# Paternal Cannabis Exposure Prior to Mating, but Not Δ9-Tetrahydrocannabinol, Elicits Deficits in Dopaminergic Synaptic Activity in the Offspring

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## ABSTRACT

The legalization and increasing availability of cannabis products raises concerns about the impact on offspring of users, and little has appeared on the potential contribution of paternal use. We administered cannabis extract to male rats prior to mating, with two different 28-day exposures, one where there was a 56-day interval between the end of exposure and mating ("Early Cannabis"), and one just prior to mating ("Late Cannabis"); the extract delivered 4 mg/kg/day of the main psychoactive component, Δ9-tetrahydrocannabinol. We then assessed the impact on dopamine (DA) systems in the offspring from the onset of adolescence (postnatal day 30) through middle age (postnatal day 150), measuring the levels of DA and its primary metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC) in various brain regions. Paternal cannabis with either regimen elicited a profound and persistent deficit in DA utilization (DOPAC/DA ratio) in the offspring, indicative of subnormal presynaptic activity. However, the two regimens differed in the underlying mechanism, with Early Cannabis reducing DOPAC whereas Late Cannabis increased DA and elicited a smaller reduction in DOPAC. Effects were restricted to male offspring. The effects of cannabis were not reproduced by equivalent exposure to its Δ9-tetrahydrocannabinol, nor did we see the effects with perinatal exposure to tobacco smoke or some of its fetotoxic contributors (benzo[a]pyrene without or with nicotine). Our studies provide some of the first evidence for adverse effects of paternal cannabis administration on neurodevelopment in the offspring, and reinforce the important consequences of paternal drug use in the preconception period.

Key words: cannabis; developmental neurotoxicity; dopamine; marijuana; paternal cannabis; tetrahydrocannabinol.

Although tobacco use during pregnancy is declining, cannabis use is rising as a consequence of its legalization, and unlike the situation with tobacco, the public has not received persistent, general warnings about potential adverse consequences of prenatal cannabis exposure on the offspring (Ryan *et al.*, 2018). The primary focus of many studies has been the consequences of maternal cannabis use or exposure to its chief psychoactive ingredient, Δ9-tetrahydrocannabinol (THC) (Abel, 1980; Fried, 2002; Huizink, 2014; Trezza *et al.*, 2008). However, relatively little is known about the potential contributions of paternal cannabis use to adverse consequences on the progeny. It is clear that premating exposure to cannabis or THC exposure causes persistent, epigenetic changes in methylation of genes associated with neurodevelopmental disorders (Murphy *et al.*, 2018; Schrott *et al.*, 2019; Szutorisz and Hurd, 2018).

In recent studies with male rats exposed to THC prior to mating, we were able to demonstrate attentional impairment and other behavioral anomalies in the offspring, associated with deficits in the development of acetylcholine systems (Holloway *et al.*, 2020a; Levin *et al.*, 2019; Slotkin *et al.*, 2020). However, the majority of cannabis consumption involves complex mixtures generated by smoking plant preparations, or by

consuming cannabis extracts, rather than just THC. Indeed, cannabis extract delivered to male rats prior to mating, elicits neurobehavioral abnormalities in the offspring (Holloway et al., 2020b), with both similarities and differences to the effects elicited by paternal exposure to THC alone. The current work focuses on the effects of paternal cannabis exposure prior to mating, on the development of dopamine (DA) systems in the offspring, an important issue given the key role of this neurotransmitter in the cognitive processing of reward, addiction, motivation, and aversion. We assessed the levels of DA and its principal metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC) so as to determine the patterns of presynaptic activity in the brain regions containing the principal DA projections; determinations were made from adolescence through young adulthood, full adulthood, and middle age, so as to examine the emergence and persistence of the effects. Specifically, the DOPAC/DA ratio enables calculation of DA turnover, an index of transmitter utilization (Powers et al., 2011). The specificity toward DA was then evaluated by contrasting the effects with those on the related catecholamine neurotransmitter, norepinephrine.

We focused on two cannabis treatment paradigms, one in which exposure terminated prior to maturation of the sperm that would subsequently be involved in insemination, and one in which cannabis was present during generation of the sperm. For the first exposure scenario (henceforth "Early Cannabis"), male rats were treated for 28 days and then cannabis was discontinued for the ensuing 56 days prior to mating, corresponding to the full length of the rat spermatogenic cycle and two seminiferous epithelium cycles. Thus, this group experienced exposure of progenitor cells including undifferentiated spermatogonia without involving exposure during the later stages of sperm maturation (de Rooij, 2017). For the second scenario ("Late Cannabis"), males received cannabis for 28 days just prior to mating, exposing the sperm used for fertilization during later maturation stages, but without exposure during their earlier proliferative stages. We then compared the latter cannabis treatment to the effects seen with comparable exposure to equivalent amounts of THC, so as to determine the relative contribution of the key psychoactive ingredient relative to the other compounds contained in cannabis.

Finally, we compared the effects of paternal cannabis exposure to those seen on the same neurotransmitter systems after perinatal exposure to tobacco smoke, or to benzo[a]pyrene, the latter alone or in combination with nicotine; these were conducted with maternal administration during gestation and early postnatal life. The comparisons were done for two reasons. First, direct fetal and neonatal exposure to tobacco, or to specific ingredients in tobacco smoke (here, benzo[a]pyrene and nicotine) are well-characterized to disrupt brain development, with the combination of benzo[a]pyrene and nicotine producing similar damage to that evoked by tobacco smoke (Slotkin, 2004; Slotkin et al., 2015, 2017, 2019). Comparing the effects of paternal cannabis to these known developmental disruptors then enables us to interpret the relative importance and magnitude of the paternal effects. Second, because the majority of human cannabis exposure involves smoking, the comparison to tobacco smoke and benzo[a]pyrene, a universal smoke product, allows some inference as to the potential for smoke components unspecific to cannabis, to contribute to adverse neurodevelopmental outcomes. Notably, the doses and exposure paradigms of these otherwise disparate agents, paternal THC, perinatal tobacco smoke, and perinatal benzo[a]pyrene in combination with nicotine, produce very similar outcomes on development of acetylcholine systems (Slotkin, 2004; Slotkin et al., 2015, 2017, 2019, 2020), indicating convergence on a common set of developmental deficits in synaptic function. Here, we establish whether that similarity extends to DA and norepinephrine systems.

## MATERIALS AND METHODS

*Cannabis treatments.* All experiments were carried out humanely and with regard for alleviation of suffering, with protocols approved by the Duke University Animal Care and Use Committee and in accordance with all federal and state guidelines. The treatment protocols have been published previously (Holloway et al., 2020b) in a study of the behavioral outcomes of paternal cannabis treatment, and the current work was carried out on littermates from the same treatment cohorts. (Holloway et al. [2020b] indicates that the material is a "cannabis smoke extract," but this is incorrect; the material is an extract of the cannabis plant, and a corrigendum has been published: https://doi.org/10.1016/j.neuro.2021.08.009. Accessed 1 October 2021) Nine-week-old, sexually mature male Sprague Dawley rats (Charles River Laboratories, Raleigh, North Carolina) were housed 2–3 per cage and were dosed daily for 28 days via subcutaneous injection. For "Early Cannabis" exposure, mating was conducted 56 days after the end of treatment, whereas for "Late Cannabis" exposure, mating occurred after only 3 days post-cannabis. Controls received vehicle only (saline containing 5% Tween80; Sigma-Aldrich, St Louis, Missouri), and experimental groups received cannabis extract (National Institute on Drug Abuse Drug Supply Program, Research Triangle Institute International, Research Triangle Park, North Carolina) in 5% Tween80 solution, calculated to deliver an amount containing 4 mg/kg of THC with each dose. The administered volume was 1 ml/kg, given by subcutaneous injection. The manufacturer's certificate of analysis indicated that the cannabis extract contained 27.3% Δ9-THC, 1.31% cannabidiol, and 1.40% cannabinol (see Supplementary Data for the analysis sheet).

We chose to administer cannabis via subcutaneous injection so as to blanket gametogenesis round the clock, for the entire treatment period, such that that all sperm were exposed to cannabis. Subcutaneous injection produces reliable bioavailability and a sustained plasma level over a 24-h span, whereas administration via smoke inhalation produces variable bioavailability with a rapid peak of THC plasma levels and an equally rapid decline (Hložek et al., 2017; Huestis, 2007); delivery via inhalation thus leads to a "valley" in between doses, in which gametogenesis could occur during the gap in exposure. Additionally, repeated smoke inhalation is potentially stressful, and we wished to avoid that confound. Bioavailability after oral cannabis dosing likewise is extremely variable and does not provide the sustained plasma levels needed for this study (Hložek et al., 2017; Huestis, 2007). To enable all three treatment groups to be compared appropriately with each other, all animals received the same number of injections on the same schedule. The controls received vehicle for both the Early and Late periods; the Early Cannabis group received cannabis injections in the first period, followed by vehicle injections in the second period; and the Late Cannabis group received vehicle injections in the first period and cannabis in the second period.

Each cannabis-exposed and control male was housed together with a drug-naive young adult female Sprague Dawley rat for 5 days, ultimately producing 11 litters per treatment group. Pregnancy was confirmed by weight gains in the female rats after the 5-day cohabitation, after which the dams were housed singly during gestation and with their litters after

delivery. Parturition occurred during gestational day 22, which was also taken as postnatal day zero (PN0), and litters were culled on PN1 to 8–10 pups to ensure standard nutrition. Weaning occurred on PN21. After weaning, the offspring were housed in same-sex, within-treatment groups with up to 3 rats per cage. On PN30, 60, 100, and 150, animals were decapitated and brain regions were dissected as follows: frontal/parietal cortex, temporal/occipital cortex, hippocampus, striatum, midbrain, brainstem and cerebellum. The two cortical regions were sectioned at the midline and the right half used for the current determinations. The left halves of the cortical regions were reserved for future studies. Tissues were frozen in liquid nitrogen and stored at −80°C until assayed. Each treatment group comprised 6–11 animals of each sex at each age point, with each litter contributing no more than one male and one female to any of the determinations at a given age.

*Assays.* Assays were conducted on each individual tissue, so that each determination represented a value from the corresponding brain region of one animal. Tissues were thawed and homogenized in ice-cold 0.1 M perchloric acid and sedimented for 20 min at 40 000 × g. The supernatant solution was collected, trace-enriched using activated alumina (Sigma-Aldrich) and was analyzed for DA, DOPAC, and norepinephrine via high-performance liquid chromatography with electrochemical detection (Slotkin et al., 2000). Concurrently-run standards, containing each of the compounds (Sigma-Aldrich) and an internal standard (dihydroxybenzylamine; Sigma-Aldrich) were used to calculate regional concentrations. DA utilization (turnover) was calculated as the DOPAC/DA ratio (Powers et al., 2011). Determinations of DA and DOPAC were limited to the two regions containing the majority of DA projections, striatum, and frontal/parietal cortex, whereas norepinephrine was evaluated in all regions except the striatum.

*Other treatments.* We compared the effects of paternal cannabis to those of other treatments, utilizing archived tissues from previous studies, so that no additional animals were required for these determinations; details of the administration paradigms are provided in previous communications (Slotkin et al., 2015, 2019, 2020). Consequently, though, we had a more limited selection of ages and brain regions for these additional studies, and thus concentrated on identifying the most persistent effects (PN150) in the key brain regions found to be affected by cannabis, as well as evaluating a restricted repertoire of samples from other ages and regions.

Paternal THC administration was carried out essentially using the same protocol as the late cannabis group, ie, 28 days of exposure followed by mating, commenced two days after the end of treatment (Slotkin et al., 2020). THC (Sigma-Aldrich) was given at either 2 or 4 mg/kg/day in the same vehicle as used for cannabis, mimicking the plasma levels achieved with moderate daily cannabis use in humans (Harte and Dow-Edwards, 2010; Irimia et al., 2015; Rubino et al., 2009).

Treatments with tobacco smoke extract, and for benzo[a]pyrene with or without nicotine, involved perinatal exposures via continuous maternal administration with implanted osmotic minipumps (Model 2ML4; Durect Corp., Cupertino, California), begun preconception and stopped during the second postnatal week (Slotkin et al., 2015, 2019). Tobacco smoke extract was prepared from Kentucky Reference cigarettes (KY3R4F) on a Rotary Smoke Machine under ISO smoke conditions. The smoke condensate was collected on 92 mm filter pads, which were then extracted by shaking for 20 min with undiluted dimethyl sulfoxide, to obtain a solution of approximately 20 mg of condensate per ml. Condensate aliquots were stored in amber vials at −80°C until used. Two cigarettes were smoked to produce each ml of extract and the final product contained 0.8 mg/ml nicotine (determined by the manufacturer; Arista Laboratories, Richmond, Virginia). The initial dose rate delivered 180 μg/kg/day of nicotine, which produces low plasma levels of nicotine, comparable to secondhand smoke exposure (Fewell et al., 2001; Okoli et al., 2007; Trauth et al., 2000). Because maternal body weights increase with gestation, the dose rate fell by approximately one-third by the end of gestation and then rose back toward the original values with postpartum weight loss.

Maternal benzo[a]pyrene (Sigma-Aldrich) was given at an initial dose of 30 μg/kg/day, with or without a second minipump set to deliver nicotine bitartrate (Sigma-Aldrich) at 2 mg/kg/day of nicotine base. The benzo[a]pyrene dose rate is at the lower end of exposures known to have significant effects on the development of ion channels (Brown et al., 2007) or on neurobehavioral endpoints (McCallister et al., 2008, 2016). The nicotine dose rate produces nicotine plasma levels achieved in moderate smokers (Fewell et al., 2001; Trauth et al., 2000). More importantly for our purposes, the combination of benzo[a]pyrene plus nicotine recapitulates neurochemical and behavioral deficits in the offspring of those seen with exposure to tobacco smoke extract (Slotkin et al., 2019).

*Data analysis.* The initial statistical comparisons were conducted by a global analysis of variance (ANOVA) (data log-transformed because of heterogeneous variance among regions, ages, and measurement types) incorporating all the factors in a single test so as to avoid an increased probability of type 1 errors that might otherwise result from multiple evaluations of the same data set. For DA systems, the variables in the global test were treatment, brain region, age and sex, with two separate measurements (DA, DOPAC), which were treated as repeated measures because they were derived from the same sample. Where we identified interactions of treatment with the other factors, data were then subdivided for lower-order ANOVAs to evaluate treatment effects for specific sexes, ages or regions, followed (where permitted) by the Tukey-Kramer test for individual effects. In the absence of interactions, we compiled only the main treatment effects, which accounted for the majority of significant outcomes. Significance was assumed at the level of $p < .05$, two-tailed. Evaluations of norepinephrine were carried out likewise, except that there was only a single measure (norepinephrine concentration).

Data were compiled as means and standard errors. To enable ready visualization of treatment effects across sexes, regions, ages, and measures, the results are given as the percent change from control values but statistical procedures were always conducted on the original data, with log transforms because of heterogeneous variance as noted above. Graphs were scaled to encompass the different dynamic ranges of the changes in the various parameters. The original values for each set of determinations appear in the Supplementary Tables.

## RESULTS

Paternal cannabis treatment had no significant effect on mating success, maternal weight gain during or after pregnancy, or on the proportion of dams giving birth and likewise, litter size and sex ratio were unaffected, nor were there any significant differences in body weight gain or brain region weights in the offspring (data not shown).

For DA systems, global ANOVA across all the factors (treatment, age, sex, region, repeated measures of DA and DOPAC) identified a main treatment effect ($p < .007$) that was interactive with the other factors ($p < .0003$ for treatment × measure, $p < .03$ for treatment × sex × region, $p < .03$ for treatment × age × region, $p < .04$ for treatment × measure × sex × region). Because the strongest interaction was for treatment × measure, we separated the values for DA and DOPAC for lower-order analyses and then evaluated the DOPAC/DA ratio to assess effects on DA utilization.

DA levels showed a significant main treatment effect across both early and late cannabis treatments ($p < .002$), indicative of an overall increase in levels relative to control values; again, the treatment effect also depended on sex and region (treatment × sex × region, $p < .03$). Separate analyses by sex indicated a significant main effect in males ($p < .003$) but not females. Accordingly, we evaluated each of the cannabis exposure paradigms separately, focusing on treatment effects and sex differences in response to cannabis treatment. Early cannabis exposure showed a significant interaction of treatment × age × region ($p < .05$) but no significant lower-order effects were identified after subdivision into separate ages or regions (Figure 1A). In contrast, the effects of late cannabis exposure were more robust and consistent (Figure 1B), with a significant overall elevation ($p < .0001$) that was again dependent on the other factors ($p < .04$ for treatment × sex × region, $p < .03$ for treatment × age × region). In light of these interactions, we examined males and females separately, and found a significant elevation in males but not females. Females showed interactions of
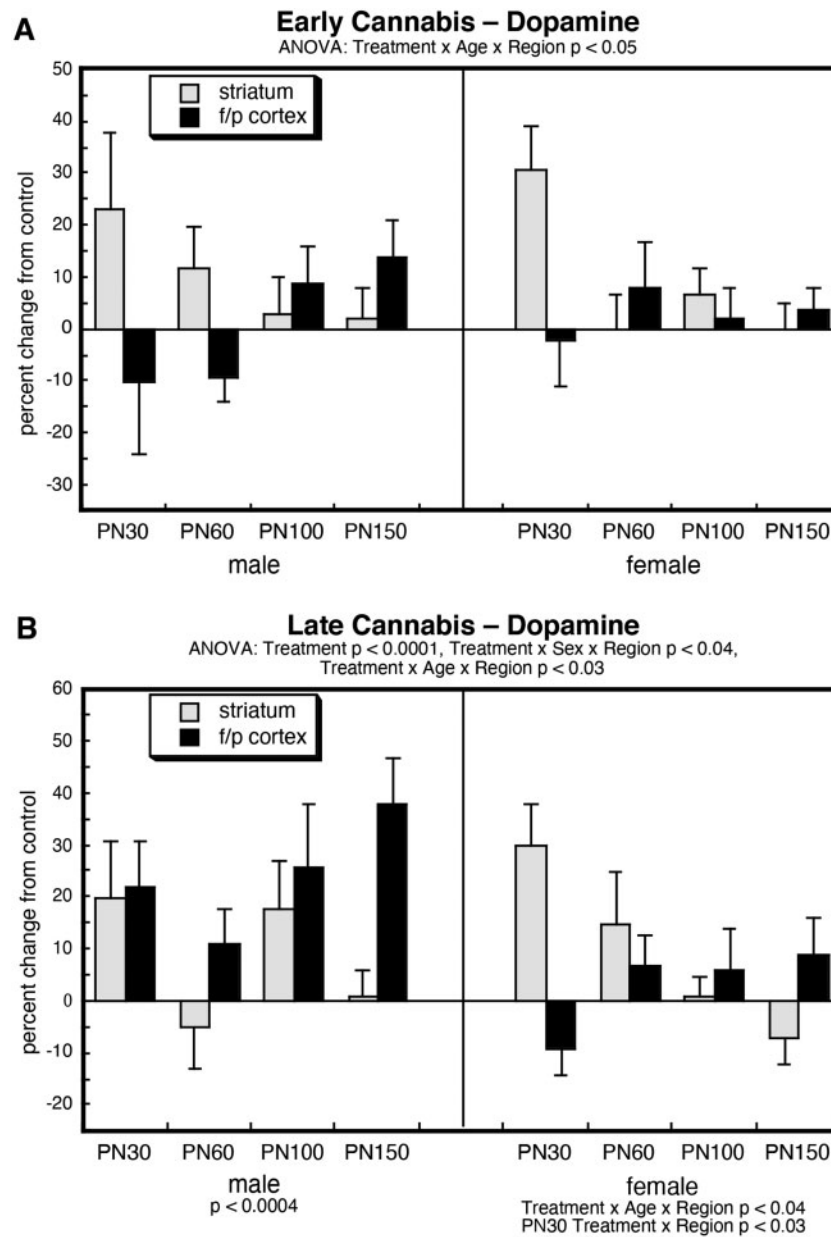


**Figure 1.** Effects of early (A) or late (B) paternal cannabis treatment on DA levels in the offspring. Data represent means and standard errors obtained from 6 to 11 animals in each treatment group for each age and sex, presented as the percent change from control values; complete original data are shown in Supplementary Table 1. Multivariate analysis of variance (ANOVA) for each treatment appears at the top of the panels, with lower order tests shown below the panels as permitted by the interactions in the overall test. Abbreviation: f/p, frontal/parietal cortex.

treatment with other variables that ultimately did not give rise to individually significant differences after subdivision by the interactive factors, and that would not have been interpretable anyway, because females showed no significant overall effect in the higher-order test.

Overall analysis of DOPAC levels likewise indicated treatment effects that interacted with the other factors: $p < .02$ for treatment $\times$ sex, $p < .002$ for treatment $\times$ age $\times$ region. Separating the analysis by sex revealed that, again, males were impacted ($p < .005$ for main treatment effect, $p < .008$ for treatment $\times$ age $\times$ region), but females were not significantly affected. Early cannabis exposure evoked a significant treatment $\times$ sex interaction ($p < .0003$), with males, but not females, showing a significant overall reduction in DOPAC (Figure 2A). Late cannabis exposure also had effects that

were statistically significant ($p < .05$ treatment $\times$ sex, $p < .002$ for treatment $\times$ age $\times$ region) but in this case, effects were less consistent in males and therefore showed no significant overall changes (Figure 2B). Females showed a complex pattern of interactions with age and region but again, this needs to be viewed with caution, because the higher-order test did not find significance for treatment effects or interactions in females. Indeed, after separation by the interactive factors, we could not identify any residual significant effects in females except for one time point in one region (striatum at PN30).

As a result of the sex-selective effects of both early and late paternal cannabis exposure on DA and DOPAC, we found a robust overall decrease in the DOPAC/DA ratio ($p < .0004$) that was strongly sex dependent (treatment $\times$ sex, $p < .0001$), with males
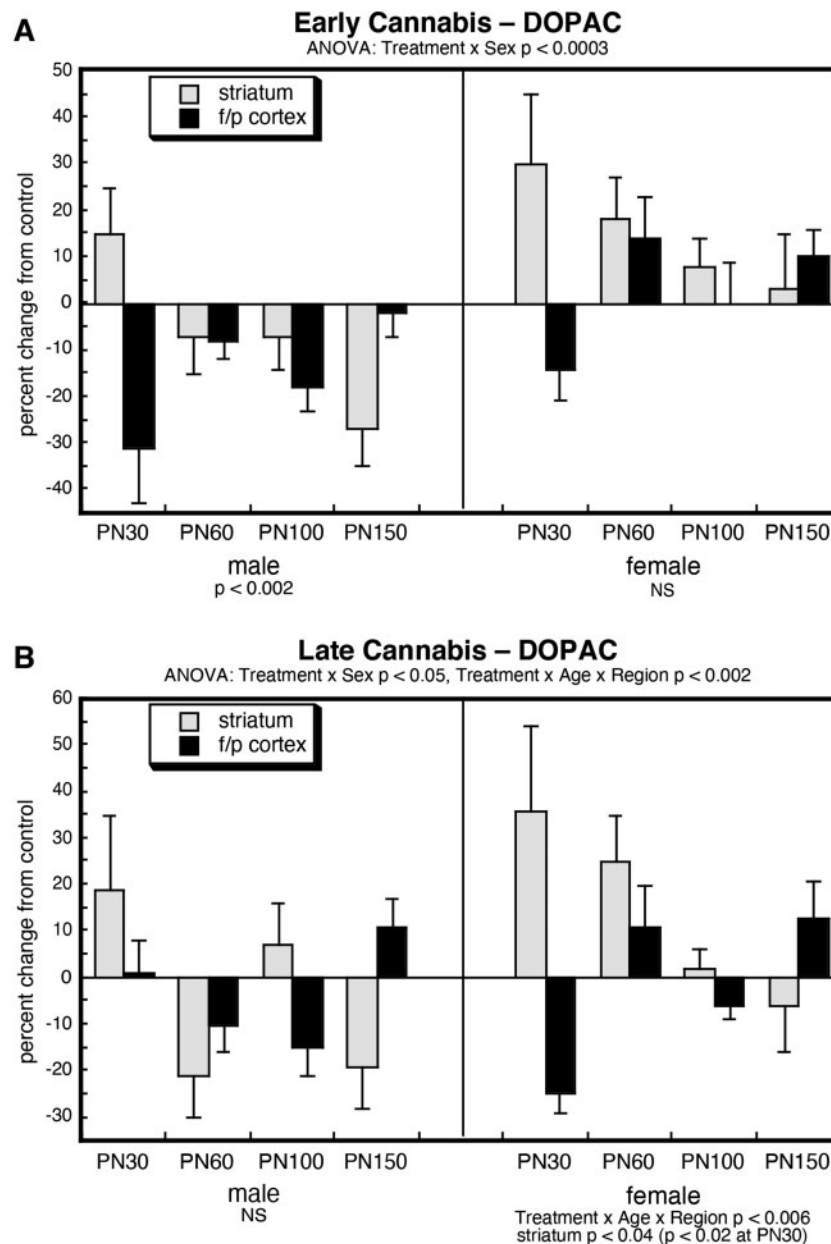


**Figure 2.** Effects of early (A) or late (B) paternal cannabis treatment on DOPAC levels in the offspring. Data represent means and standard errors obtained from 6 to 11 animals in each treatment group for each age and sex, presented as the percent change from control values; complete original data are shown in Supplementary Table 1. Multivariate ANOVA for each treatment appears at the top of the panels, with lower order tests shown below the panels as permitted by the interactions in the overall test. Abbreviations: f/p, frontal/parietal cortex; NS, not significant.

showing significance ($p < .0001$) but not females. Early cannabis treatment elicited a robust and consistent decrease in turnover ($p < .009$ for treatment, $p < .0001$ for treatment $\times$ sex), with the effect confined to males ($p < .0001$; Figure 3A). Likewise, late cannabis elicited both an overall reduction ($p < .0001$ for treatment) that was sex specific ($p < .0002$ for treatment $\times$ sex), reflecting an overall reduction in males ($p < .0001$) but not females (Figure 3B). The effects in males were not statistically different between the two cannabis regimens (no significant treatment $\times$ regimen interaction).

Turning to the effects on norepinephrine systems, we found a significant overall reduction in levels across the two cannabis regimens (main treatment effect, $p < .0001$) but no interactions of treatment with the other factors, and specifically, no

interaction with sex that would have been indicative of a preferential effect in males as we found for DA systems. Both early cannabis exposure (Figure 4A) and late cannabis exposure (Figure 4B) evinced significant overall reductions, but the magnitude of effect was substantially lower than that seen for DA systems.

### Other Treatments
As with paternal cannabis, paternal THC administration did not evoke any deficits in mating success, litter parameters, or offspring growth (data not shown). Paternal THC treatment elicited a significant sex-dependent effect on DA systems ($p < .05$ for treatment $\times$ sex $\times$ measure), with effects once again restricted to males (Figure 5A). At the lower THC dose, males displayed a
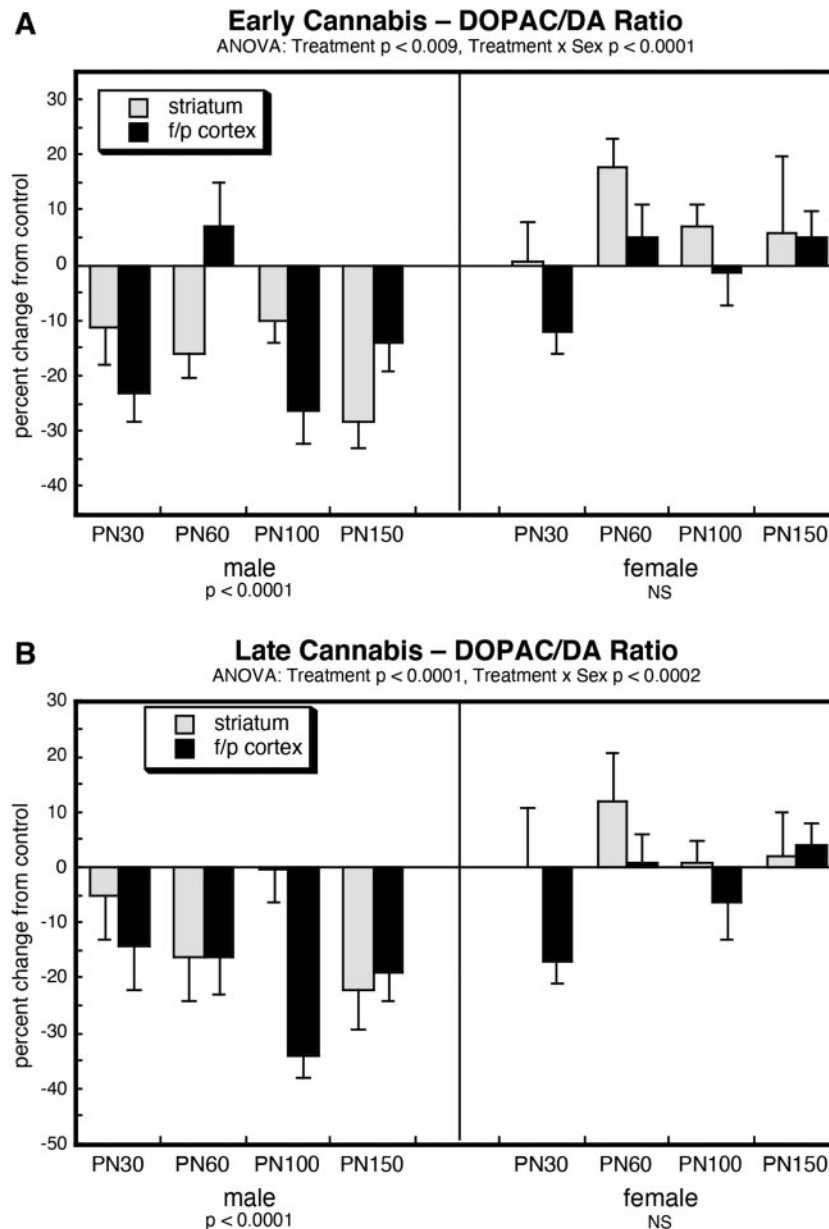


**Figure 3.** Effects of early (A) or late (B) paternal cannabis treatment on DA turnover (DOPAC/DA ratio) in the offspring. Data represent means and standard errors obtained from 6 to 11 animals in each treatment group for each age and sex, presented as the percent change from control values; complete original data are shown in Supplementary Table 1. Multivariate ANOVA for each treatment appears at the top of the panels, with lower order tests shown below the panels as permitted by the interactions in the overall test. Abbreviations: f/p, frontal/parietal cortex; NS, not significant.
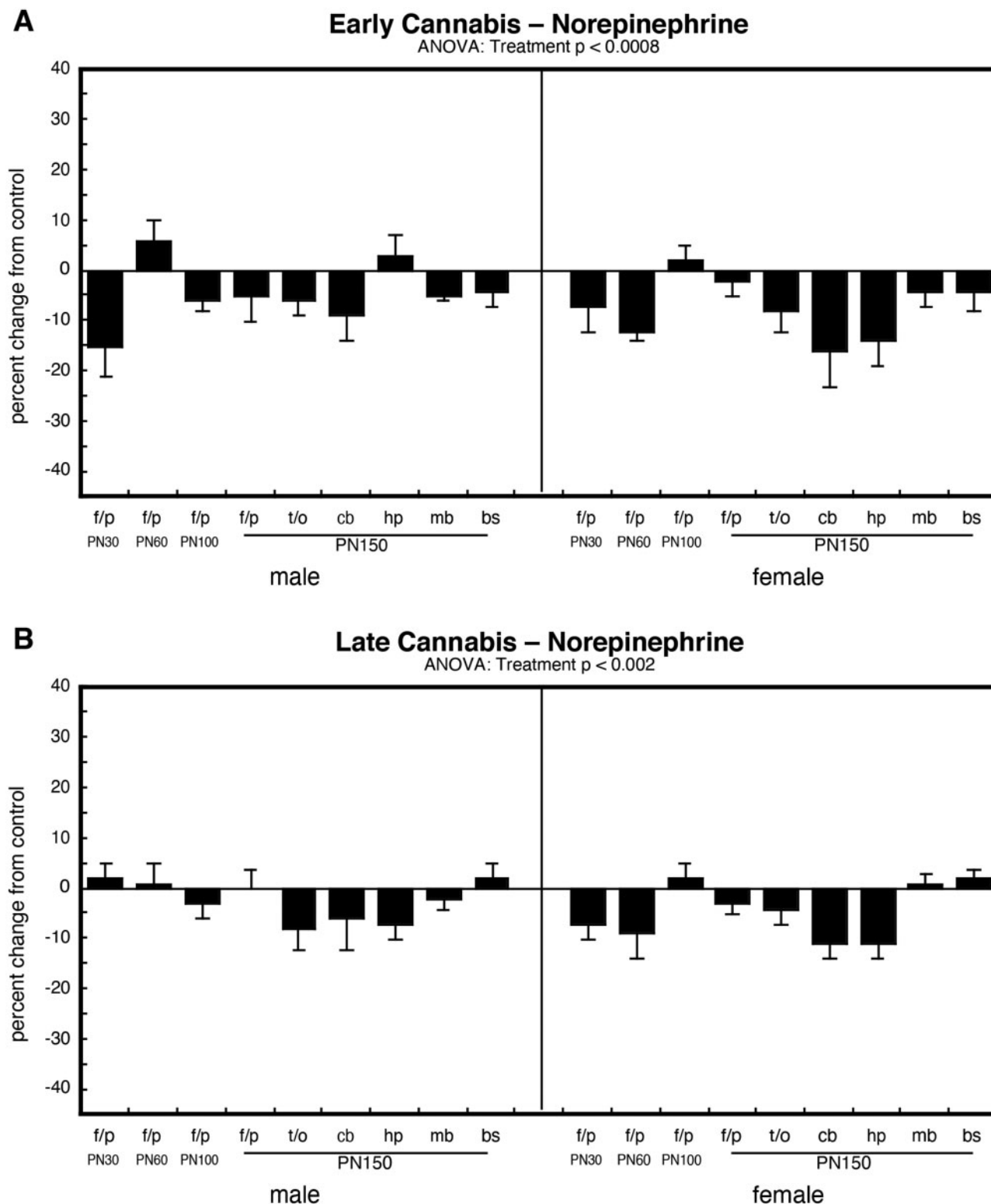
**Figure 4.** Effects of early (A) or late (B) paternal cannabis treatment on norepinephrine levels in the offspring. Data represent means and standard errors, presented as the percent change from control values; complete original data are shown in Supplementary Table 1. Multivariate ANOVA for each treatment appears at the top of the panels. Only the main treatment effects are shown; lower order tests were not carried out because of the absence of interactions between treatment and other factors. Abbreviations: f/p, frontal/parietal cortex; t/o, temporal/occipital cortex; cb, cerebellum; hp, hippocampus; mb, midbrain; bs, brainstem.

significant elevation in DOPAC levels, leading to a comparable increase in the DOPAC/DA ratio, an effect opposite to that seen with cannabis. Somewhat surprisingly, the higher dose of THC, which matched the THC in the cannabis extract, had no significant effect. We did not observe any significant effects of paternal THC on norepinephrine levels at either dose (Figure 5B).
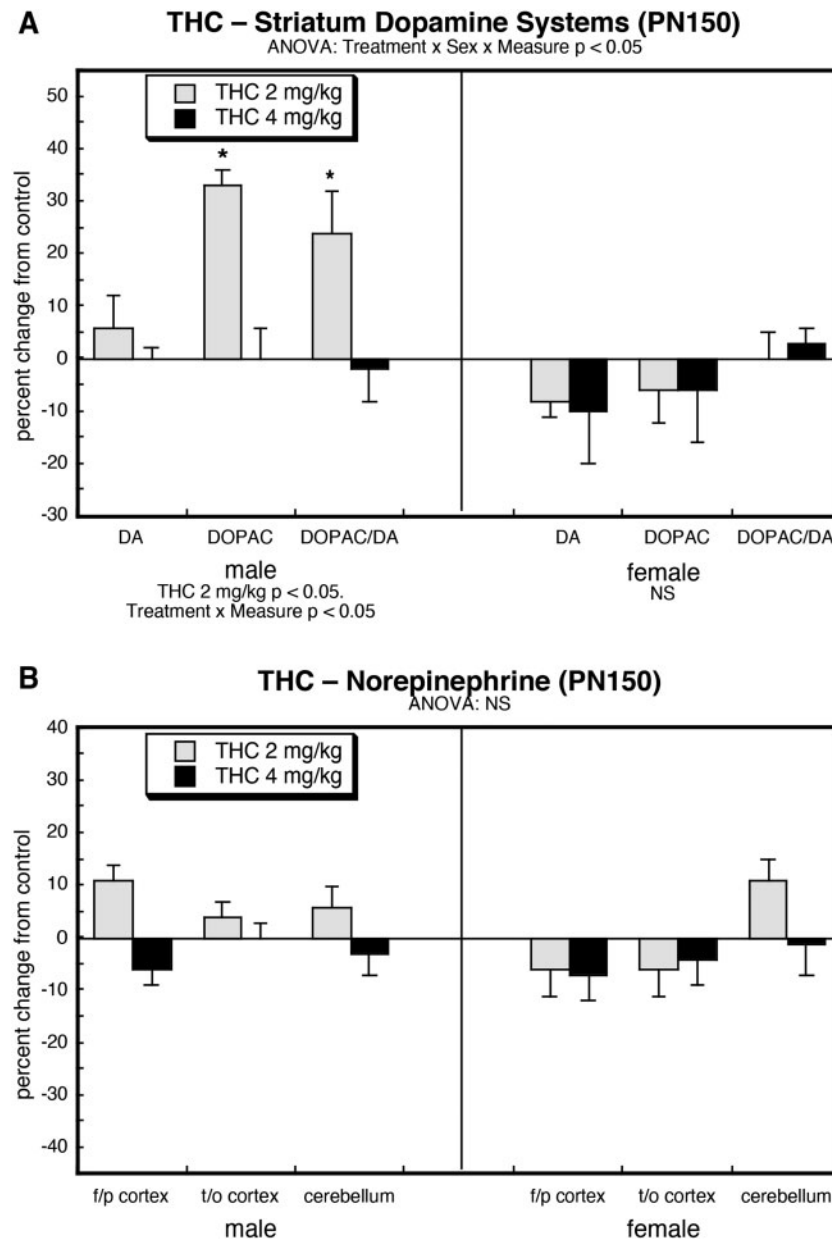
**Figure 5.** Effects of paternal THC treatment on (A) DA, DOPAC, and DA turnover (DOPAC/DA ratio) and (B) norepinephrine in the offspring. Data represent means and standard errors obtained from 4 to 6 animals in each treatment group for each age and sex, presented as the percent change from control values; complete original data are shown in Supplementary Table 2. Multivariate ANOVA for each treatment appears at the top of the panels, with lower order tests shown below the panels as permitted by the interactions in the overall test; asterisks denote individual values that differ significantly from corresponding controls. Abbreviations: f/p, frontal/parietal cortex; t/o, temporal/occipital cortex; NS, not significant.

Comparing the results of the cannabis series of experiments to those of the lower dose of THC (the one that exhibited significant treatment effects), we found a significant distinction between the two when comparing the matched ages and regions for DOPAC ($p < .006$ for treatment × series, $p < .0002$ for treatment × series × sex), the DOPAC/DA ratio ($p < .03$ for treatment × series, $p < .03$ for treatment × series × sex), and norepinephrine ($p < .05$ for treatment × series).

In contrast to the effects of either cannabis extract or THC, perinatal exposure to tobacco smoke extract had no significant effect on either DA (Figure 6A) or norepinephrine (Figure 6B) systems. Again, to ensure that these negative findings were interpretable, we assessed whether they were statistically distinguishable from the significant effects of cannabis,

comparing the two series of experiments matched for region and age. Each of the DA measures was distinguishable between tobacco smoke extract and cannabis: treatment × series, $p < .05$ for DA, $p < .05$ for DOPAC, $p < .02$ for DOPAC/DA. However, for norepinephrine, the difference between the effects of tobacco smoke extract and cannabis was not significant, which is not surprising, given the much smaller net effect of cannabis on this parameter and the lower power of the determinations (only a single age point).

Likewise, we did not find any significant effects of perinatal exposure to benzo[a]pyrene, without or with nicotine, on DA, DOPAC, or turnover (Figure 7A) or on norepinephrine levels (Figure 7B). Again, these negative results were statistically distinguishable from the positive findings of the cannabis series of
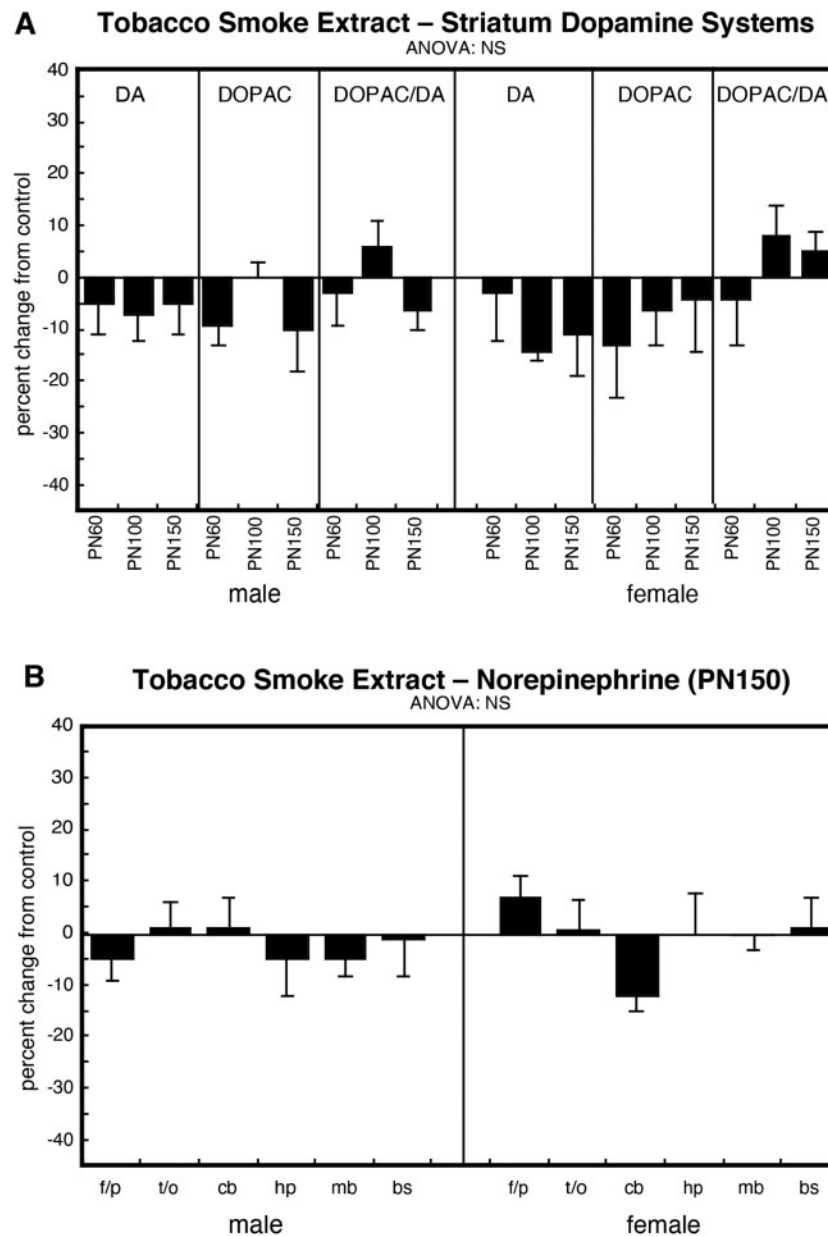
**Figure 6.** Effects of perinatal tobacco smoke extract treatment on (A) striatal DA, DOPAC, and DA turnover (DOPAC/DA ratio) and (B) norepinephrine in the offspring. Data represent means and standard errors obtained from 4 to 6 animals for each age and sex, presented as the percent change from control values; complete original data are shown in Supplementary Table 3. Multivariate ANOVA for each treatment appears at the top of the panels; lower order tests were not carried out because of the absence of interactions between treatment and other factors. Abbreviations: f/p, frontal/parietal cortex; t/o, temporal/occipital cortex; cb, cerebellum; hp, hippocampus; mb, midbrain, bs, brainstem; NS, not significant.

experiments when matched for region and age: treatment × series, $p < .0002$ for DA, $p < .04$ for DOPAC/DA, $p < .006$ for norepinephrine. The significant difference for norepinephrine reflected the tendency for *increases* in the benzo[a]pyrene and nicotine series (albeit not statistically significant), compared with significant *decreases* with cannabis exposure.

## DISCUSSION

Our results show that paternal cannabis exposure prior to mating evokes deficits in DA utilization in male offspring throughout maturation from adolescence through middle age, indicative of reduced overall presynaptic activity. The adverse effect was not regionally selective, pointing to an underlying impact on the development of DA systems in general, rather than an alteration in the regulation of specific DA synaptic circuits. There were two additional, notable features of the effect. First, the reduction in turnover in the offspring was seen with both of the paternal cannabis regimens, despite the fact that, for the Early Cannabis treatment group, mating occurred after a prolonged, drug-free hiatus. Worrisomely, this indicates a persistent effect on developing sperm even with abstinence for more than a full spermatogenerational cycle. Indeed, a recent study in human cannabis users found that abstinence for a complete spermatogenic cycle reduced, but did not eliminate cannabis effects on methylation of genes related to neurodevelopment, suggesting persistent, perhaps permanent epigenetic changes in the spermatogonia (Schrott et al., 2021). Our results
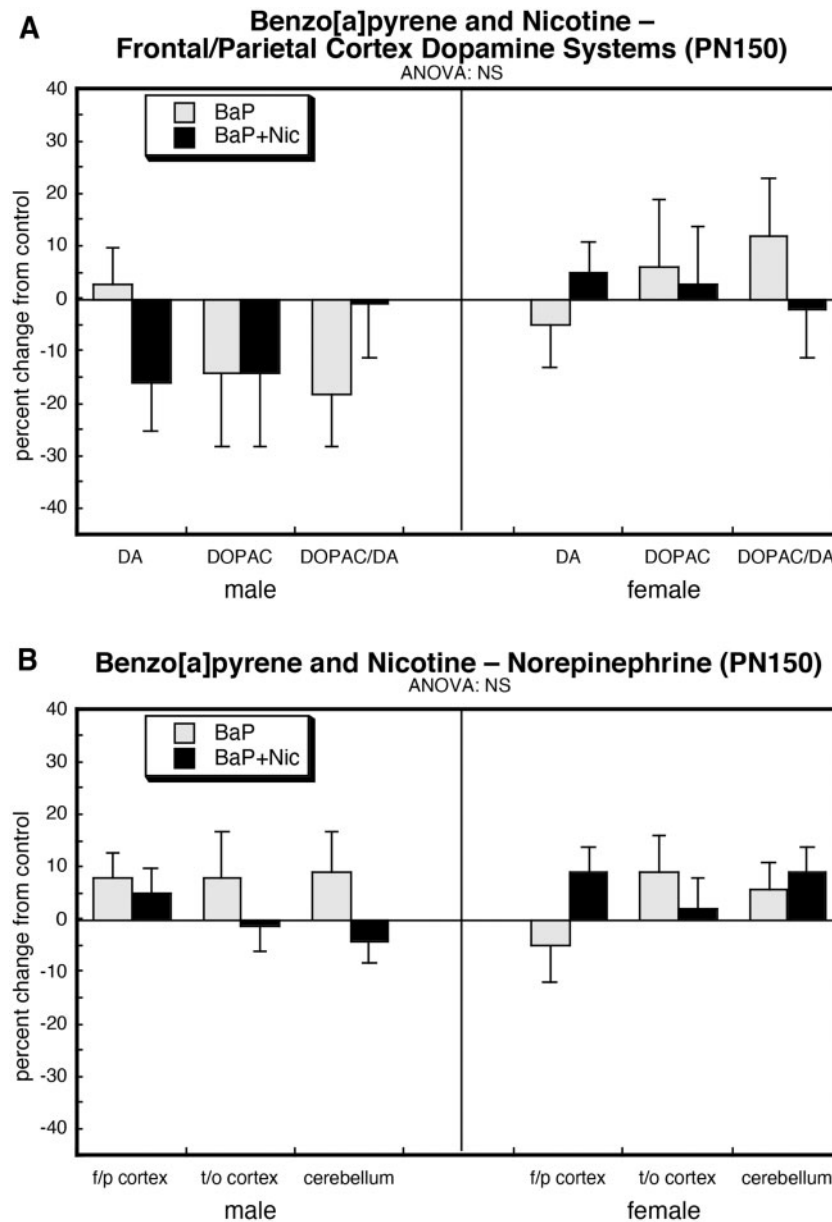
**Figure 7.** Effects of perinatal benzo[a]pyrene treatment, without and with nicotine, on (A) frontal/parietal cortex DA, DOPAC, and DA turnover (DOPAC/DA ratio) and (B) norepinephrine in the offspring. Data represent means and standard errors obtained from 8 to 11 animals in each treatment group for each age and sex, presented as the percent change from control values; complete original data are shown in Supplementary Table 4. Multivariate ANOVA for each treatment appears at the top of the panels; lower order tests were not carried out because of the absence of interactions between treatment and other factors. Abbreviations: BaP, benzo[a]pyrene; Nic, nicotine; f/p, frontal/parietal cortex; t/o, temporal/occipital cortex; NS, not significant.

also indicate potentially different underlying mechanisms for the long-term effects after Early versus Late Cannabis paternal exposure. For Early Cannabis, the subnormal turnover ratio reflected a primary deficit in DOPAC, whereas for Late Cannabis, the effect was generated by a combination of a significant overall increase in DA and a nonsignificant decrease in DOPAC.

The second critical feature was the restriction of effects to males. Because the cannabis exposure occurred during spermatogenesis, this points to specific involvement of factors that differentiate between male and female gametes. A likely prospect is the targeting of genes on the Y-chromosome itself. Given the unlikely possibility of actual mutations induced selectively in the Y-chromosome, our results point to the need to explore epigenetic targets for cannabis that are specific to that chromosome. In that regard, there are Y-chromosome genes that are known to have a specific impact on the development and function of DA neurons, notably SRY (Kopsida et al., 2009; Lee et al., 2019), and future studies should investigate whether paternal cannabis affects the methylation status and/or expression of such genes. However, there are likely to be other target genes that are not located on the Y-chromosome: we found significant (albeit smaller) deficits for the related catecholamine neurotransmitter, norepinephrine, but these effects were not sex selective. All that would be required for sex-selective effects on DA systems is that the effects on gene expression interact with hormonal status; indeed, this is the case for male-pattern baldness, which involves multigenic effects including some involving genes on the X-chromosome (Yap et al., 2018).

Prior work with the same premating paternal cannabis treatments identified a number of behavioral anomalies that likewise involved both early and late exposure paradigms (Holloway et al., 2020b) but there was not one-to-one match of these effects with the sex-selective actions seen here for the impact on DA synaptic function. It is highly likely that the adverse effects of paternal cannabis extend beyond DA systems, and can thus impact a variety of behavioral outcomes beyond those involving this neurotransmitter. In particular, though, it would be worthwhile to examine behaviors known to have a high dependence on DA, such as reward and addiction liability.

The comparison of paternal cannabis effects to those of a matched-dose of THC (4 mg/kg) is striking: Paternal THC when given alone did not produce a defect in offspring DA utilization. Obviously, other constituents in cannabis, either by themselves or in combination with each other or THC, are responsible for the adverse impact on dopaminergic activity. When we lowered the THC dose to 2 mg/kg, we were thus surprised to find an effect opposite to that seen with cannabis, namely an *increase* in DA utilization in the offspring, again with specificity for males. As posited above, this points to potential epigenetic modifications involving genes on the Y-chromosome or on genes that interact with hormonal status, but obviously that are distinct from the genes targeted by cannabis. There are likely explanations both for the differences between cannabis and THC, and for THC's "inverted U" dose-response curve. Cannabinoid receptors are expressed in male germ cells, which contain a complete endocannabinoid signaling system (Barchi et al., 2020); the other cannabinoids in cannabis extract may interfere with the effects of THC, shifting the nonmonotonic dose-response curve to the right. But also, nonmonotonic dose-response curves are typical of cannabinoids in general (Guimarães et al., 1990; Levin et al., 2014; Zuardi et al., 2017). Indeed, just as found in our study, when given directly to adult animals, THC can evoke behavioral effects opposite to those of other cannabinoids (Levin et al., 2014). In any case, the hyperdopaminergic outcome evoked by the lower dose of THC resembles that seen with direct prenatal or adolescent THC exposure (Frau et al., 2019; Renard et al., 2017a,b), implying that developmental vulnerability to this cannabis component extends from preconception through end stages of brain development. It would therefore be useful to determine whether the effects we observed here for premating paternal cannabis exposure likewise extend to exposures in later developmental stages, including prenatal and postnatal periods.

The primary purpose of our comparing paternal cannabis to perinatal exposure to tobacco smoke extract and to benzo[a]pyrene without and with nicotine, was to provide a benchmark to interpret the magnitude of the effects of cannabis with those of widely known neurodevelopmental disruptors. Indeed, the exposure paradigms used here for all the perinatal treatments, perinatal tobacco smoke, and perinatal benzo[a]pyrene in combination with nicotine (as well as that of paternal THC), all produce clear-cut damage to development of cholinergic pathways in the developing brain (Slotkin, 2004; Slotkin et al., 2015, 2017, 2019, 2020). It is therefore particularly notable that the cannabis effects were substantially greater than those of the perinatal treatments, reinforcing the unique sensitivity of DA systems to the impact of paternal cannabis. The perinatal treatments also provide some inference as to components of cannabis that may or may not contribute to the adverse effects, especially in light of the fact that the majority of cannabis exposure in humans comes from smoking marijuana. The fact that neither tobacco smoke nor the universal combustion product, benzo[a]pyrene, reproduced the effect of paternal cannabis, points away from

smoke itself as a likely contributor, especially when one considers the ordinarily greater importance of direct in utero exposure as compared with premating paternal treatment. This interpretation is limited, however, by the different exposure period for the smoke products (perinatal) as compared with our cannabis study (premating paternal). Nevertheless, our results indicate the need for a future focus on components of cannabis other than just THC or smoke per se.

In conclusion, our results reinforce the fact that the consequences of parental drug use are not restricted to maternal exposure, nor to the period of gestation, but rather extend to contributions from the father over an extended period prior to conception. The greater effect of cannabis as compared with THC alone provides an important cautionary note in light of the increasing availability of THC-free cannabis extracts that are promoted as "safe" Alternative Medicines. Just because an extract is not intoxicating does not mean that it is not toxic. Indeed, the legalization of cannabis products and their increased availability and promotion requires a parallel effort to inform the public about potential adverse effects on children of users (Nashed et al., 2021), just as we do for other legal drugs such as alcohol and tobacco. Equally important, our findings suggest that these warnings need to extend to paternal, not just maternal use, and reinforce the increasing awareness of the importance of chemical exposures in the preconception period as a contributor to neurodevelopmental disorders (Rimawi et al., 2021).

## SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

## DECLARATION OF CONFLICTING INTERESTS

T.A.S. has received consultant income in the past 3 years from the State of Arizona, Matthews & Associates (Houston, Texas), Gjording Fouser (Boise, Idaho), Thorsnes Bartolotta McGuire (San Diego, California), and Cracken Law (Dallas, Texas).

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