the typical staining pattern seen. Numerous large THir neurons were observed (black cells with white ❖) that had numerous processes with varicosities typical of DA neurons (black arrows). Cells reacting to NeuN were not developed with nickel enhancement and therefore appear brown. The NeuN reacted primarily with the cell bodies of non-dopaminergic cells (sampled cells are labeled with white *). (Magnification Bar=15 μm).

in THir cells, no change in NeuNir cells, and the reduction in THir cells would be positively correlated with the changes seen in total cells.

The double stains of the mesencephalic sections revealed clear segregation of the two different cell types that readily allowed for stereological assessment (Fig. 2). THir cells were jet black and exhibited fine, thread-like, extensive processes with varicosities typical of DA neurons in the compacta region of the SN. In contrast, the NeuNir cells were rich brown, and since the NeuN immunoreacts only with neuronal nuclei, there was no confusion between cell types. The sum of the THir and NeuNir cell counts thus generated the total neuron cell counts. In the compacta and reticulata regions of the SN in the Saline/Sham control animals, THir cells represented 33.17% of the total cell counts.

Both prenatal LPS exposure and postnatal treatment had significant effects on the apparent THir immunoreactivity (Fig. 3) as well as the THir cell counts (Fig. 4). The photomicrographs of the SN clearly reveled that the most pronounced THir cell loss was present in the lateral regions of the nigra, as well as the ventral regions of the compact and within the reticulata. Both prenatal LPS and postnatal 6OHDA had this effect. However, postnatal treatment with 6OHDA in animals exposed to LPS prenatally, produced a more profound THir cell loss. Statistically, the effects of pre- and postnatal treatments were both highly significant (prenatal exposure: $F_{1,22}=35.036$, $P<0.001$; postnatal treatment: $F_{1,22}=92.246$, $P<0.001$). In addition, the pre- vs. postnatal treatment interaction was also statistically significant ($F_{3,22}=7.36$, $P=0.014$). This, together with an examination of the means, suggested that the combined effects of prenatal LPS exposure and postnatal toxin treatment produced a less than additive effect, albeit

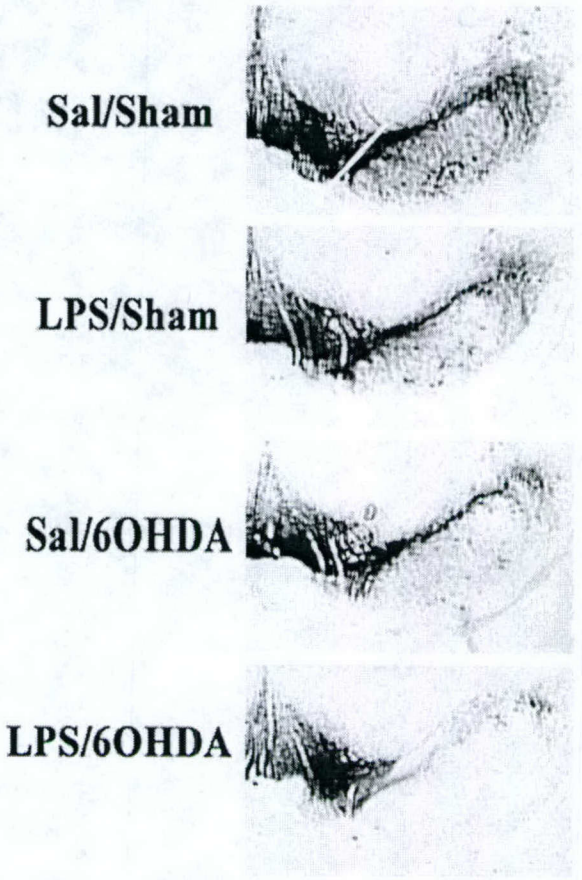

Fig. 3. Photomicrographs of the mesencephalons. Representative photomicrographs (20×) of the right side of the mesencephalon of animals exposed to the various combinations of treatments (Sal=saline; Sham=Vehicle treatment). The sections depicted were immunostained for TH only and were adjacent to those used to generate the data in Figs. 4 and 5. The white line transecting the stained area in the Sal/Sham photomicrograph depicts the dividing point between the VTA (medial to) and the SN (lateral to).

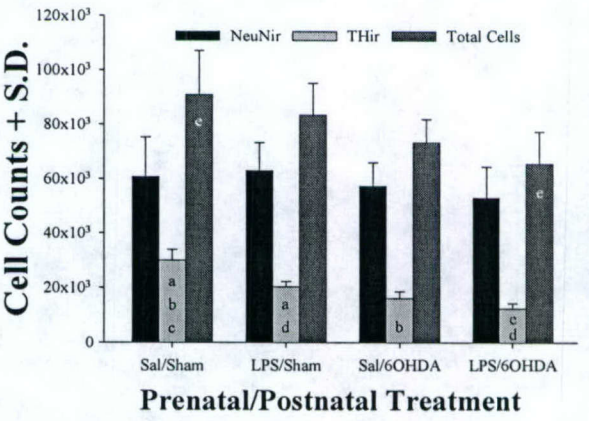

Fig. 4. Stereological cell counts from the SN. The mean THir, NeuNir, and Total Cells (THir+NeuNir) cell counts from the four different treatment groups are shown. Bars which share the same letter are significantly different from one another ($P<0.05$). Sal=saline; LPS=lipopolysaccharide; 60HDA=6-hydroxydopamine.

Table 1. Stereology data on THir cells in the SN

Prenatal/postnatal	Cell counts	Density	Volume (mm^3)	Cell size (μm^2)
Saline/Sham	$30,152 \pm 3,916^{abc}$	$9,683 \pm 1,150^{ab}$	$3.13 \pm 0.39^{*}$	180.77 ± 13.51
LPS/Sham	$20,293 \pm 2,005^{ad}$	$7,734 \pm 1,118^{a}$	2.64 ± 0.23	174.43 ± 16.00
Saline/6OHDA	$16,084 \pm 2,443^{b}$	$8,819 \pm 920^{b}$	2.36 ± 0.23	189.45 ± 24.67
LPS/6OHDA	$12,423 \pm 1,933^{cd}$	$5,299 \pm 934^{a}$	2.35 ± 0.16	$227.70 \pm 22.10^{*}$

Values which share a common letter are significantly different from one another in the same column. * = $P<0.05$ relative to all other treatment groups in the same column. LPS=lipopolysaccharide; 60HDA=6-hydroxydopamine.

significantly greater than either of the two treatments alone (Fig. 4). The LPS/Sham animals exhibited a 32.7% decrease in THir cells, which was significantly reduced relative to control animals. The Saline/6OHDA animals similarly exhibited a statistically significant 46.7% THir cell count reduction, which was in excellent agreement with the 50% cell loss anticipated from the 150 μg dose of 6OHDA injected. However, the combined effects of prenatal LPS and postnatal 6OHDA resulted in further statistically significant cell loss (58.8% decrease). As anticipated, the density of the THir cells decreased proportionately with cell loss (Table 1). Interestingly, the cross-sectional cell size of the THir cells was dramatically increased ($P<0.05$) in the animals exposed to LPS prenatally and injected with 6OHDA postnatally. No other pair-wise comparisons were statistically significant.

In contrast to the effects of pre- and postnatal treatment on THir cell counts, neither of these treatments produced significant changes in the SN NeuNir cell counts (prenatal exposure: $F_{1,22}=0.034$; postnatal treatment: $F_{1,22}=1.971$). Prenatal treatment did not affect total cell counts (i.e. THir+NeuNir: $F_{1,22}=2.188$, $P=0.156$), while postnatal treatment did reduce the total number of cells slightly ($F_{1,22}=11.697$, $P=0.003$) probably reflecting the significant loss of THir cells. The only statistically significant pair-wise comparison for total cells was between the control animals and the LPS/6OHDA animals. Pearson's two-tailed test revealed that the THir cell counts and NeuNir were not significantly correlated ($r^2=0.162$), whereas the THir and the total cell counts were ($r^2=0.622$; $P=0.001$), suggesting that the THir cell loss seen was not simply phenotypic suppression, but actual cell loss.

VTA cell counts

Cell counts in the VTA were assessed in an effort to determine if the prenatal or postnatal treatments produced similar effects to those seen in the SN. Although cell losses were seen in the VTA, the overall effects were significantly less obvious than those seen in the SN (Fig. 3). In the VTA, the average THir cell count was 18,723 in the Saline/Sham group which was 37.8% lower than that seen in the SN. However, the number of THir cells represented 70.75% of the total cells. This percentage is significantly higher than that seen in the SN (33.17%), and likely reflects the fact that the total cell counts for the SN were combined from the THir-rich compacta and THir-poor reticulata region. Regardless, approximately 30% of the cells in the VTA were non-dopaminergic.

Both pre- ($F_{1,22}=4.884$; $P=0.04$) and postnatal ($F_{1,22}=6.081$; $P=0.024$) treatments had a statistically significant effect on the VTA THir cells counts, although the magnitudes of the effects were less than those seen in the SN (Fig. 5). THir cell counts were only reduced by 10.97% by prenatal LPS exposure and 12.2% by 6OHDA, neither of which was significantly reduced relative to controls. The combined effects of both treatments led to a THir cell reduction of only 22.4%, although pair-wise comparisons revealed that this value was significantly reduced relative to the Saline/Sham group. Unlike the effects seen in the SN, the interaction between pre- and postnatal treatments was not statistically significant ($F_{3,22}=0.006$), suggesting that both pre- and postnatal treatment contributed equally to the cell loss seen. Prenatal treatment did not alter NeuNir cell counts, although the effect of postnatal treatment was statistically different ($F_{1,22}=11.26$; $P=0.004$). Total cells were similarly affected only by postnatal treatment ($F_{1,22}=10.997$; $P=0.004$), while no other statistically significant effects were seen.

Striatal DA biochemistry

Striatal DA measurements were made in order to determine if any changes in mesencephalic cell counts were associated with biochemical changes in this target structure. The mean striatal DA content of 221 ng/mg protein in the Saline/Sham group was in excellent agreement with

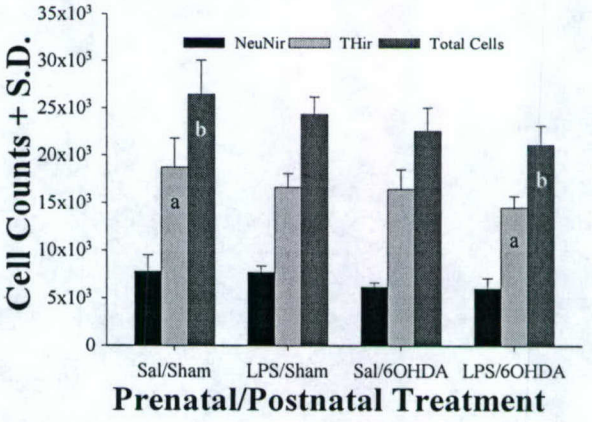

Fig. 5. Stereological cell counts from the VTA. The mean THir, NeuNir, and Total Cells (THir+NeuNir) cell counts from the four different treatment groups are shown. Bars which share the same letter are significantly different from one another ($P<0.05$). Sal=saline; LPS=lipopolysaccharide; 60HDA=6-hydroxydopamine.

Table 2. Striatal biochemistry and TNFa levels in the four treatment groups

Prenatal/postnatal	DA (ng/mg protein)	HVA (ng/mg protein)	HVA/DA ratio	Mean striatal TNFa (pg/mg protein)
Saline/Sham	211.51±9.96[a]	21.93±1.63[a]	0.099±0.007[a]	71.49±6.26[a]
LPS/Sham	186.70±18.13[a]	19.79±2.18[b]	0.107±0.014[c]	92.65±4.85[a]
Saline/6OHDA	116.05±9.45[a]	16.33±0.76[a]	0.141±0.008[ac]	86.81±20.13[b]
LPS/6OHDA	73.65±34.52[a]	13.31±3.73[ab]	0.193±0.040[ac]	129.90±23.62[ab]

Values which share a common letter are significantly different from one another ($P<0.05$ relative to all other treatment groups in the same column). The mean striatal levels for each of the measures were determined from pooled (left and right) tissue punches taken from each of the four treatment groups. LPS=lipopolysaccharide; 6OHDA=6-hydroxydopamine.

normal levels seen in several previous studies from our laboratory (Vu et al., 2000). Similar to the results seen for the cell counts in the SN, both prenatal ($F_{1,22}=21.998$; $P<0.001$) and postnatal treatments ($F_{1,22}=176.18$; $P<0.001$) had statistically significant effects on striatal DA, but unlike the SN cell count data, the interaction term was not significantly altered (Table 2). DA levels in the striatum of the animals exposed to LPS prenatally were reduced 11.7%, while postnatal 6OHDA treatment led to a more pronounced 45.16% DA loss. The combined effects of prenatal LPS exposure and postnatal 6OHDA led to a 65.1% DA loss. All four treatment groups were significantly different from one another. The DA levels in the striatum were highly correlated with the THir cell counts in the SN ($r^2=0.833$; $P<0.001$). Similar reductions were seen in HVA levels (Table 2).

DA activity (i.e. [HVA]/[DA]) is a widely used index of DA release (Thiblin et al., 1999; D'Astous et al., 2003; Yurek et al., 1998) with increases in this ratio ostensibly reflecting increased DA release and metabolism to HVA. The effects on DA activity were opposite to those seen on DA (Table 2). The ratio in the Saline/Sham animals was 0.099, while the effects of prenatal LPS increased the ratio only 7.7% ($P>0.05$), yet the overall effects of prenatal treatment were significantly altered ($F_{1,22}=12.066$; $P=0.003$). Postnatal 6OHDA increased the ratio by 42.3% ($F_{1,22}=56.29$; $P<0.001$), while the combined effects of both treatments increased the ratio dramatically (95.15%) yielding a significant interaction term ($F_{3,22}=6.733$; $P=0.017$) consistent with a super-additive effect of prenatal and postnatal treatments on this index.

Striatal cytokine protein levels

Cytokine levels were assessed in the striatum to determine if prenatal LPS was associated with long-term elevations in TNFα, and if the combined effects of prenatal LPS and postnatal 6OHDA produced further increases in this pro-inflammatory cytokine. IL-1β levels were also assessed in striatal tissue punches. However, ELISA determinations revealed that all IL-1β levels were below the lower level of quantification (lower level of quantification=5 pg/ml lysate) for the cytokine kit. In contrast, levels of TNFα were not only detected in all samples studied, but significantly increased by the respective treatments (Table 2). Thus, prenatal LPS exposure led to a statistically significant increase of 29.61% ($F_{1,21}=16.41$; $P<0.001$), and while TNFα levels in the striatum of animals treated with 6OHDA were only 21.4% increased ($P>0.05$), postnatal treatment overall was statistically significant ($F_{1,21}=24.51$; $P<0.001$). The combined effects of prenatal LPS exposure and postnatal 6OHDA led to a 81.71% increase in TNFα although the interaction term between pre- and postnatal treatments was not statistically significant ($F_{3,21}=2.85$), suggesting that both pre- and postnatal treatments contributed equally to the overall increases seen.

DISCUSSION

Overall, the effects seen in the present study confirm those seen at P10 and P21 in that the prenatal LPS-induced THir cell losses reported in younger animals were still present in P120 animals. This suggests that prenatal LPS treatment produces long-lived effects in the mesencephalon. Similarly, the reductions in DA and increases in DA activity seen at P10 and P21 were still present at P120 as would be suggested by the THir cell losses seen at all time points. TNFα protein levels were also statistically elevated in animals prenatally treated with LPS as was also seen in animals killed at P10 and 21. This later point is significant given the fact that TNFα is known to be increased in the brains of patients with PD (Boka et al., 1994; Hunot et al., 1999). In contrast, IL-1β levels were elevated in the striatum of P10 animals, but not in P21 animals. In animals killed at P120, these levels were not even detectable, suggesting that the IL-1β elevations seen in younger animals in response to prenatal LPS exposure were transient.

THir cell loss vs phenotype suppression

The THir and NeuNir cell count data supports our previous assertion that LPS produces actual DA neuron cell loss, and not just phenotypic suppression. Prior studies have shown that neurotrophic factors can rescue THir cells in the SN of animals treated with 6OHDA (Bowenkamp et al., 1995, 1996; Kearns and Gash, 1995; Winkler et al., 1996). These studies suggested that simply assessing THir cell counts can give the false impression that THir cell loss is indicative of DA neuron death. However, combining TH staining with NeuN staining, as was done in the present study, circumvents this problem. So, if the effect of prenatal LPS was simply to suppress TH expression and not kill the cell, the number of THir cell counts would have decreased while the NeuNir cell counts in that same animal would have increased proportionately. This was clearly not the case, and therefore argues that the decreases in the

THir cell counts seen in the P10, P21 and P120 animals reflect the birth of animals with fewer than normal DA neurons.

Pattern of cell loss

The current findings demonstrate that the pattern of LPS-induced DA cell loss is not only preferentially in the lateral nigra, its ventral tier, and in the reticulata, but that the cells of the VTA are relatively spared. Thus, despite the fact that prenatal LPS produced a 33% DA cell loss in the SN, the magnitude of the DA cell loss in the VTA was only 11%. The pattern of cell loss in the SN together with the apparent reduced sensitivity of the VTA is significant because it is similar to the cell loss pattern seen in the mesencephalon of PD patients (Javoy-Agid et al., 1981; Hornykiewicz and Kish, 1987). Furthermore, the decreases in striatal DA and HVA, and increases in DA activity in the animals exposed to LPS prenatally, are similar to those seen in PD patients. This is significant since an increase in the DA activity ratio implies increased DA metabolism, which is thought to lead to increased free radical production and further compromises in DA neuron function (Jenner, 1998). This, together with the increases in the TNFα seen in these animals, could lead to further DA neuron loss over time.

Absolute THir cell counts in control animals

In the current study, the average number of THir cells in the SN of control animals was 30,152 and in the VTA, 18,732. Previously, Banrezes et al. (2001) reported numbers of 13,518 and 20,372, respectively, while German and Manaye (1993) reported 20,912 and 20,418, respectively. Our average total THir cell count of 48,500 is not dramatically different from that of German and Manaye (1993), but significantly larger than that reported by Banrezes et al. (2001). These differences could reflect differences in animal strain, counting techniques, antibody penetration, and sampling issues. However, a major discrepancy occurs in the cell numbers of cells in the SN relative to the VTA. In the present study we designated the SN as the stained cells lateral to the accessory optic track or, when this landmark was not present, lateral to a line connecting the most inferior point on the dorsal curvature between the THir cells of the SN and the VTA and the most superior point of the ventral curvature. This could under and over-estimate SN cells in some sections which could have contributed to the differences in the cell counts seen. In addition, and of greatest importance, is that we routinely included the lateral regions of the parabrachial pigmented nucleus with our SN assessments. Located immediately dorsal to the compacta (Rodriguez et al., 2001), including this cell-rich, probable A10 region could easily lead to over-estimation of the THir cell counts. Regardless, it was still noteworthy that in both structures, a large percentage of the cells were not THir. This was particularly true in the SN where the non-THir cells counted in the reticulata likely contributed to the low percentage of THir cells. However, even in the compacta where Fig. 2 was taken, it was clear that a large number of cells (NeuNir) in the compacta region were not dopaminergic and likely GABA neurons. Regardless, statistical analysis revealed that the number of NeuNir cells was not affected by any of the treatments employed suggesting that the toxicity of both prenatal LPS and postnatal i.c.v. 6OHDA were specific to the DA neuron.

Effects of pre- and postnatal toxin exposures on THir cells

The elevations in TNFα seen in P10 and P21 animals as a result of prenatal LPS exposure, together with the reduced numbers of THir cells, suggested that such animals would be more susceptible to the effects of DA neurotoxins. Since 6OHDA is thought to induce THir cell loss, at least in part, through increased TNFα (Nagatsu et al., 2000; Cicchetti et al., 2002), we hypothesized that inflammatory synergy between the effects of prenatal LPS and postnatal neurotoxin exposure would produce a greater THir cell loss than LPS or 6OHDA exposure alone. Interestingly, the results from the present study do not support this hypothesis. Although 6OHDA treatment led to DA cell loss in the SN and decreased DA in the striatum, the combined effects of prenatal LPS exposure and postnatal 6OHDA treatment did not lead to greater than expected THir cell loss. The interaction term for THir cell loss and examination of the group means argued that the combined effects of the two toxins were less than additive. This might suggest that prenatal LPS exposure conferred partial resistance to subsequent 6OHDA exposure. Thus, DA neurons within the mesencephalon remaining after prenatal LPS exposure might be less susceptible to 6OHDA, or represent a different subtype of DA neuron that is more resistant to this neurotoxin (e.g. calbindin ir; Rodriguez, 2003). However, although the combined effects of LPS and 6OHDA on DA cell counts were less than additive, the effects on DA and TNFα were additive, and the effects on DA activity were synergistic. Thus, further studies on the interactions between prenatal LPS and postnatal toxin exposure are warranted in order to adequately characterize the true nature of multiple toxin exposures. These should include time-course studies as well as studies in older animals, both of which could influence the nature of the interaction. Regardless, the data clearly show that animals born with fewer than normal DA neurons will exhibit further loss of DA neurons following exposure to a second toxin like 6OHDA in adult life. This suggests that animals born with fewer than normal DA neurons are at greater risk for compromised DA function in response to subsequent neurotoxin exposure. This is consistent with the often considered notion that several factors might contribute to PD (multifactorial), but that cell loss sufficient to produce PD may require multiple exposures ("multiple hit" concept; Di Monte et al., 2002). Therefore, whether the effect of multiple exposures to environmental toxins is additive or synergistic, the end result is greater compromise to DA function and subsequent increased risk for PD. A study by Gao and colleagues (2003) demonstrated that combining the effects of LPS and the DA neurotoxin rotenone produced synergistic DA neuron loss in tissue culture. They also demonstrated that whether or not an additive or synergistic

effect was produced, was a consequence of dosage and duration of treatment with optimal synergistic effects being seen at low dosages for longer durations (Gao et al., 2003). Thus it is possible that had the animals been exposed to a lower dosage of 6OHDA or had the kill interval been extended longer than three weeks, a synergism may have been seen.

Interestingly, the cell body sizes of the DA neurons in the combined treatment group were significantly enlarged relative to all other treatment groups. We interpret this increase to indicate compensatory increases in neuronal activity and function of remaining neurons, since we have also seen it previously in response to DA depletion-induced increased trophic activity (Vu et al., 2000). If so, then had a larger dosage of 6OHDA been used, or had exposure occurred in older animals where compensatory capacity is attenuated (Ling et al., 2000), a more profound cell loss might have occurred. Regardless, the data clearly shows that animals born with fewer than normal DA neurons will exhibit further loss of DA neurons following exposure to a second toxin like 6OHDA in adult life.

Effects of pre- and postnatal toxin exposures on striatal TNFα levels

The striatal TNFα levels were not increased statistically in the group treated with 6OHDA alone. Previous studies (Mogi et al., 1999, 2000) showed that 6OHDA led to increased levels of striatal TNFα. Failure to detect changes in TNFα in response to 6OHDA in the present study could simply reflect differences in dosage and the time to kill between the two studies. Regardless, the combined effects of both prenatal LPS and postnatal 6OHDA did produce a dramatic 82% increase in TNFα within the striatum. Again, however, this increase was at best additive. Despite this lack of synergism, it is remarkable that a single injection of LPS into a gravid female at E10.5 produced increases in TNFα in her offspring that lasted 132 days. If this increase were to continue into advanced age, it is possible that the combined effects of reduced compensatory activity associated with aging (Yurek and Fletcher-Turner, 2001; Calne and Peppard, 1987) together with the reduced anti-inflammatory environment seen in the aged brain (Saurwein-Teissl et al., 2000) might then combine to produce synergistic DA cell losses.

Body temperature of LPS-exposed gravid females

The mechanism(s) responsible for the LPS induced DA cell loss are unknown at this time, although the data from the P21 animals (Ling et al., 2002b) suggested that the DA neuron loss seen was likely not a consequence of alterations of programmed cell death (apoptotic culling) during development. However, since LPS is a well known inducer of hyperthermia, it was possible that the DA neuron loss seen in the animals was a consequence of hyperthermia, which is a known generalized teratogen (Edwards, 2003) and known to alter neurotransmitters systems (Colado et al., 1999) including DA (Albers and Sonsalla, 1995). However, the results indicated only transient body temperature fluctuations in some LPS-treated gravid dams that were short-lived and not statistically significant. The inability of LPS to alter body temperature was not a function of gender or gravidity, since neither male rats nor non-gravid females, respectively, exhibited alterations in rectal temperature in response to LPS. The failure of LPS to induce the expected increases in body temperature has been described previously (Fraifeld and Kaplanski, 1998), and was likely a consequence of the very low dosage used in the present study relative to those typically used to produce hyperthermia. Other factors associated with the LPS-induced DA neuron loss that are currently being considered are alterations in the DA neuron progenitor cell population and alterations in fetal TNFα transcription in glia within the developing brain. Regardless, prenatal LPS does appear to produce long-lived reductions in DA cell counts in the mesencephalon coupled with DA biochemical alterations and increases in TNFα that are similar to those seen in patients with PD.

Implications for PD

The current results suggest that prenatal LPS exposure may serve as a risk factor for PD. That prenatal LPS could be a potential risk factor for PD becomes more plausible given that a common condition of pregnancy, BV, leads to fetal LPS exposure. Approximately 14% of pregnant women have BV (Thorsen et al., 1998). Numerous studies have consistently shown that BV leads to increased levels of IL-1α/β, IL-6, and TNF-α in the chorioamniotic environment (Dammann and Leviton, 1997; Purwar et al., 2001; Haefner, 1999; Romero et al., 1989). Moreover BV is known to affect the CNS and has been associated with white matter damage (periventricular leukomalacia), intraventricular hemorrhage, and cerebral palsy (Dammann and Leviton, 1997, 1998; Dammann et al., 2002; Yoon et al., 1997). Thus, it is likely that the brains of many patients who reach adult age will have been exposed to LPS prenatally, and the results from the present study suggest that even mild exposure can disrupt normal DA neuron development when that exposure occurs just prior to the development of the DA neuron. Although it is intriguing to speculate that such patients might exhibit progressive loss of DA neurons which could lead to PD in advanced age, it seems more likely that such individuals would be at increased risk to the DA-depleting effects of DA neurotoxin exposure encountered in later life. This would support the notion that PD is a consequence of exposure to multiple environmental toxins, and further suggests that prenatal toxin exposure should be considered in the pathogenesis of PD. If prenatal LPS exposure is taken into consideration, it could then be argued that the adult toxin dosages needed to produce clinically significant DA depletion in later life might not need to be as large as previously thought, elevating the potential role of environmental DA toxins in the etiopathogenesis of PD.

Acknowledgements—This work was supported by ES10776, NS045316, ES012307, DAMD 17-01-1-0771, The Michael J. Fox Foundation, and the American Parkinson's Disease Association.

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(Accepted 15 December 2003)

Available online at www.sciencedirect.com

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Experimental Neurology 190 (2004) 373–383

Experimental Neurology

www.elsevier.com/locate/yexnr

Rotenone potentiates dopamine neuron loss in animals exposed to lipopolysaccharide prenatally

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Received 3 March 2004; revised 26 July 2004; accepted 12 August 2004
Available online 30 September 2004

Abstract

We previously demonstrated that treating gravid female rats with the bacteriotoxin lipopolysaccharide (LPS) led to the birth of offspring with fewer than normal dopamine (DA) neurons. This DA neuron loss was long-lived and associated with permanent increases in the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα). Because of this pro-inflammatory state, we hypothesized that these animals would be more susceptible to subsequent exposure of DA neurotoxins. We tested this hypothesis by treating female Sprague–Dawley rats exposed to LPS or saline prenatally with a subtoxic dose of the DA neurotoxin rotenone (1.25 mg/kg per day) or vehicle for 14 days when they were 16 months old. After another 14 days, the animals were sacrificed. Tyrosine hydroxylase-immunoreactive (THir) cell counts were used as an index of DA neuron survival. Animals exposed to LPS prenatally or rotenone postnatally exhibited a 22% and 3%, respectively, decrease in THir cell counts relative to controls. The combined effects of prenatal LPS and postnatal rotenone exposure produced a synergistic 39% THir cell loss relative to controls. This loss was associated with decreased striatal DA and increased striatal DA activity ([HVA]/[DA]) and TNFα. Animals exposed to LPS prenatally exhibited a marked increase in the number of reactive microglia that was further increased by rotenone exposure. Prenatal LPS exposure also led to increased levels of oxidized proteins and the formation of α-Synuclein and eosin positive inclusions resembling Lewy bodies. These results suggest that exposure to low doses of an environmental neurotoxin like rotenone can produce synergistic DA neuron losses in animals with a preexisting pro-inflammatory state. This supports the notion that Parkinson's disease (PD) may be caused by multiple factors and the result of "multiple hits" from environmental toxins.

Keywords: Parkinson's diseases; LPS; Rotenone; Prenatal; Environment; Lewy body; Dopamine

Introduction

Environmental factors are thought to contribute to the development of nonfamilial Parkinson's disease (PD). Von Economo's pan encephalitis (Casals et al., 1998) and manganese (Zheng et al., 1998) are considered long-standing members of a growing list of risk factors, while more recent additions include pesticides and fungicides such as dieldrin (Corrigan et al., 2000) and maneb (Thiruchelvam et al., 2000), respectively (see Adams and Odunze, 1991; Dawson et al., 1995; Di Monte, 2003; for reviews). That environmental factors serve as risk factors for PD was formalized by Calne and Langston (1983). They proposed that exposure to an environmental toxin such as 1-methyl, 4-phenyl, 1,2,3,6 tetrahydropyridine (MPTP) killed a portion of the DA neurons; but not enough cells were lost to produce PD. However, additional age-related DA neuron losses eventually produced sufficient cell losses to cause clinical symptoms. Unfortunately, the vast majority of PD patients cannot identify a specific exposure in their past that was sufficient enough to produce their disease. Although this might suggest that environmental factors are not responsible, it might also

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doi:10.1016/j.expneurol.2004.08.006

imply the involvement of low doses of toxins acting synergistically. This latter possibility is consistent with the emerging hypothesis that the cause of PD is multifactorial and a result of interacting "multiple hits" involving different toxins (Di Monte, 2003) or gene–toxin interactions (Greenamyre et al., 2003; Le Couteur et al., 2002).

The multiple hit hypothesis is attractive because it raises the possibility of synergistic interactions. This would imply that toxin exposure levels could be low or sporadic, and therefore difficult to detect epidemiologically. It has been widely assumed that these exposures occurred during adult life. However, we previously demonstrated that exposure to a low dose of the bacteriotoxin lipopolysaccharide (LPS) during a critical window of vulnerability during fetal development led to the birth of rat pups with fewer than normal DA neurons (Ling et al., 2002a,b). This suggests toxin exposure relevant to PD can also occur in utero. Such exposure frequently occurs in humans as a result of bacterial vaginosis (Dammann and Leviton, 1998; Paige et al., 1998), which is known to complicate approximately 14% of normal births (Thorsen et al., 1998). Interestingly, animals born following prenatal exposure to LPS have long-lived elevations in the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα). This is potentially significant because TNFα can kill DA neurons (McGuire et al., 2001), is increased by several DA neurotoxins (Hornykiewicz and Kish, 1987; Nagatsu et al., 2000), and is elevated in the brains of patients with PD (Boka et al., 1994; Mogi et al., 2000a). If animals exposed to LPS prenatally were exposed as adults to a neurotoxin that increased TNFα or other pro-inflammatory factors, synergistic DA neuron loss might occur.

We previously tested this hypothesis in vivo. Four-month-old male rats exposed to LPS prenatally received a moderate dose of the DA neurotoxin 6-hydroxydopamine (6OHDA; icv) (Ling et al., 2004). Both prenatal LPS exposure and postnatal 6OHDA produced significant DA neuron losses, although the combined effects of both factors were, at best, additive. Although the results supported the hypothesis that "multiple hits" to the DA system can combine to produce significant DA neuron loss, synergism was not seen. The young age of animals and the toxin used were discussed as possible reasons why synergistic cell losses were not seen. In the present study, we evaluated a different DA neurotoxin, the complex I inhibitor rotenone (Fiskum et al., 2003; Sherer et al., 2003), and evaluated it in aged female rats (17 months old) to explore the possibility of synergistic cell loss further. Rotenone is a pesticide that is found naturally in the environment and has been distributed even more widely by man (Gosalvez, 1983; Soloway, 1976). It selectively kills DA neurons in rats (Betarbet et al., 2000, 2002). Here we show that synergistic DA neuron losses do result from exposure to rotenone in animals treated with LPS prenatally.

Materials and methods

Animals

Thirty-six timed-gravid, Sprague–Dawley (Zivic-Miller, Allison Park, PA) female rats were delivered to Rush's animal facility at gestational day nine ± 12 h. All animals and their offspring were allowed access to food and water ad libitum and were maintained in an environmentally regulated animal facility for the duration of the study (lights on 0600–1800). The survival surgery was carried out under ketamine and xylazine anesthesia to minimize pain. The protocols and procedures used in these studies were approved by the Institutional Animal Care and Utilization Committee (IACUC) of Rush University.

Prenatal treatments and postnatal groupings

LPS was purchased from Sigma (St. Louis) and dissolved in saline (Hank's balanced salt solution (HBSS)) at 10,000 endotoxin units (EU)/ml. At embryonic day (E) 10.5, each gravid female received a single injection (intraperitoneal) of either 10,000 EU/kg LPS or HBSS (1 ml/kg; $n = 18$ for each group). This very low dose of LPS was chosen based on prior studies where it was shown to be well tolerated, did not produce fetal demise (Ling et al., 2000), and did not increase the gravid female's body temperature (Ling et al., 2004). All the animals were monitored daily for obtundation or other signs of distress, and were allowed to deliver their pups normally. The pups were weaned at P21 and housed two per cage with same-sex partners.

The offsprings from these gravid females were used in several studies. In the present study, 32 female offsprings were used. Fourteen of the 32 animals were from seven different litters that had been exposed to saline (Sal) prenatally and litter pairs were randomly assigned to one of two postnatal treatment groups. One group received rotenone (Rot) infusion (Sal/Rot; $n = 7$) and the other group received vehicle (Veh) infusion (Sal/Veh; $n = 7$). Fourteen animals from seven different litters that had been exposed to LPS prenatally were similarly randomly assigned to the two postnatal treatment groups (LPS/Rot; $n = 7$) or vehicle infusions (LPS/Veh; $n = 7$). Additional two female littermates from each prenatal treatment group were sacrificed and assessed using OxyBlot analysis when they were 16 months old.

Intrajugular rotenone infusion

Rotenone (Sigma) was dissolved in a dimethyl sulphoxide and polyethylene glycol vehicle [DMSO/PEG (1:1)]. Jugular cannulation (right side in all animals) was performed as described previously (Betarbet et al., 2000) under ketamine (100 mg/kg) and xylazine (5 mg/kg) anesthesia. The cannulae were attached to Alzet osmotic

minipumps (2ML1, Alzet Osmotic Pumps, Cupertino, CA) implanted subcutaneously in the napes of the neck. The pumps were designed to deliver 1.25 mg rotenone/kg per day or vehicle for 14 days. At the end of 14 days, the pumps were removed and the skin was sutured. Animals were allowed to recover for an additional 14 days to provide time for development of a stable lesion. After 14 days they were injected with pentobarbital (65 mg/kg; Sigma). Once fully anesthetized, the abdomens were opened, the chests invaded, and the brains perfused with ice-cold saline following insertion of a standard feeding tube (attached to a saline reservoir) into the left common carotid with the vena cava and aorta clamped. Following perfusion, the brains were removed and frozen immediately by immersion in dry ice-cooled 2-methylbutane. Once frozen, the frontal part of the brain (containing the striatum) was cut off and passed to enzyme-linked immunosorbent assay (ELISA) and DA biochemistry. The posterior part of brain (containing the mesencephalon) was immediately submerged in Zamboni's fixative (7.5% saturated picric acid, 12 mM NaH_2PO_4, 88 mM Na_2HPO_4, and 4% paraformaldehyde) for stereology studies. Animals destined for assessment using OxyBlot were sacrificed using the same protocol and perfused. However, the entire brain was frozen.

Tyrosine hydroxylase and microglia immunohistochemistry

Tyrosine hydroxylase (TH) is the rate-limiting, DA-producing enzyme in DA neurons and is widely used as an index of DA neurons (Fishell and Van Der, 1989). The number of TH immunoreactive (THir) cells was assessed using a stereological procedure described in detail elsewhere (Ma et al., 1997; Vu et al., 2000). Briefly, the entire mesencephalon was sliced on a cryostat into consecutive 40-μm sections. Every sixth section was immunohistochemically processed for TH using mouse anti-rat TH antibody overnight (Immunostar, Stillwater MN; 1:20,000). The endogenous peroxidase activity was eliminated using a 1-h incubation with 0.1% periodate. The immunohistochemical procedure was continued by using a biotinylated horse anti-mouse IgG (0.5%; Vector Laboratories, Burlingame CA) for 1 h and peroxidase conjugated avidin–biotin complex (Vector Laboratories) for 1 h. The chromogen solution used to complete the reaction consisted of 0.05% 3,3′-diaminobenzidine (DAB), 0.5% nickel sulfate, and 0.003% H_2O_2 in I/A solution (10 mM imidazole/50 mM sodium acetate) to obtain a black stain. The sections were washed, mounted on gelatin-coated slides, dehydrated through graded alcohols, cleared in xylenes, and cover-slipped with Permount.

Reactive microglia were revealed using the major histocompatibility complex class II (MHC II) immunoreactivity marker (Ox-6) (Ng and Ling, 1997; Shigematsu et al., 1992). MHC II immunohistochemistry was performed using a primary antibody purchased from NOVUS Biologicals (Littleton, CO). Mesencephalic sections were immunostained overnight using Ox-6 (diluted 1:5,000). The sections were then processed using the secondary antibody as described above for TH.

Stereology

The estimation of the total number of THir neurons in the SN was determined using the computerized optical dissector method, which allows for the stereological estimation of THir cells in the entire structure independent of size, shape, orientation, tissue shrinkage, or anatomical level using MicroBrightField software (Ling et al., 2004). The antibody penetration throughout the whole tissue section was assessed by dissectors using an imaging capture technique (Kordower, 1996; Ma et al., 1999). The total number (N) of SN neurons was calculated using the formula $N = N_V \cdot V_{SN}$, where N_V is the numerical density and V_{SN} is the volume of the SN, as determined by Cavalieri's principle. The MicroBrightField software was also used to determine the total volume of the SN (in mm^3), the density of the cells in that volume (cell counts/mm^3), and the cell body size (μm^2).

The SN was segregated from the ventral tegmental area for stereology as described previously (Ling et al., 2004). Briefly, the cells lateral to the accessory optic track were designated as SN, while those medial to the track were considered part of the ventral tegmental area (see Fig. 2).

Biochemistry

DA and its metabolite homovanillic acid (HVA) were measured using HPLC (Lipton et al., 2002) with electrochemical detection in pooled (left and right) tissue punches taken from the center of each striatum as previously described (Vu et al., 2000). DA and HVA were expressed as ng/mg tissue protein (assessed using the Bio-Rad Protein Assay Kit) and the ratio of [HVA]/[DA] was used as an index of DA activity.

Cytokine assessments using ELISA

The cytokine levels were determined as previously described (Ling et al., 2000). Briefly, pooled tissue punches from the left and right striata were submerged in 200 μl homogenate buffer (Trizma/HCl pH 7.2, 0.1 M, sodium chloride 0.9%, Aprotinin 2%, leupeptin 0.5 μg/ml, pepstatin 0.7 μg/ml, and PMSF 200 μM; all reagents were purchased from Sigma) and homogenized. To each tube, 200-μl homogenate buffer containing 0.5% NP-40 (Sigma) was then added. The tubes were shaken, centrifuged (10,000 × g), and the supernatants collected. The levels of TNFα were assessed in each supernatant by an ELISA that was run according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The plates were read in a Dynatech ELISA plate reader (Dynatech Laboratories, Chanlity, VA) and the concentrations of the cytokines were determined

against a six-point standard curve. The total protein content of each homogenate was determined using the Bio-Rad Protein Assay kit and the quantity of TNFα was expressed as pg/mg total protein.

OxyBlot

Four females, two exposed to LPS prenatally and two exposed to saline, were left in the colony after assigning the other animals to their respective treatment groups. Even though we did not have sufficient animals to perform a formal study, we evaluated the relative levels of oxidized protein in the striata and mesencephalons of these animals using the Oxyblot technique. The OxyBlot technique assesses total protein oxidation and was performed according to the manufacturer's instructions (Intergen, Purchase, NY). Briefly, the striata and mesencephalons from each animal were dissected out and homogenized in lysis buffer containing NP-40 detergent, 2-Me, and proteinase inhibitors. The homogenate was adjusted to 2 μg/μl of protein concentration. Protein was derivatized in a 2,4-dinitriphenylhydrazine (DNPH) solution or control solution for 20 min and neutralized. The samples were loaded onto a 12% polyacrylamide gel. Upon completion of electrophoresis, the protein was blotted overnight onto a piece of nitrocellulose membrane. The membranes then were blocked with a 2% fat-free dry milk/TBS solution followed by sequential incubations with primary and secondary antibodies that were conjugated with horseradish peroxidase enzyme. The protein bands were visualized in a dark room following exposure of X-ray films to the membranes incubated with chemiluminescent reagent (Pharmigen).

α-Synuclein immunohistochemistry and H and E staining

The protocol used for α-Synuclein immunohistochemistry was the same as that used for THir immunohistochemistry except anti-α-Synuclein (1:1,000; Pharmingen) was used as a primary antibody and the DAB was not nickel enhanced. For hematoxylin and eosin (H and E; Sigma) staining, tissue sections were mounted onto coated slides and sequentially stained in hematoxylin and eosin according to the manufacturer's protocol (Sigma). All sections were covered with Permount and cover slips.

Statistics

All data were expressed as the mean ± SD. Initial examination of the data revealed that the variances across groups were not homogeneous and therefore the raw data were transformed using a log scale after which homogeneity was achieved. The data were then analyzed using two-way ANOVA with prenatal treatment (LPS vs. HBSS) and postnatal treatment (Rot vs. Veh) as factors. Individual differences between treatment groups were determined using Tukey's HSD post hoc test following one-way ANOVA with significance accepted if $P < 0.05$. Because the biochemical and cell count data were gathered from the same brains, it was possible to determine the relationship, if any, between these two variables. Pearson correlation of striatal DA and THir cell count data was assessed using two-tailed test.

Results

Animals

Overall, the animals tolerated the LPS treatments without difficulty. At birth, the female pups were all full-term and no differences in birth weights were detected (data not shown). The animals developed normally and did not exhibit overt behavioral effects. We had performed a study similar to the current experiment except that 6-month-old males were used and 2.5 mg rotenone/kg per day was used. Unfortunately, at the dosage used, the animals became very sick, several died, and the experiment had to be terminated early. We tested additional animals and eventually chose a dosage of 1.25 mg/kg per day. Overall, the animals tolerated this dosage reasonably well, although one Sal/Veh and one LPS/Rot animal became sick immediately after surgery and another two animals became sick after 10 days and were dropped from the study. The remaining animals (Sal/Veh, $n = 5$; Sal/Rot, $n = 6$; LPS/Veh, $n = 7$; and LPS/Rot, $n = 6$) survived the protocol with no adverse effects or no overt behavioral changes until sacrifice (28 days after surgery) when they were 17 months old. At that time there were no significant differences in their body weights ($F(1,23) = 0.052$).

SN THir cell counts

Overall, the effects of prenatal LPS and postnatal rotenone treatment had a profound and highly significant effect on THir cell counts ($F(3,23) = 15.04$; $P < 0.001$). Prenatal LPS exposure produced a significant reduction in THir cell counts ($F(1,23) = 34.842$; $P < 0.0001$) reducing the average THir cell counts by 22% (Fig. 1; Table 1). In contrast, postnatal rotenone treatment did not statistically alter THir cell counts (3% decrease; $F(1,23) = 3.005$; $P = 0.098$). However, the combined effects of prenatal exposure to LPS together with postnatal exposure to rotenone produced a further, dramatic significant THir cell loss of 39%. The interaction term between the pre- and postnatal treatments was significant ($F(1,23) = 5.717$; $P < 0.05$), suggesting that the effects of LPS and rotenone were synergistic. In addition to alterations in cell counts, the prenatal and postnatal treatments had a similar, statistically significant effect on the density of THir cells (Table 1), but had no significant effects on the volume of the nigra or the size of the individual THir cell bodies.

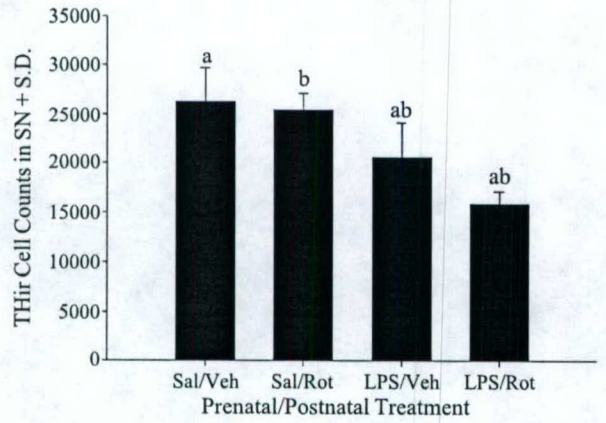

Fig. 1. Tyrosine hydroxylase-immunoreactive (THir) cell counts in the substantia nigra (SN). Stereological cell counts were gathered from animals exposed to lipopolysaccharide (LPS) or Hank's balanced salt solution (Sal) prenatally at E10.5. When they were 16 months old, they were infused for 14 days with rotenone (Rot) or vehicle (Veh). (Bars that share a common letter are significantly different from one another ($P < 0.05$).)

As we have reported in previous studies, the THir cell loss appeared to be primarily in the lateral regions of the substantia nigra and its ventral tier (Fig. 2). This cell loss pattern was similar in animals exposed only to prenatal LPS and those exposed to both prenatal LPS and postnatal rotenone. Interestingly, the apparent intensity of the TH immunoreactivity in the animals treated only with rotenone was decreased, although few cells were lost. This might suggest that rotenone treatment reduced expression of TH without killing the cells. No apparent loss of cells was seen in the ventral tegmental area in any of the treatment groups (data not shown).

Striatal biochemistry

Like the cell count data, the overall effect of treatments had a significant effect on DA levels in the striatum ($F(3,23) = 10.196$; $P < 0.001$). However, unlike the cell count data, two-way ANOVA revealed that both factors (prenatal LPS; $F(1,23) = 8.78$; $P < 0.01$; postnatal Rot exposure; $F(1,23) = 16.76$; $P < 0.001$) significantly reduced striatal DA levels (Fig. 3). Prenatal LPS reduced striatal DA by 12% from that seen in the Sal/Veh group (204.63 ng/mg), while rotenone reduced levels by 22%. Post hoc analysis revealed that these two treatment groups were not significantly different from controls. However, the combined effects of both treatments reduced levels to 47% of normal. Yet, despite the magnitude of this change, the two-way ANOVA interaction term was not statistically significant ($F(1,23) = 3.309$), suggesting that the change in DA was not synergistic. Regardless, the striatal DA levels were highly correlated with the SN THir cell counts ($r^2 = 0.855$; $P < 0.001$), verifying the relationship between striatal DA loss and reductions in THir cell counts in the SN.

Striatal HVA levels were not statistically different among the four groups ($F(1,23) = 2.203$; data not shown). As expected, as THir cells decreased, DA activity increased proportionately (Fig. 3) and the overall effect on DA activity was highly significant ($F(3,23) = 9.633$; $P < 0.001$). As was true with DA, both prenatal ($F(1,23) = 20.281$; $P < 0.001$) and postnatal treatments ($F(1,23) = 7.034$; $P < 0.05$) significantly increased DA activity, although again, the interaction term was not statistically significant. Post hoc analysis of the ratio of [HVA]/[DA] revealed that the 67.5% increase seen in the LPS/Veh group was significantly elevated relative to that seen in the Sal/Veh group. Although the 40% increase in DA activity seen in postnatal rotenone animals was not statistically significant, the combined effects of both treatments were significantly increased by 85%. Interestingly, the 0.04 DA activity ratio seen in the control females (Sal/Veh) is significantly lower than that normally seen in males, which generally runs around 0.09. Thus, in our previous studies using 4-month-old males, the DA activity ratio was 0.099 (Ling et al., 2004). Because the DA levels in females (204.63 ng/mg protein) were similar to those seen in 4-month-old males (211.51 ng/mg protein), the reduction in DA activity was primarily a consequence of reduced HVA.

Striatal TNFα

Overall, the combined effects of prenatal LPS and postnatal Rot produced significant alterations in striatal TNF levels ($F(3,23) = 19.17$; $P < 0.0001$; Fig. 3). However, only the prenatal effects reached statistical significance ($F(1,23) = 51.02$; $P < 0.001$), and while the effects of Rot on TNFα levels were minimal and not statistically significant ($F(1,23) = 3.459$; $P = 0.075$), the combined effects of pre- and postnatal treatments were 91% increase.

Reactive microglia

Reactive microglia were recognized using the OX-6 monoclonal antibody. Microscopic examination of the brain

Table 1
The effect of prenatal LPS and postnatal rotenone on stereological measures in the substantia nigra

Group	Cell count	Density (cells/mm^3)	Volume (mm^3)	Size (μm^3)
Sal/Veh	26,202.64 ± 3501.01^a	10,666.27 ± 1659.55^a	2.47 ± 0.12	157.04 ± 11.16
Sal/Rot	25,394.31 ± 1677.72^b	10,177.67 ± 837.86^b	2.50 ± 0.15	184.32 ± 17.50
LPS/Veh	20,527.72 ± 3613.72^{ab}	8347.35 ± 1267.30^{ab}	2.45 ± 0.13	170.54 ± 17.49
LPS/Rot	15,901.04 ± 1286.86^{ab}	6307.88 ± 452.97^{ab}	2.53 ± 0.20	177.08 ± 22.87

Values that share the same letter are significantly different from one another at the $P < 0.05$ level.

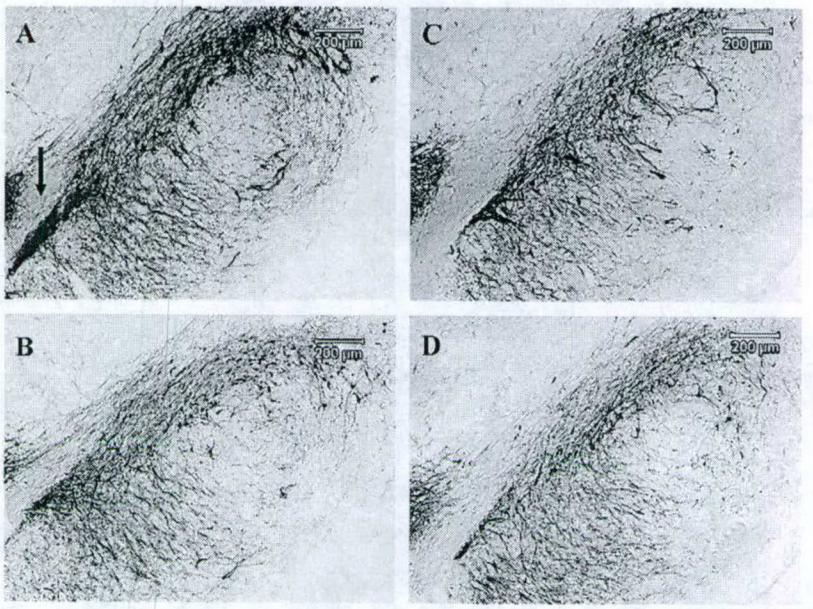

Fig. 2. Photomicrographs of TH immunocytochemistry from the right SN of the four treatment groups. (A) Sal/Veh; (B) Sal/Rot; (C) LPS/Veh; (D) LPS/Rot. Note the reduced intensity of immunostaining in the B and D sections due to exposure to postnatal rotenone infusion. The arrow in A identifies the accessory optic track that defined the medial demarcation point separating the SN from the ventral tegmental area. Scale bar = 200 μm.

sections from animals exposed to saline prenatally and sham treatment postnatally revealed an occasional OX-6ir cell such that in most sections no activated microglia could be seen (Fig. 4). In contrast, the numbers of microglia were markedly elevated in animals exposed to LPS prenatally. These cells were rod shape and possessed numerous reactive processes indicative of activated microglia. Although the numbers of OX-6ir cells were more common than controls throughout the LPS brains, their numbers were markedly elevated in the SN, and to a lesser extent, in the striatum. A similar pattern was seen in saline animals exposed postnatally to rotenone, although the magnitude of increase was not as profound. However, in animals exposed to LPS prenatally and rotenone postnatally, the number of OX-6ir cells was so markedly increased in the SN that it was visible on gross inspection of the sections (Fig. 4). Again, although

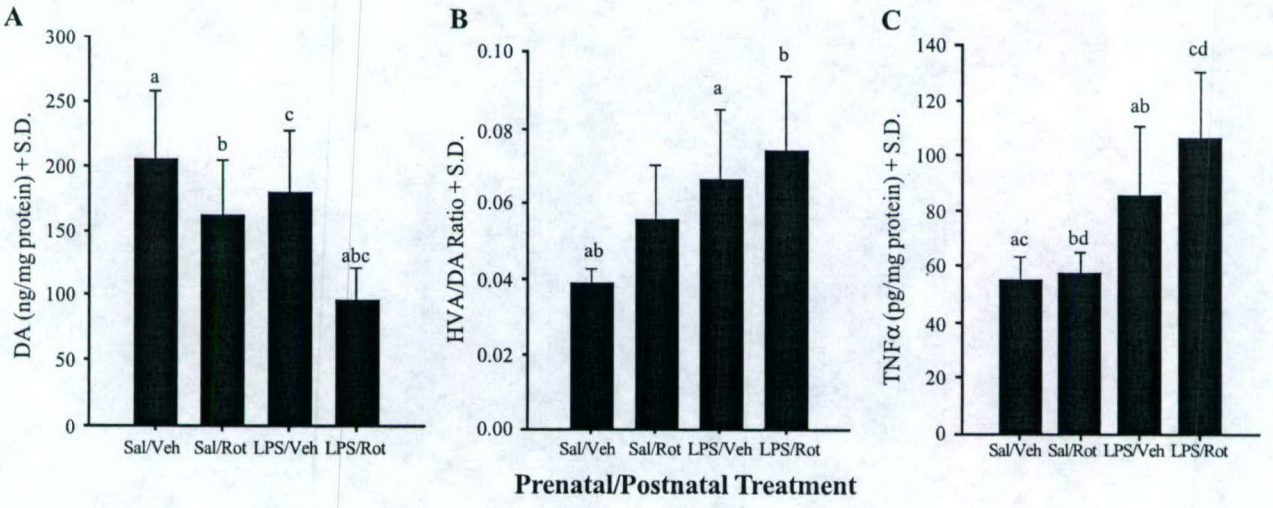

Fig. 3. Dopamine (DA), DA activity, and tumor necrosis factor alpha (TNFα) levels in the striata of the four treatment groups. Animals were exposed to lipopolysaccharide (LPS) or saline (Sal) prenatally and rotenone (Rot) or vehicle (Veh) when they were 16 months old. Striatal DA (A) and DA activity ([HVA]/[DA]) (B) were assessed using HPLC. Striatal TNFα (C) was assessed using ELISA. (Bars that share the same letter are statistically different from one another ($P < 0.05$).)

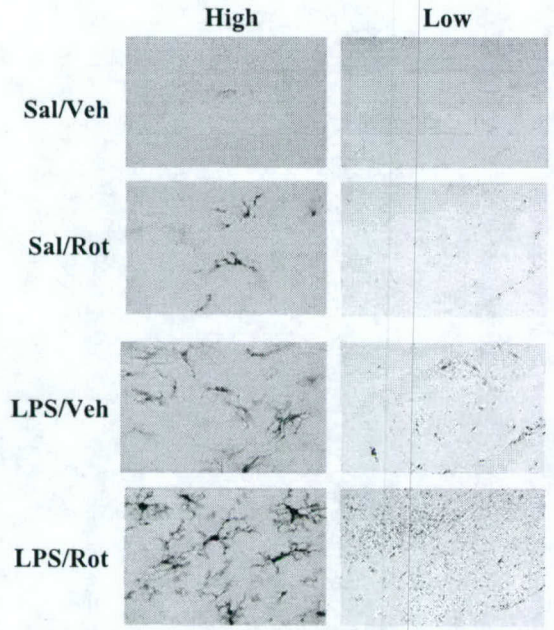

Fig. 4. OX-6-immunoreactive cells in the substantia nigra histochemistry. This photomicrograph represents typical sections in the SN of the four treatment groups. Low: low power magnification (×10); High: high power magnification (×100). Sal/Veh: prenatal saline exposure and postnatal vehicle infusion; Sal/Rot: prenatal saline exposure and postnatal rotenone infusion; LPS/Veh: prenatal LPS exposure and postnatal vehicle infusion; LPS/Rot: prenatal LPS exposure and postnatal rotenone infusion. Note that in LPS-exposed animals treated with rotenone, the number of reactive microglia was so pronounced they were overtly visible upon gross inspection.

OX-6ir cells were found throughout the brain in these animals, the density was profoundly elevated in the SN with significant increases also seen in the striatum.

OxyBlot

Two separate sets of animals were assessed using the OxyBlot technique to evaluate the overall oxidation status of animals exposed to LPS or HBSS prenatally just before rotenone treatment. The idea here was that the level of oxidized protein in the 16-month-old animals would be indicative of the overall accumulated protein damage arising from oxidant stress. Overall, there was a dramatic increase in the density of, and number of, protein bands in both the mesencephalons and the striata of the females exposed to LPS prenatally compared with controls (Fig. 5). This suggested that in both structures, the accumulated level of oxidant stress was such that it led to extensive protein oxidation.

α-Synuclein and H and E-positive inclusions

α-Synuclein immunostains of the tissue sections revealed a diffuse, mottled background staining with occasional deep-brown, rounded immunopositive intracellular objects in animals prenatally exposed to LPS (Fig. 6B). These α-Synuclein-ir objects were similarly present in animals exposed to LPS prenatally and rotenone postnatally. However, these objects were not seen in any of the control animals, although the mottled background staining typical of the treated animals was seen (Fig. 6A).

The H and E sections revealed numerous eosin-positive inclusions in animals exposed to LPS prenatally (Figs. 6D–F). These inclusions were seen in numerous brain regions including cortex, hippocampus, and SN. However, inspection of the sections revealed that the inclusions were more abundant in the SN than in other regions. Interestingly, their distribution appeared to parallel that of microglia. Many of these inclusions were clearly located intracellulary (Fig. 6F). They were smaller than typical Lewy bodies seen in human brain, and did not have the traditional halo often seen around Lewy bodies. Animals exposed to prenatal LPS and postnatal rotenone infusion also exhibited these Lewy-like bodies. Interestingly, the number of inclusion bodies was not noticeably increased in any of brains of animals exposed to both toxins. These inclusions were not seen in any of the control animals.

Discussion

Results from the present study showed that exposure to rotenone, at a dose that failed to alter THir cell counts and had minimal effects on striatal DA in control animals, produced a synergistic loss of THir cells in animals exposed to LPS prenatally. This loss was accompanied by and was correlated with striatal DA losses. The combination of both treatments also increased DA activity and TNFα. This study extends those reported previously in that the DA cell losses and increases in TNFα seen in young animals perinatally (P10 and 21; Ling et al., 2000, 2004) and in young adults (P120; Ling et al., 2004) are still present in aged adults (P510). In addition, the effects of prenatal LPS are not

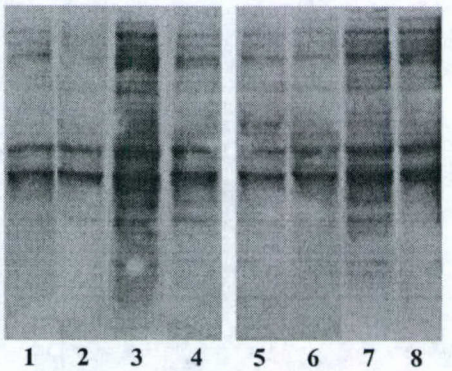

Fig. 5. Chemiluminescence patterns of oxidized protein in animals exposed to Sal (1–2; 5–6) or LPS (3–4; 7–8) prenatally. Gels were loaded with equal amounts of protein from the striata (lanes 1–4) and SN (lanes 5–8), and then exposed to X-ray film. Striata and SNs from animals exposed to LPS prenatally have more oxidized proteins as indicated by the apparent darker bands.

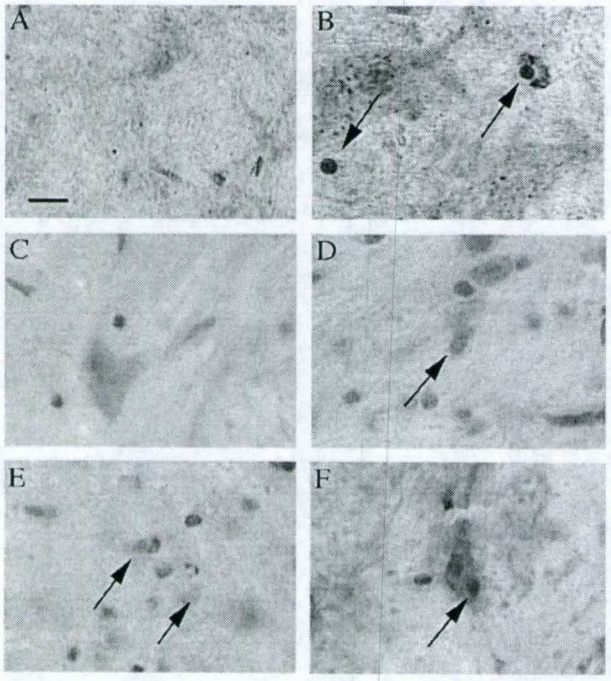

Fig. 6. The α-Synuclein (A and B) and H and E stained sections (C–F) of the SN. α-Synuclein immunoreactivity produced a mottled background staining in control sections (A). In animals exposed to LPS prenatally, deep-brown, spheroid α-Synuclein inclusions were often seen (arrows in B). Hematoxylin and eosin (H and E) processed sections revealed typical backgrounds of reddish-gray interrupted by bluish-black nuclei typical of normal tissue (C). In animals exposed to LPS prenatally (D and F) or LPS and rotenone (E), numerous reddish inclusions were detected (arrows in D and E) and were sometimes seen inside of cells (arrow in F) (scale bar = 5 μm).

restricted to males, but can be seen in females as well. Furthermore, prenatal LPS exposure was associated with increases in oxidized proteins in both the striatum and mesencephalon, significant increases in microglia activation, and the formation of Lewy-like bodies.

We previously examined animals exposed to LPS prenatally for possible synergistic cell losses following icv 6OHDA treatment. However, in that study, the THir cell loss seen was additive. We suggested that the failure to produce synergistic toxicity was, in part, a consequence of the young age of the animals studied. The present results support this contention. A recent study by Thiruchelvam et al. (2003) further supports this argument. Thus, they showed that the combined neurotoxicity of paraquat and maneb produced greater DA neuron losses in aged mice than in young animals. They also showed that this synergistic toxicity was associated with an attenuated compensatory response to DA neuron loss (i.e., increased DA activity). We have shown that several compensatory responses to DA cell loss including increased DA activity and trophic factor production are reduced in aged animals (Ling et al., 2000). Moreover, pro-inflammatory cytokines such as interleukin-1 and TNFα increase in the aged brain (Kyrkanides et al., 2001; Terao et al., 2002). Indeed, Kalehua et al. (2000) demonstrated that the pro-inflammatory response of the aged brain to LPS was greater than in young animals. Thus, it is possible that a synergistic response was seen at 17 months, but not after 4 months, because the aged brain is more sensitive to toxins due to reduced compensation coupled with an increased pro-inflammatory response.

It is also possible that differences in toxicity were a consequence of the toxin used. Thus, 6OHDA might not be capable of producing a synergistic response regardless of the age of the animals it is used in, and studies are currently underway to assess this possibility. Another explanation involves dosage. Gao et al. (2003a,b) demonstrated in tissue cultures pretreated with LPS that synergistic THir cell loss was more readily produced when lower concentrations of MPTP or rotenone were used. Interestingly, low dosages of paraquat and maneb were used in studies by Thiruchelvam et al. (2000), and a low dosage of rotenone was used in the present study. Using lower dosages, preferably ones that do not produce overt toxicity, clearly allows greater room for additive and synergistic effects.

As part of the 6OHDA study, we stereologically assessed the loss of THir cells as well as other neurons in the mesencephalon. Those results clearly showed that prenatal LPS, as well as adult exposure to 6OHDA, produced actual DA cell loss and not simply a suppression of the DA neuron phenotype. In addition, the cell loss was almost exclusively in the lateral regions of the substantia nigra and its ventral tier, sparing the ventral tegmental area. This is significant because this pattern of cell loss mimics that seen in PD patients (Hornykiewicz and Kish, 1987; Javoy-Agid et al., 1981). Although not specifically assessed here, the pattern of cell loss was similar to that in the 4-month-old study, and we further assume that the decrease in THir cell counts seen here represents true DA cell loss.

The timing of exposures in the current study differs from the more traditional exposure paradigms where two toxins are often co-administered or administered close together during adulthood. Thus, exposure to the first toxin (LPS) occurred in utero while exposure to the second toxin (rotenone) occurred 17 months later. The prenatal LPS model used here assumes that some type of permanent alteration was produced by exposure to LPS in utero that rendered the animal more susceptible to a toxin exposure that occurred 17 months later. Thiruchelvam et al. (2002) showed a similar effect in mice. They exposed mice to paraquat or maneb perinatally and showed that the mice exhibited a greater than expected DA cell loss to a subsequent challenge to the same toxin during adulthood several months later. They referred to this as a "silent toxicity". Thus, in their study, as was true in the LPS animals studied here, early exposure did not produce an overtly affected animal, but nonetheless, an animal that exhibited synergistic DA losses following low dose toxin treatment during adulthood. The mechanism(s) responsible for the silent toxicity in the paraquat/maneb model, unfortunately, remains unknown. However, we are beginning to gain

insight into the potential mechanisms involved in the LPS model.

In the present model, prenatal LPS exposure increased DA activity that is known to produce significant free radical production and DA cell death (Jenner, 2003; Spina and Cohen, 1988). Thus, it is possible that the life-long increases in DA activity seen in animals exposed to LPS prenatally perpetuates a cycle of continuous free-radical production that was elevated even further by exposure to a low dose of rotenone. This was not only supported by the significant increases in DA activity seen in animals exposed to LPS alone, but by the increased levels of oxidized proteins seen in the striata and mesencephalons of animals exposed to LPS prenatally. However, it is hard to envision this type of feed-forward inflammatory process lasting for 17 months. An alternative hypothesis involves pro-inflammatory cytokines.

LPS is a known inflammogen and inducer of pro-inflammatory cytokines (Gayle et al., 2002; Paludan, 2000). It is thus possible that elevated levels of TNFα are responsible for, or at least involved with the DA neuron loss, because the levels of this pro-inflammatory cytokine remain elevated for life following prenatal LPS exposure. This hypothesis is particularly attractive because TNFα kills DA neurons (McGuire et al., 2001), is increased by several DA neurotoxins (Hebert et al., 2003; Nagatsu et al., 2000), and is elevated in the brains of patients with PD (Boka et al., 1994; Mogi et al., 2000b). However, one again has to determine how such a phenomenon could occur for life. One potential mechanism involves epigenetic alterations in the genes responsible for TNFα production (Li et al., 2003; Sutherland and Costa, 2003). Thus, if LPSs were able to alter the methylation status of the TNFα promoter in utero, protein levels of TNFα would remain elevated for the life of the animal. Such an elevation would be particularly interesting if it were present in microglia given that their numbers were markedly elevated in LPS animals and that these cells are also the primary source of TNFα in brain (Kielian et al., 2002; Kim et al., 2000). Moreover, because rotenone also appeared to activate microglia, it is possible that microglial activation already present in animals exposed to LPS prenatally produced further increases in TNFα resulting in further DA neuron loss. We are currently exploring this possibility.

Regardless of the cause of the DA cell loss in the animals exposed to LPS prenatally and the synergism associated with subsequent exposure to rotenone, it is clear that the animals described in this study share many characteristics with patients with PD. We examined the sections to determine whether these toxins also induced formation of Lewy-like bodies seen in similar animal model (Betarbet et al., 2000). Alpha-Synuclein-immunoreactive inclusions were detected in the animals exposed to prenatal LPS or animals exposed to prenatal LPS and postnatal rotenone. Moreover, traditional H and E stains of the sections from the LPS animals also revealed the presence of inclusion bodies resembling Lewy bodies in LPS-exposed animals as well as the animals exposed to LPS and rotenone. Many of these inclusions were present in cell bodies, were found in several brain regions including cortex (although they were more prevalent in the SN), but lacked the halo traditionally seen in human Lewy bodies (Forno, 1996). Rotenone infusion alone was reported to be able to induce Lewy-like inclusion in rat (Betarbet et al., 2000). Therefore, a synergism of prenatal LPS and postnatal rotenone in the inclusion formation was originally predicted. A careful evaluation had demonstrated that this was not the case. The absence of such synergism was believed due to the lower dose of rotenone (1.25 mg/kg per day) and shorter infusion duration (14 days) being used in our current study compared to higher dose (2.5 mg/kg per day) and longer infusion duration (28 days) by other group (Betarbet et al., 2000). The presence of Lewy-like and other inclusion bodies like these was once considered very rare, but is becoming more common in a variety of animal models (Betarbet et al., 2000; Chapman et al., 2003; Fornai et al., 2004; Song et al., 2004). Regardless, they represent an important finding that further validates the animal model. The presence of alpha-Synuclein inclusions and Lewy-like bodies might suggest an abnormality in proteosomal processing (Chung et al., 2003) secondary to increased oxidant stress present in these animals (Jenner and Olanow, 1996). Regardless, the presence of alpha-Synuclein inclusions and Lewy-like bodies suggests that prenatal LPS produces numerous characteristics in animals that are similar to those seen in human PD patients.

Taken together, these studies are consistent with the notion that exposure to multiple DA neurotoxins can combine to produce synergistic DA cell loss supporting the idea that the etiology of PD may be multifactorial and a result of multiple "hits" from environmental toxins. From the perspective of the patient, the notion of synergistic cell losses makes it easier to understand how a cell loss of the magnitude needed to produce PD can occur from environmental events. A synergistic response between two or more toxins, especially resulting from low, subthreshold dosages, could produce the heterogeneous cell loss typical of PD, but not be sufficient enough to produce clinical symptoms. Such an event would be difficult to detect epidemiologically and likely not be recalled by the patient. Homogeneous age-related DA neuron losses could then bring the patient to the symptom threshold as originally proposed by Calne and Langston (1983). Alternatively, exposure to low doses of a second toxin as described in the present study could produce synergistic DA neuron loss that starts 4–7 years before actual symptom expression, and thereby produce the exponential pattern of DA neuron loss predicted by imaging studies of PD patients (Calne et al., 1997; Morrish et al., 1998; Nurmi et al., 2000). Regardless, it is clear that synergistic cell losses resulting from multiple hits with environmental toxins as proposed here or genetic–environ-

mental interactions are consistent with the current clinical phenomenology of PD and are likely to be relevant to our understanding of its pathogenesis.

Acknowledgments

This work was supported by ES10776, NS045316, and The Michael J. Fox Foundation.

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