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TOXICOLOGICAL SCIENCES, 174(2), 2020, 210–217

doi: 10.1093/toxsci/kfaa004
Advance Access Publication Date: February 20, 2020
Research Article

Paternal Δ9-Tetrahydrocannabinol Exposure Prior to Mating Elicits Deficits in Cholinergic Synaptic Function in the Offspring

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ABSTRACT

Little attention has been paid to the potential impact of paternal marijuana use on offspring brain development. We administered Δ9-tetrahydrocannabinol (THC, 0, 2, or 4 mg/kg/day) to male rats for 28 days. Two days after the last THC treatment, the males were mated to drug-naïve females. We then assessed the impact on development of acetylcholine (ACh) systems in the offspring, encompassing the period from the onset of adolescence (postnatal day 30) through middle age (postnatal day 150), and including brain regions encompassing the majority of ACh terminals and cell bodies. Δ9-Tetrahydrocannabinol produced a dose-dependent deficit in hemicholinium-3 binding, an index of presynaptic ACh activity, superimposed on regionally selective increases in choline acetyltransferase activity, a biomarker for numbers of ACh terminals. The combined effects produced a persistent decrement in the hemicholinium-3/choline acetyltransferase ratio, an index of impulse activity per nerve terminal. At the low THC dose, the decreased presynaptic activity was partially compensated by upregulation of nicotinic ACh receptors, whereas at the high dose, receptors were subnormal, an effect that would exacerbate the presynaptic defect. Superimposed on these effects, either dose of THC also accelerated the age-related decline in nicotinic ACh receptors. Our studies provide evidence for adverse effects of paternal THC administration on neurodevelopment in the offspring and further demonstrate that adverse impacts of drug exposure on brain development are not limited to effects mediated by the embryonic or fetal chemical environment, but rather that vulnerability is engendered by exposures occurring prior to conception, involving the father as well as the mother.

Key words: acetylcholine; cannabis; developmental neurotoxicity; marijuana; paternal Δ9-tetrahydrocannabinol.

The increasing use of marijuana by women of childbearing age raises a concern for potential adverse outcomes in the offspring (Ryan *et al.*, 2018). There have been numerous studies of the consequences of maternal cannabis use in humans or Δ9-tetrahydrocannabinol (THC) administration during pregnancy in animals (Abel, 1980; Fried, 2002; Huizink, 2014; Trezza *et al.*, 2008), but little or no attention has been paid to the role of paternal exposure for adverse consequences to the developing fetus or child. Animal studies where both males and females were exposed to THC in adolescence identified persistent changes in gene methylation in association with neurobehavioral anomalies in the offspring (Szutorisz and Hurd, 2018) ; however, these did not distinguish whether there was a specific paternal contribution, which would presumably involve epigenetic changes in sperm. Indeed, recent work found that cannabis exposure in humans, or THC exposure in rats, produces persistent changes in sperm DNA methylation, including the genes that were affected by combined paternal and maternal exposure, as well as genes associated with risk of autism spectrum disorder (Murphy *et al.*, 2018; Schrott *et al.*, 2020). The effects in rats were associated with long-lasting attentional impairment in the offspring (Levin *et al.*, 2019).

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In the present study, we provide one of the first demonstrations that paternal THC administration, prior to mating, results in abnormalities of offspring brain development, specifically targeting acetylcholine (ACh) systems, which provide essential inputs for learning, memory, reward, and mood. We exposed male rats for 28 days to THC at doses commensurate with moderate cannabis use in humans, mated them to drug-naïve females, and then assessed biomarkers of ACh synaptic function in the offspring. We conducted our evaluations longitudinally from adolescence through adulthood, so as to capture early and late stages of brain development and function, and made our assessments in brain regions comprising all the major ACh projections and their corresponding cell bodies. We evaluated the concentration of presynaptic high-affinity choline transporters (hemicholinium-3 [HC3] binding), the activity of choline acetyltransferase (ChAT), and the concentration of α4β2 nicotinic ACh receptors (nAChRs). High-affinity choline transporters and ChAT are both constitutive components of ACh nerve terminals but they differ in their regulatory mechanisms and hence in their functional significance. Choline acetyltransferase is the enzyme that synthesizes ACh, but is not regulated by nerve impulse activity, so that its presence provides an index of the density of ACh innervation (Slotkin, 2008). In contrast, HC3 binding to the choline transporter is directly responsive to neuronal activity (Klemm and Kuhar, 1979), so that comparative effects on HC3 binding and ChAT enable the characterization of both the concentration of ACh terminals and presynaptic impulse activity. We then calculated the HC3/ChAT ratio as an index of presynaptic activity relative to the number of cholinergic nerve terminals (Slotkin, 2008). Finally, the α4β2 nAChR is the most abundant subtype in the mammalian brain and regulates the ability of ACh systems to release other neurotransmitters involved in reward, cognition, and mood (Dani and De Biasi, 2001). These indices have been used successfully to characterize the impact of diverse neurotoxicants and diseases on ACh systems: neuroactive pesticides (Slotkin *et al.*, 2013, 2019b), nicotine or tobacco smoke (Slotkin *et al.*, 2015), polycyclic aromatic hydrocarbons (Slotkin *et al.*, 2019a), and glucocorticoids (Slotkin et al., 2013); and terminal stages of Alzheimer's disease (Slotkin *et al.*, 1994).

MATERIALS AND METHODS

Animal 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. Nine-week-old, sexually mature male Sprague Dawley rats were divided into 3 groups that were dosed daily for 28 days via subcutaneous injection (1 ml/kg) with vehicle only (5% TWEEN-80 in saline), 2 mg/kg/day THC or 4 mg/kg/day THC. These doses of THC mimic the levels achieved with moderate daily cannabis use in humans (Harte and Dow-Edwards, 2010; Irimia *et al.*, 2015; Rubino *et al.*, 2009). We chose to administer THC via this route of exposure so as to blanket gametogenesis round-the-clock, for the entire treatment period, such that that all sperm were exposed to THC. 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 plasma levels and an equally rapid decline (Hložek *et al.*, 2017; Huestis, 2007); the latter route thus leads to a "valley" in between doses, in which gametogenesis could occur during the gap in THC exposure. Additionally, repeated smoke inhalation is potentially stressful, and we wished to avoid that confound. Likewise, bioavailability after oral THC dosing is extremely variable and does not provide the sustained plasma levels needed for this study. Oral THC administration has similar issues (Hložek *et al.*, 2017; Huestis, 2007): irregular bioavailability leading to a lack of sustained plasma levels, and repeated handling stress.

Two days after the last injection, mating was initiated by housing each male rat with a drug-naïve female for 5 days in a breeding cage. After mating, dams were housed singly through gestation and postnatally with their litters. 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 which offspring were housed in same-sex groups. On PN30, 60, 100, and 150, animals were decapitated and brain regions were dissected for determination of ACh synaptic markers: frontal/parietal cortex, temporal/occipital cortex, hippocampus, striatum, midbrain, and brainstem. The 2 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, along with the cerebellum, which is sparse in ACh projections. Tissues were frozen in liquid nitrogen and stored at −80°C until assayed. Each treatment group composed 6 animals of each sex at each age point, with each litter contributing no more than 1 male and 1 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. The techniques have been described in detail previously (Slotkin *et al.*, 2006, 2008a,b), and accordingly, will be presented only in brief. Tissues were thawed and homogenized (Polytron; Brinkmann Instruments, Westbury, New York) in 19 volumes of ice-cold 10 mM sodium-potassium phosphate buffer (pH 7.4). Aliquots of the homogenate were assayed for ChAT using 50 μM [^{14}C]acetyl-coenzyme A as a substrate and activity was determined as the amount of labeled ACh produced relative to tissue protein. For binding measurements, the cell membrane fraction was prepared from the same tissue homogenate and aliquots were assayed for HC3 binding, using a ligand concentration of 2 nM [^{3}H]HC3 with or without 10 μM unlabeled HC3 to displace specific binding; and nAChR binding, using 1 nM [^{3}H]cytisine with or without 10 μM nicotine as a displacer. Ligand binding was calculated relative to the membrane protein concentration. The striatum had insufficient amounts of tissue to permit determination of nAChR binding.

Data analysis. The initial statistical comparisons were conducted by a global analysis of variance (ANOVA; data log transformed because of heterogeneous variance among regions and ages) 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 tests of the same data set. The variables in the global test were treatment (vehicle, THC 2 mg/kg/day, THC 4 mg/kg/day), brain region, age, and sex. Where we identified interactions of treatment with the other factors, data were then subdivided for lower-order ANOVAs to evaluate treatment effects at specific ages or for specific regions. In the absence of interactions, we compiled only the main treatment effects. Significance was assumed at the level of $p < .05$, two tailed.

Data were compiled as means and standard errors. To enable ready visualization of treatment effects across different

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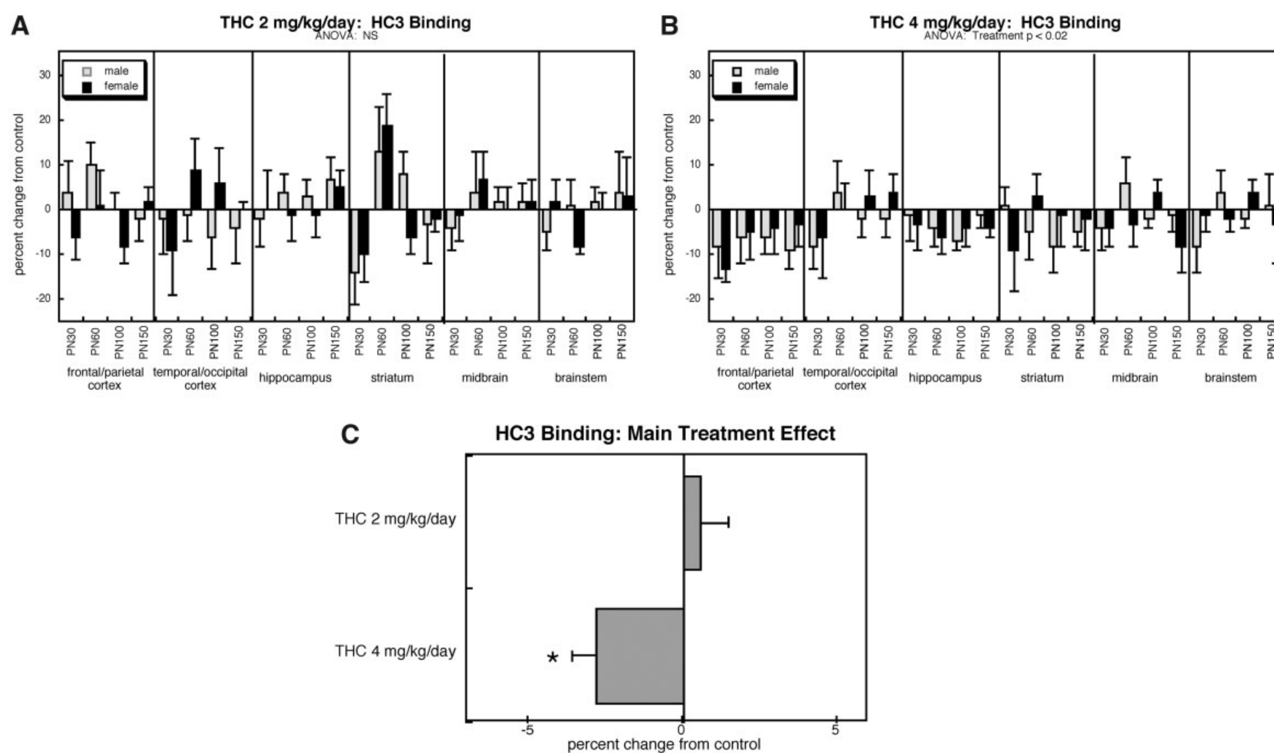

Figure 1. Effects of paternal Δ9-tetrahydrocannabinol (THC) exposure on hemicholinium-3 (HC3) binding: (A) 2 mg/kg/day and (B) 4 mg/kg/day. Data represent means and standard errors, presented as the percent change from control values; complete original data are shown in Supplementary Table S2. Multivariate ANOVA for each treatment appears at the top of the panels. Lower-order tests were not carried out because of the absence of interactions of treatment with other factors. C, The simple main treatment effects, collapsed across region, age, and sex; asterisk denotes the group that is significantly different from control. Abbreviation: NS, not significant.

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. In addition, the log transform evaluates the treatment differences as a proportion to control values, rather than as an arithmetic difference. This was important because of technical limitations: On any single day, we could conduct assays for all treatment groups and both sexes, but for only one region at one age point. Accordingly, representing the data as proportional differences (percent control) enables a full comparison of treatment effects and treatment interactions with all the other variables. 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.

Materials. Animals were purchased from Charles River Laboratories (Raleigh, North Carolina). PerkinElmer Life Sciences (Boston, Massachusetts) was the source for [^{3}H]HC3 (specific activity, 125 Ci/mmol), [^{3}H]cytisine (specific activity 35 Ci/mmol), and [^{14}C]acetyl-coenzyme A (specific activity 6.7 mCi/mmol). Δ9-Tetrahydrocannabinol and all other chemicals came from Sigma-Aldrich (St Louis, Missouri).

RESULTS

Maternal, Litter, and Growth Effects

Paternal THC treatment had no significant effect on maternal weight gain during or after pregnancy or on the proportion of dams giving birth and likewise, litter size and sex ratio were unaffected (data not shown). The offspring displayed no significant treatment-related changes in body weight throughout adolescence and adulthood (Supplementary Table S1). For brain region weights, there was a slight (2%) overall increase in the group receiving the higher dose of THC that was statistically significant compared with either the control or the low-dose THC groups (Supplementary Table S1).

HC3 Binding

Across all groups, global ANOVA (factors of treatment, age, region, and sex) identified a main treatment effect ($p < .02$) but no interactions of treatment with the other factors (Figure 1). Accordingly, we examined each THC dose group for differences from the control, disregarding any potential dependence on the other factors. The group receiving 2 mg/kg/day of THC did not show statistically significant changes in HC3 binding (Figure 1A), whereas the higher dose produced a significant ($p < .02$) overall decrement (Figure 1B). Because of the complexity of the results, we developed a simplified graphical representation of the data, calculating the mean values for main treatment effects, collapsed across region and age (Figure 1C). To do this, we normalized the values to eliminate the scalar differences attributable to region, age, and sex, leaving in only the treatment parameter. This streamlined picture dilutes the effects seen for specific regions or ages by averaging them with data points for which there was no effect or an opposite effect, so that the absolute magnitude appears smaller. Despite this limitation, there was an obvious overall pattern that corresponded to the net outcomes presented in Figures 1A and 1B: little or no change for the low dose of THC but a reduction in the group receiving high dose THC.

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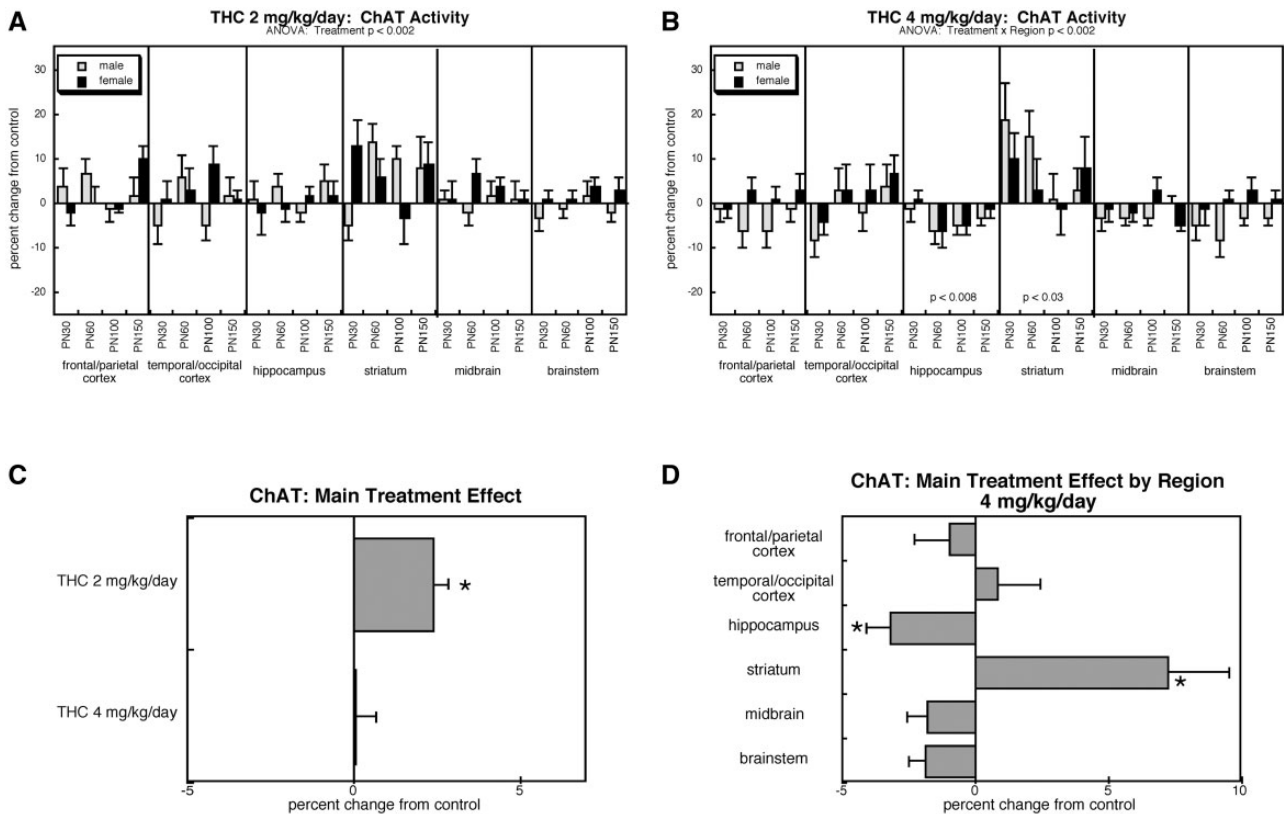

Figure 2. Effects of paternal THC exposure on choline acetyltransferase (ChAT) activity: (A) 2 mg/kg/day and (B) 4 mg/kg/day. Data represent means and standard errors, presented as the percent change from control values; complete original data are shown in Supplementary Table S3. Multivariate ANOVA for each treatment appears at the top of the panels. Lower-order tests were carried out where permitted by the interaction of treatment × region (B). C, The simple main treatment effects, collapsed across region, age, and sex. D, The simple main effects by region, collapsed across age and sex, for the 4 mg/kg/day treatment, which showed a significant treatment × region interaction. In panels (C) and (D), asterisks denote the groups that are significantly different from control.

ChAT Activity

For ChAT activity, the global ANOVA displayed both a main treatment effect ($p < .002$) and a treatment × region interaction ($p < .03$) (Figure 2). Consequently, we examined the two THC doses for treatment effects and the dependence of those effects on brain region. For the group receiving 2 mg/kg/day, we detected only a main treatment effect ($p < .002$), reflecting overall increases in ChAT (Figure 2A). At the higher doses, overall increases were also evident but in a regionally selective manner (treatment × region interaction, $p < .002$; Figure 2B). There were significant increases in the striatum and smaller decreases in the hippocampus. Again, these patterns are most readily seen by examining the main treatment effects collapsed across region and age to highlight main treatment effects or across age and sex to highlight regional differences in the treatment effects at the higher dose (the dose that showed a significant treatment × region interaction). For the main treatment effect, the increases were more consistent at the lower THC dose because of the uniformity across brain regions, whereas the higher dose was inconsistent because of the mixed increases and decreases across the various regions (Figure 2C). The regional selectivity at the higher dose was readily apparent in the values collapsed across age and sex, with a robust increase in the striatum and a decrease of smaller magnitude in the hippocampus (Figure 2D).

HC3/ChAT Ratio

Just as for ChAT activity, global ANOVA for the HC3/ChAT ratio identified a main THC treatment effect ($p < .05$) and a treatment × region interaction ($p < .05$) (Figure 3). At the lower dose, there was an overall lowering of the ratio ($p < .05$ for the main treatment effect; Figure 3A). At the higher dose, THC elicited a larger overall decrease (main treatment effect, $p < .0003$) that was also regionally selective (treatment × region, $p < .0001$), with the largest deficits seen in the striatum and frontal/parietal cortex (Figure 3B). As before, these effects were readily evident when collapsing the results across several variables. The main treatment effect collapsed across region and age showed a dose-dependent overall decrease (Figure 3C). At the higher dose, which displayed a treatment × region interaction, values collapsed across age and sex displayed large decrements in the striatum and frontal/parietal cortex, with sparing of the other regions (Figure 3D). It should be noted that, as with the higher dose, the lower dose also showed a trend toward greater deficits in the striatum but we were not justified in performing a statistical analysis to compare this with the other regions, given the lack of statistical significance for the treatment × region interaction. However, we did compare the effect in the striatum across the two doses and found that the net deficits were indistinguishable from each other (no significant difference between doses, but a main treatment effect distinguishing both from the corresponding control).

nAChR Binding

Because of limitations in the amount of tissue available for assay, evaluations of nAChR binding were not conducted in the striatum, the region showing the greatest changes for ChAT and

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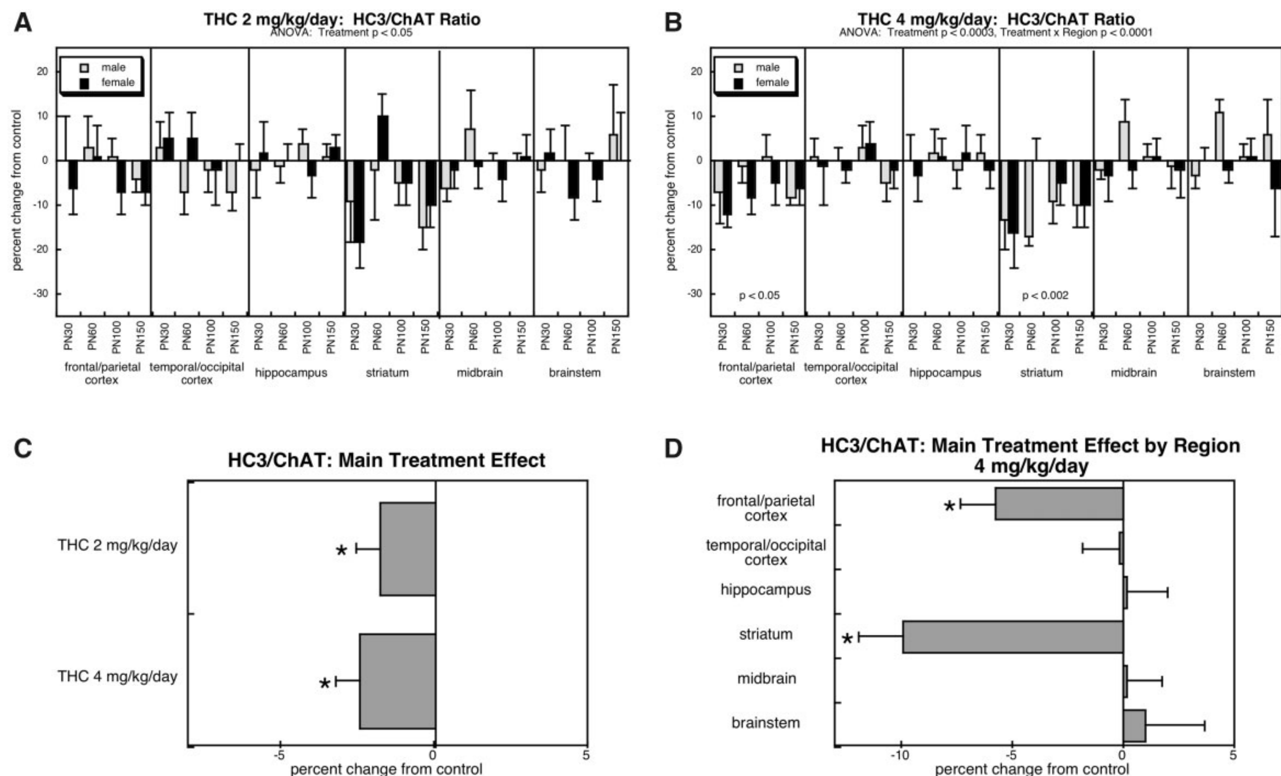

Figure 3. Effects of paternal THC exposure on the HC3/ChAT ratio: (A) 2 mg/kg/day and (B) 4 mg/kg/day. Data represent means and standard errors, presented as the percent change from control values; complete original data are shown in Supplementary Table S4. Multivariate ANOVA for each treatment appears at the top of the panels. Lower-order tests were carried out only where permitted by the interaction of treatment × region (B). C, The simple main treatment effects, collapsed across region, age, and sex. D, The simple main effects by region, collapsed across age and sex, for the 4 mg/kg/day treatment, which showed a significant treatment × region interaction. In panels (C) and (D), asterisks denote the groups that are significantly different from control.

the HC3/ChAT ratio (Figure 4). Nevertheless, we were readily able to identify both a main treatment effect of THC ($p < .0001$) and a treatment × age interaction ($p < .009$). At the lower dose of THC, the overall direction of change was upward, but the primary statistical indication was a significant treatment × age interaction ($p < .05$; Figure 4A). After separation into the various ages, we found statistical significance individually on days PN100 and PN150, representing net effects in opposite directions, ie, a robust decrease on PN100 and a small, but statistically significant increase on PN150. At the higher dose, there was both a significant main treatment effect ($p < .003$), representing a robust overall decrease in nAChR binding, as well as an interaction of treatment × age ($p < .02$), indicating the need for examination of individual age points (Figure 4B). Here, the subdivision by age indicated individual significance at PN60 and PN100, with a consistent decrease across both age points. The main treatment effect collapsed across region and age readily illustrates the difference between the low and high dose groups, with net increases at 2 mg/kg/day THC but decreases at 4 mg/kg/day (Figure 4C). We characterized the age course of effects (dictated by the treatment × age interactions for both dose groups) by collapsing the values across sex and region (Figure 4D). At the low dose, values tended to be above normal initially, dropped precipitously to subnormal values at PN100, and then recovered by PN150. The high dose displayed the same U-shaped pattern but displaced in the direction of greater deficits. Thus, values were approximately normal on PN30, nosedived at PN60 and PN100, and then recovered by PN150.

DISCUSSION

Our results provide some of the first evidence for adverse effects of paternal THC administration on neurodevelopment in the offspring. A chief finding was a dose-dependent deficit in presynaptic ACh activity; at low-dose THC exposure, this defect was partially compensated by upregulation of nAChRs, but at the higher dose, nAChRs were subnormal, an effect that would serve to exacerbate the impairment of presynaptic input. We therefore anticipate significant disparities in neurobehavioral outcomes with the two different exposures, predictions that are currently being verified with a battery of cognitive tests. Importantly, the magnitudes of the effects of paternal THC on ACh synaptic parameters seen here were comparable to those seen with direct fetal exposure known developmental neurotoxicants, including organophosphate pesticides (Slotkin *et al.*, 2013, 2019b), nicotine or tobacco smoke (Slotkin *et al.*, 2015), polycyclic aromatic hydrocarbons (Slotkin *et al.*, 2019a), or glucocorticoids (Slotkin *et al.*, 2013); not surprisingly, though, the effects of paternal THC or all the direct developmental neurotoxicants are decidedly smaller than the drastic changes seen in terminal stages of Alzheimer's disease (Slotkin *et al.*, 1994).

If the effect of paternal THC exposure were simply to block emergence of the ACh phenotype or to interfere with subsequent development of ACh neurons, then we would have expected to see decrements in both ChAT activity and HC3 binding (because both are constitutive proteins of ACh neurons), without regional selectivity. Likewise, if the effects were directed toward general expression of either of the proteins, then

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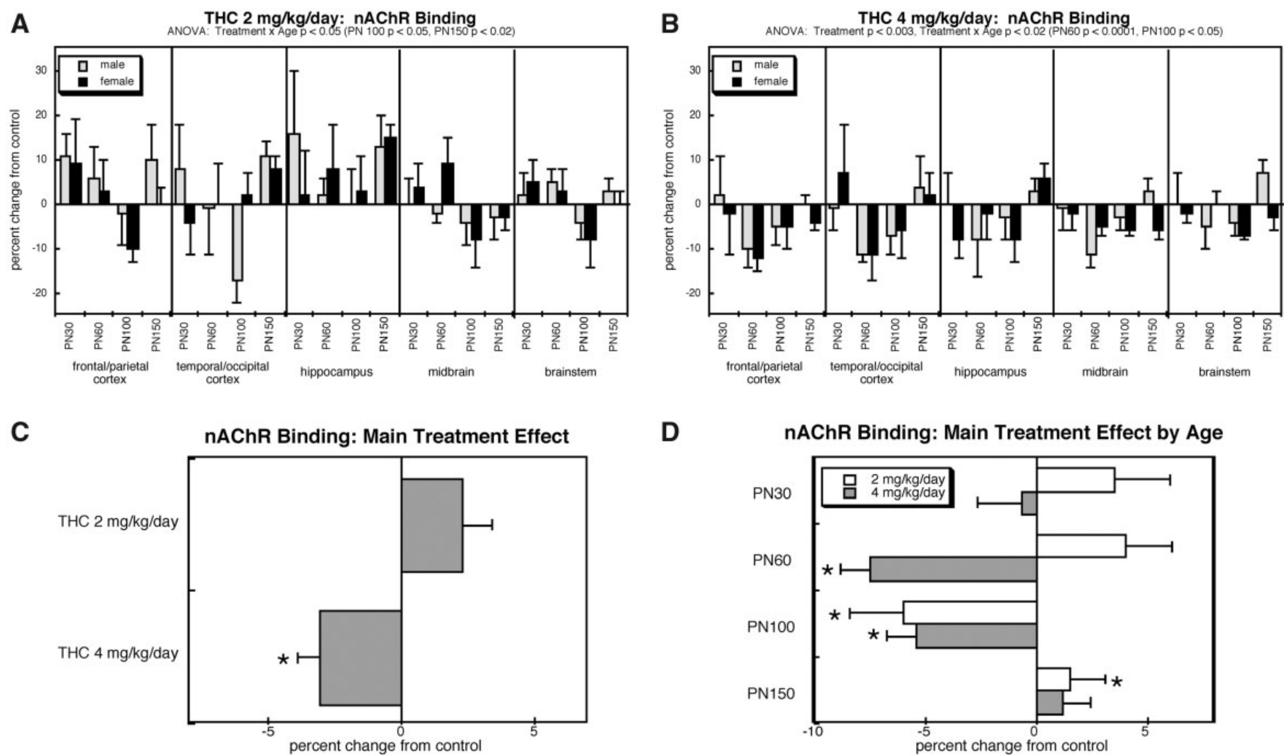

Figure 4. Effects of paternal THC exposure on nicotinic acetylcholine receptors (nAChR) binding: (A) 2 mg/kg/day and (B) 4 mg/kg/day. Data represent means and standard errors, presented as the percent change from control values; complete original data are shown in Supplementary Table S5. Multivariate ANOVA for each treatment appears at the top of the panels. Lower-order tests were carried where permitted by the interaction of treatment × age. C, The simple main treatment effects, collapsed across region, age, and sex. D, The simple main effects by age, collapsed across region and sex. In panels (C) and (D), asterisks denote the groups that are significantly different from control.

there would be no specificity for any particular brain region. Neither of these cases was true. We found net increases in ChAT but decreases in HC3 binding, with targeting of specific brain regions for ChAT and for the HC3/ChAT ratio. Further, effects on ChAT in the striatum and hippocampus were in opposite directions, and reductions in the HC3/ChAT ratio were selective for two regions (frontal/parietal cortex and striatum), results that are incompatible with global interference with the development of ACh neurons or of specific proteins. Instead, our data point to long-term regulatory changes in ACh presynaptic activity (HC3 binding and HC3/ChAT ratio), superimposed on regionally selective effects in the density of ACh terminals (ChAT activity).

The effects on nAChRs further support the conclusion that paternal THC disrupts developmental patterns of ACh synaptic function. As noted, the two doses produced opposite effects: nAChR upregulation at the lower dose, but suppression at the higher dose. Again, superimposed on these main treatment effects, there were changes indicative of other factors, in this case age dependence. Despite the different directions in the net treatment effect for the low versus high dose, both showed a pronounced deficit in young adulthood that resolved by PN150. It is important to note that, in all brain regions, nAChRs decline by about one-third from PN60 to PN150 (Supplementary Table S5), so that the temporal pattern for effects of THC actually presents an accelerated loss of nAChRs. It would therefore be interesting to pursue the effects of paternal THC on ACh systems in senescence of the offspring, specifically evaluating whether the exposure likewise speeds up the functional decline associated with aging.

Although our studies did not directly assess the mechanism(s) underlying the adverse effects of paternal THC on development of ACh systems, there is a reason to believe that a likely cause is epigenetic changes in the sperm for genes that control subsequent nervous system development (Murphy *et al.*, 2018; Schrott *et al.*, 2020; Szutorisz and Hurd, 2018). It would obviously be useful to identify which genes are affected and whether the epigenetic changes are transient or permanent. If they are transient, it would be necessary only for gene expression to be altered during a critical phase of ACh neuronal development, but the effects would essentially be “hit-and-run,” making their identification problematic. If they are permanent, then detection of the relevant changes would be much easier. Future work will be needed to resolve these possibilities.

To our knowledge, ours is the first study to demonstrate an effect of paternal THC administration on specific neural pathways in the offspring that are involved in cognition and reward. Indeed, although paternal effects have been studied for a number of psychoactive drugs, nearly all have involved behavioral measurements alone, with only a few articles focusing on underlying mechanisms involving synaptic function or structure (Goldberg and Gould, 2019). Even where these have been evaluated, no prior studies have followed multiple indices of specific neurotransmitter pathways over a full temporal-spatial progression. As seen here, paternal THC alters the developmental *trajectory* of ACh systems, and thus, examination of a single region or time point can give a misleading sense of safety; further, even small (but significant) main treatment effects can contain and mask much more sizeable differences at specific time points or in particular brain regions. Behavioral studies confirm

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the importance of the impact on ACh systems. Paternal THC administration elicits persistent attentional deficits in the offspring (Levin *et al.*, 2019), and ACh is known to play a critical role in attention (Klinkenberg *et al.*, 2011).

Perhaps most importantly, our studies demonstrate that the adverse impacts of drug exposure on brain development are not limited to effects mediated by the embryonic or fetal chemical environment, but rather that vulnerability is engendered by exposures occurring prior to conception, involving the father as well as the mother. Ensuring an environment that produces healthy neurodevelopment is a responsibility of both parents.

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 Pardieck Law (Seymour, Indiana), Gjording Fouser (Boise, Idaho), Thorsnes Bartolotta McGuire (San Diego, California), and Cracken Law (Dallas, Texas).

FUNDING

John Templeton Foundation.

ACKNOWLEDGMENTS

The authors thank Dr Zade Holloway and Dr Andrew Hawkey for assistance with the animal treatments.

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SUPPLEMENT

Paternal Δ9-Tetrahydrocannabinol Exposure Prior to Mating, Elicits Deficits in Cholinergic Synaptic Function in the Offspring

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Edward D. Levin and Frederic J. Seidler

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TABLE S1: Body and Brain Region Weights (mean ± SE)

	Postnatal Age (days)	Male			Female		
		Control	THC 2 mg/kg	THC 4 mg/kg	Control	THC 2 mg/kg	THC 4 mg/kg
Body Weight (g)	30	120 ± 2	117 ± 2	123 ± 6	113 ± 3	102 ± 5	107 ± 4
	60	330 ± 3	327 ± 9	324 ± 23	232 ± 3	228 ± 2	231 ± 10
	100	524 ± 8	488 ± 12	526 ± 18	287 ± 6	283 ± 8	289 ± 7
	150	544 ± 19	533 ± 12	532 ± 17	316 ± 7	306 ± 8	306 ± 6
Region Weight (mg)							
frontal/parietal cortex	30	230 ± 8	226 ± 8	233 ± 10	225 ± 8	217 ± 7	230 ± 7
	60	240 ± 21	264 ± 6	268 ± 9	260 ± 14	251 ± 5	254 ± 3
	100	262 ± 6	270 ± 4	273 ± 7	255 ± 2	246 ± 12	242 ± 10
	150	265 ± 12	280 ± 12	270 ± 7	248 ± 9	258 ± 10	254 ± 5
temporal/occipital cortex	30	166 ± 7	164 ± 5	167 ± 4	153 ± 4	160 ± 6	154 ± 5
	60	175 ± 4	176 ± 9	192 ± 2	163 ± 10	159 ± 7	172 ± 6
	100	193 ± 11	194 ± 9	189 ± 11	174 ± 5	178 ± 4	170 ± 6
	150	183 ± 11	184 ± 7	199 ± 8	170 ± 6	162 ± 7	182 ± 10
hippocampus	30	128 ± 5	138 ± 2	127 ± 5	126 ± 6	121 ± 4	128 ± 4
	60	150 ± 6	145 ± 6	160 ± 4	144 ± 6	145 ± 8	144 ± 7
	100	161 ± 4	156 ± 12	160 ± 8	146 ± 8	147 ± 8	152 ± 7
	150	157 ± 8	158 ± 5	164 ± 9	150 ± 4	143 ± 8	154 ± 7
striatum	30	95 ± 5	87 ± 8	80 ± 6	87 ± 5	80 ± 3	93 ± 4
	60	105 ± 3	106 ± 6	107 ± 4	109 ± 9	95 ± 5	93 ± 8
	100	100 ± 2	101 ± 4	126 ± 11	106 ± 8	98 ± 5	100 ± 10
	150	111 ± 12	124 ± 13	123 ± 8	110 ± 12	87 ± 9	105 ± 8
midbrain	30	285 ± 9	269 ± 9	285 ± 8	258 ± 9	250 ± 10	262 ± 7
	60	321 ± 13	327 ± 8	346 ± 5	311 ± 10	298 ± 19	325 ± 7
	100	359 ± 11	375 ± 6	374 ± 8	325 ± 4	343 ± 8	336 ± 10
	150	363 ± 16	348 ± 20	369 ± 10	315 ± 7	355 ± 6	347 ± 6
brainstem	30	169 ± 4	166 ± 4	163 ± 4	157 ± 3	152 ± 2	157 ± 5
	60	209 ± 5	210 ± 5	220 ± 12	199 ± 7	213 ± 17	202 ± 8
	100	244 ± 7	239 ± 4	241 ± 3	235 ± 4	221 ± 4	233 ± 9
	150	243 ± 12	258 ± 8	261 ± 5	250 ± 5	238 ± 6	248 ± 12

Data represent mean ± SE obtained from 6 animals in each treatment group for each age and sex. For body weight, three-factor ANOVA (treatment, age, sex) indicates no significant treatment effects or interactions of treatment with the other factors. For brain region weight, four-factor ANOVA (treatment, age, sex, region) indicates a significant main treatment effect ($p < 0.005$) but no interactions of treatment with the other factors. The THC 4 mg/kg group was significantly different from either control ($p < 0.002$) or low dose THC ($p < 0.03$), representing an overall increase of approximately 2%.

Note that weights for frontal/parietal cortex and temporal/occipital cortex are for the right hemisphere only, the portion used in this study.

TABLE S2: HC3 Binding (mean ± SE)

Region	Postnatal Age (days)	Male (fmol/mg protein)			Female (fmol/mg protein)		
		Control	THC 2 mg/kg	THC 4 mg/kg	Control	THC 2 mg/kg	THC 4 mg/kg
frontal/parietal cortex	30	16.5 ± 1.2	17.1 ± 1.1	15.2 ± 1.1	18.2 ± 0.7	17.1 ± 1.0	15.8 ± 0.6
	60	18.1 ± 1.5	19.9 ± 1.0	17.1 ± 1.4	21.1 ± 1.6	21.4 ± 1.6	20.1 ± 1.2
	100	16.3 ± 0.4	16.3 ± 0.7	15.4 ± 0.7	19.2 ± 0.6	17.6 ± 0.7	18.5 ± 1.1
	150	17.3 ± 0.9	17.0 ± 0.8	15.7 ± 0.7	17.3 ± 0.9	17.6 ± 0.5	16.8 ± 0.9
temporal/occipital cortex	30	11.1 ± 0.8	10.9 ± 0.9	10.2 ± 0.5	12.1 ± 1.6	11.0 ± 1.2	11.4 ± 1.1
	60	10.8 ± 0.4	10.7 ± 0.6	11.2 ± 0.7	11.7 ± 0.6	12.8 ± 0.8	11.7 ± 0.7
	100	11.2 ± 0.5	10.5 ± 0.8	11.4 ± 0.9	10.8 ± 0.3	11.5 ± 0.8	11.6 ± 0.8
	150	10.0 ± 0.3	9.6 ± 0.8	9.8 ± 0.4	10.2 ± 0.3	10.2 ± 0.2	10.6 ± 0.4
hippocampus	30	14.2 ± 0.9	14.0 ± 0.9	14.1 ± 0.9	15.6 ± 1.4	15.6 ± 1.4	15.2 ± 1.0
	60	13.0 ± 0.8	13.5 ± 0.5	12.5 ± 0.5	14.3 ± 0.6	14.1 ± 0.9	13.5 ± 0.6
	100	12.8 ± 0.8	13.2 ± 0.5	11.9 ± 0.3	13.7 ± 0.6	13.5 ± 0.7	13.2 ± 0.6
	150	13.0 ± 0.5	13.9 ± 0.6	12.9 ± 0.4	13.5 ± 0.3	14.2 ± 0.6	13.0 ± 0.3
striatum	30	102 ± 7	88 ± 7	103 ± 4	117 ± 6	105 ± 7	106 ± 10
	60	104 ± 4	117 ± 10	99 ± 6	95 ± 6	113 ± 7	98 ± 5
	100	64 ± 3	69 ± 3	59 ± 4	70 ± 2	66 ± 3	69 ± 5
	150	58 ± 3	56 ± 5	55 ± 2	61 ± 3	60 ± 2	60 ± 4
midbrain	30	14.0 ± 0.3	13.4 ± 0.7	13.5 ± 0.7	14.1 ± 0.7	13.9 ± 0.8	13.6 ± 0. 5
	60	10.6 ± 0.5	11.0 ± 0.9	11.2 ± 0.6	11.4 ± 0.6	12.2 ± 0.7	11.1 ± 0.6
	100	10.6 ± 0.4	10.8 ± 0.3	10.4 ± 0.2	11.1 ± 0.4	11.1 ± 0.5	11.5 ± 0.3
	150	9.4 ± 0.4	9.6 ± 0.4	9.3 ± 0.4	9.7 ± 0.4	9.9 ± 0.5	8.9 ± 0.6
brainstem	30	9.9 ± 0.5	9.4 ± 0.4	9.1 ± 0.6	9.9 ± 0.6	10.1 ± 0.5	9.8 ± 0.4
	60	8.1 ± 0.4	8.2 ± 0.5	8.4 ± 0.4	8.6 ± 0.3	7.9 ± 0.2	8.4 ± 0.3
	100	7.8 ± 0.2	7.8 ± 0.3	7.9 ± 0.5	7.9 ± 0.3	7.9 ± 0.3	7.8 ± 0.3
	150	6.7 ± 0.4	7.0 ± 0.6	6.8 ± 0.5	6.6 ± 0.4	6.8 ± 0.6	6.4 ± 0.6

Data represent mean ± SE obtained from 6 animals in each treatment group for each age and sex. Results of multivariate ANOVA are provided in the main text.

TABLE S3: ChAT Activity (mean ± SE)

Region	Postnatal Age (days)	Male (nmol/min per mg protein)			Female (nmol/min per mg protein)		
		Control	THC 2 mg/kg	THC 4 mg/kg	Control	THC 2 mg/kg	THC 4 mg/kg
frontal/parietal cortex	30	0.95 ± 0.02	0.99 ± 0.04	0.94 ± 0.03	1.03 ± 0.04	1.01 ± 0.03	1.02 ± 0.02
	60	0.99 ± 0.05	1.06 ± 0.03	0.93 ± 0.04	1.07 ± 0.04	1.07 ± 0.04	1.10 ± 0.03
	100	1.42 ± 0.02	1.41 ± 0.05	1.34 ± 0.06	1.49 ± 0.06	1.48 ± 0.02	1.50 ± 0.05
	150	1.46 ± 0.06	1.49 ± 0.06	1.44 ± 0.05	1.44 ± 0.06	1.58 ± 0.04	1.49 ± 0.06
temporal/occipital cortex	30	0.76 ± 0.02	0.72 ± 0.03	0.70 ± 0.03	0.76 ± 0.04	0.77 ± 0.03	0.73 ± 0.02
	60	0.63 ± 0.03	0.67 ± 0.03	0.65 ± 0.03	0.66 ± 0.01	0.68 ± 0.03	0.68 ± 0.04
	100	1.09 ± 0.03	1.04 ± 0.03	1.07 ± 0.04	1.07 ± 0.01	1.17 ± 0.04	1.10 ± 0.06
	150	1.01 ± 0.03	1.03 ± 0.04	1.05 ± 0.05	1.02 ± 0.04	1.03 ± 0.02	1.09 ± 0.04
hippocampus	30	0.83 ± 0.03	0.84 ± 0.03	0.82 ± 0.02	0.88 ± 0.03	0.86 ± 0.04	0.89 ± 0.02
	60	1.34 ± 0.04	1.39 ± 0.04	1.26 ± 0.04	1.38 ± 0.02	1.36 ± 0.04	1.30 ± 0.05
	100	1.29 ± 0.01	1.27 ± 0.02	1.22 ± 0.03	1.34 ± 0.01	1.37 ± 0.03	1.27 ± 0.03
	150	1.31 ± 0.02	1.38 ± 0.05	1.27 ± 0.03	1.32 ± 0.02	1.35 ± 0.04	1.31 ± 0.02
striatum	30	2.27 ± 0.07	2.16 ± 0.08	2.70 ± 0.18	2.41 ± 0.13	2.73 ± 0.15	2.65 ± 0.15
	60	2.29 ± 0.11	2.61 ± 0.09	2.63 ± 0.14	2.46 ± 0.15	2.61 ± 0.09	2.53 ± 0.18
	100	2.95 ± 0.11	3.25 ± 0.10	2.99 ± 0.18	3.28 ± 0.20	3.19 ± 0.19	3.27 ± 0.19
	150	2.95 ± 0.24	3.19 ± 0.22	3.03 ± 0.14	3.04 ± 0.12	3.31 ± 0.15	3.28 ± 0.20
midbrain	30	1.15 ± 0.02	1.16 ± 0.03	1.12 ± 0.04	1.12 ± 0.03	1.13 ± 0.05	1.11 ± 0.03
	60	1.23 ± 0.03	1.21 ± 0.04	1.19 ± 0.03	1.23 ± 0.03	1.31 ± 0.04	1.21 ± 0.03
	100	0.99 ± 0.02	1.01 ± 0.03	0.96 ± 0.02	1.01 ± 0.02	1.05 ± 0.02	1.04 ± 0.03
	150	1.11 ± 0.02	1.12 ± 0.04	1.11 ± 0.02	1.18 ± 0.02	1.19 ± 0.02	1.12 ± 0.01
brainstem	30	1.71 ± 0.02	1.66 ± 0.05	1.63 ± 0.05	1.69 ± 0.02	1.71 ± 0.03	1.68 ± 0.06
	60	2.19 ± 0.04	2.18 ± 0.04	2.02 ± 0.08	2.15 ± 0.04	2.16 ± 0.04	2.17 ± 0.05
	100	1.66 ± 0.03	1.68 ± 0.03	1.65 ± 0.03	1.74 ± 0.03	1.77 ± 0.06	1.72 ± 0.02
	150	1.87 ± 0.05	1.84 ± 0.04	1.82 ± 0.04	1.86 ± 0.04	1.92 ± 0.05	1.87 ± 0.04

Data represent mean ± SE obtained from 6 animals in each treatment group for each age and sex. Results of multivariate ANOVA are provided in the main text.

TABLE S4: HC3/ChAT ratio (mean ± SE)

Region	Postnatal Age (days)	Male			Female		
		Control	THC 2 mg/kg	THC 4 mg/kg	Control	THC 2 mg/kg	THC 4 mg/kg
frontal/parietal cortex	30	17.5 ± 1.5	17.5 ± 1.7	16.3 ± 1.2	17.8 ± 1.2	16.7 ± 1.1	15.6 ± 0.5
	60	18.4 ± 1.6	19.0 ± 1.3	18.2 ± 0.8	19.8 ± 1.5	20.0 ± 1.4	18.2 ± 0.8
	100	11.5 ± 0.4	11.6 ± 0.4	11.6 ± 0.6	12.9 ± 0.4	12.0 ± 0.6	12.3 ± 0.6
	150	11.9 = 0.6	11.4 ± 0.3	10.9 ± 0.3	12.0 ± 0.4	11.1 ± 0.4	11.3 ± 0.5
temporal/occipital cortex	30	14.5 ± 0.9	15.0 ± 0.9	14.7 ± 0.7	15.7 ± 1.7	14.0 ± 0.9	15.5 ± 1.4
	60	17.1 ± 0.9	15.9 ± 0.9	17.1 ± 0.5	17.9 ± 0.9	18.8 ± 1.0	17.3 ± 0.6
	100	10.3 ± 0.5	10.1 ± 0.5	10.6 ± 0.5	10.1 ± 0.3	9.9 ± 0.8	10.5 ± 0.5
	150	9.9 ± 0.4	9.2 ± 0.4	9.4 ± 0.4	10.0 ± 0.3	10.0 ± 0.4	9.8 ± 0.4
hippocampus	30	17.1 ± 1.2	16.8 ± 1.1	17.1 ± 1.0	17.7 ± 1.6	18.1 ± 1.2	17.1 ± 1.1
	60	9.8 ± 0.8	9.7 ± 0.4	10.0 ± 0.5	10.3 ± 0.5	10.3 ± 0.4	10.4 ± 0.4
	100	10.0 ± 0.6	10.4 ± 0.3	9.8 ± 0.4	10.2 ± 0.4	9.9 ± 0.5	10.4 ± 0.6
	150	10.0 ± 0.4	10.1 ± 0.3	10.2 ± 0.4	10.2 ± 0.2	10.5 ± 0.3	10.0 ± 0.4
striatum	30	45 ± 3	41 ± 4	39 ± 3	49 ± 3	40 ± 3	41 ± 4
	60	46 ± 2	45 ± 5	38 ± 1	39 ± 2	43 ± 2	39 ± 2
	100	22 ± 1	21 ± 1	20 ± 1	22 ± 2	21 ± 1	21 ± 1
	150	20 ± 2	17 ± 1	18 ± 1	20 ± 1	18 ± 1	18 ± 1
midbrain	30	12.2 ± 0.4	11.5 ± 0.4	12.0 ± 0.3	12.7 ± 0.7	12.4 ± 0.5	12.3 ± 0.7
	60	8.6 ± 0.4	9.2 ± 0.8	9.4 ± 0.4	9.4 ± 0.7	9.3 ± 0.5	9.2 ± 0.4
	100	10.7 ± 0.6	10.7 ± 0.2	10.8 ± 0.3	11.0 ± 0.4	10.6 ± 0.6	11.1 ± 0.4
	150	8.5 ± 0.5	8.5 ± 0.2	8.4 ± 0.4	8.2 ± 0.3	8.3 ± 0.4	8.0 ± 0.5
brainstem	30	5.8 ± 0.3	5.7 ± 0.3	5.6 ± 0.2	5.8 ± 0.4	5.9 ± 0.3	5.8 ± 0.2
	60	3.7 ± 0.2	3.7 ± 0.3	4.1 ± 0.1	4.0 ± 0.1	3.7 ± 0.2	3.9 ± 0.1
	100	4.7 ± 0.1	4.7 ± 0.2	4.8 ± 0.3	4.6 ± 0.1	4.5 ± 0.3	4.5 ± 0.1
	150	3.6 ± 0.2	3.8 ± 0.4	3.8 ± 0.3	3.6 ± 0.2	3.6 ± 0.4	3.4 ± 0.4

Data represent mean ± SE obtained from 6 animals in each treatment group for each age and sex. Results of multivariate ANOVA are provided in the main text.

TABLE S5: nAChR Binding (mean ± SE)

Region	Postnatal Age (days)	Male (fmol/mg protein)			Female (fmol/mg protein)		
		Control	THC 2 mg/kg	THC 4 mg/kg	Control	THC 2 mg/kg	THC 4 mg/kg
frontal/parietal cortex	30	56 ± 2	62 ± 3	57 ± 5	58 ± 3	63 ± 6	57 ± 5
	60	70 ± 2	74 ± 5	63 ± 3	69 ± 3	71 ± 5	61 ± 2
	100	55 ± 2	54 ± 4	52 ± 2	60 ± 3	54 ± 2	57 ± 3
	150	51 ± 2	56 ± 4	51 ± 1	52 ± 2	52 ± 2	50 ± 1
temporal/occipital cortex	30	72 ± 4	78 ± 7	71 ± 4	72 ± 8	69 ± 5	77 ± 8
	60	81 ± 3	80 ± 8	72 ± 2	85 ± 3	85 ± 8	76 ± 5
	100	72 ± 6	60 ± 4	67 ± 3	64 ± 3	65 ± 3	60 ± 4
	150	57 ± 3	63 ± 3	59 ± 4	61 ± 3	66 ± 2	62 ± 3
hippocampus	30	44 ± 2	51 ± 6	44 ± 3	48 ± 3	49 ± 5	44 ± 2
	60	46 ± 3	47 ± 2	42 ± 2	49 ± 2	53 ± 5	48 ± 3
	100	37 ± 3	37 ± 3	36 ± 2	39 ± 2	40 ± 3	36 ± 2
	150	32 ± 2	36 ± 2	33 ± 1	33 ± 3	38 ± 1	35 ± 1
midbrain	30	77 ± 3	77 ± 5	76 ± 4	80 ± 3	83 ± 4	78 ± 3
	60	90 ± 3	88 ± 2	80 ± 3	88 ± 3	96 ± 5	84 ± 2
	100	67 ± 2	64 ± 3	65 ± 2	72 ± 3	66 ± 4	67 ± 1
	150	66 ± 2	64 ± 3	68 ± 2	69 ± 3	67 ± 2	65 ± 1
brainstem	30	42 ± 1	43 ± 2	42 ± 3	42 ± 1	44 ± 2	41 ± 1
	60	39 ± 1	41 ± 1	37 ± 2	40 ± 1	41 ± 2	40 ± 1
	100	30 ± 1	30± 1	31 ± 1	31 ±	32 ± 1	31 ± 1
	150	29 ± 1	30 ± 1	31 ± 1	31 ± 1	31 ± 1	30 ± 1

Data represent mean ± SE obtained from 6 animals in each treatment group for each age and sex. Results of multivariate ANOVA are provided in the main text.