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Running Head: Gestational exposure to nicotine

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Abstract

Cigarette smoking is associated with a wide variety of adverse reproductive outcomes, including increased infant mortality and decreased birth weight. Prenatal exposure to tobacco smoke, of which nicotine is a major teratogenic component, has also been linked to the acceleration of the risk for different psychiatric disorders, including conduct disorder and attention deficit hyperactivity disorder (ADHD). Whether this increased risk is influenced by the direct effects of gestational nicotine exposure on the developing fetus remains uncertain. Here we provide experimental evidence for the effects of prenatal nicotine exposure on measures of attention and impulsivity in adult male rats. Offspring of females exposed during pregnancy to 0.06 mg/ml nicotine solution as the only source of water (daily consumption: 69.6±1.4 ml/kg; nicotine blood level: 96.0±31.9 ng/ml) had lower birth weight and delayed sensorimotor development measured by negative geotaxis, righting reflex and grip strength. In the 5-choice serial reaction time test, adult rats showed increased numbers of anticipatory responses and omissions errors, more variable response times and lower accuracy with evidence of delayed learning of the task demands when the 1 s stimulus duration was introduced. In contrast, prenatal nicotine exposure had no effect on exploratory locomotion or delay-discounting test. Prenatal nicotine exposure increased expression of the D5 dopamine receptor gene in the striatum, but did not change expression of other dopamine-related genes (DRD4, DAT1, NR4A2, TH) in either the striatum or the prefrontal cortex. These data suggest a direct effect of prenatal nicotine exposure on important aspects of attention, inhibitory control or learning later in life.

Key words: nicotine, gestation, attention, impulsivity, dopamine system, ADHD.

Introduction

Cigarette smoking is associated with a wide variety of adverse reproductive outcomes (Jauniaux and Burton, 2007), including increased infant mortality and decreased birth weight (Ernst, *et al* 2001; Winzer-Serhan, 2008). Prenatal exposure to nicotine, a major teratogenic component of tobacco, modulates neurotransmitter release, gene expression, neuronal outgrowth, cell survival and synapse formation and maturation (Dwyer, *et al* 2008); and has also been linked to increased risk for childhood onset psychiatric disorders including attention deficit hyperactivity disorder (ADHD) (for review, see Pauly and Slotkin, 2008; Cornelius and Day, 2009). Recent literature suggests that the association with ADHD might be mediated by genetic effects rather than the direct toxic effects of nicotine (Thapar, *et al* 2009; D'Onofrio, *et al* 2008), but this has yet to be evaluated in an animal model.

ADHD is characterized by developmentally inappropriate and impairing levels of inattentive, hyperactive and impulsive behaviors (Kuntsi, *et al* 2006) affecting approximately 5% of children (Polanczyk, *et al* 2007) and persisting into adult life in around 65% of cases (Faraone, *et al* 2006). Heritability for ADHD is around 76% (Faraone, *et al* 2005). Candidate gene studies have identified associations with genetic variants within or close to dopamine (DA) system genes including the D4 and D5 receptor genes (Li, *et al* 2006). Other DA system genes potentially associated with ADHD include the D1 receptor (DRD1; Misener, *et al* 2004), the DA transporter (DAT1; Asherson, *et al* 2007) and the DA-related intracellular transcription factor (NR4A2; Smith, *et al* 2005). More recently rare copy number variants greater than 500 kb were found to be over-represented in ADHD cases compared to controls, implicating neurodevelopmental processes in the etiology of ADHD (Williams et al. 2010).

A range of neuropsychological performance deficits is associated with ADHD, although none have been unequivocally implicated in the etiology of ADHD symptoms (Johnson et al.,

2009). Furthermore, there is considerable heterogeneity in the pattern of associated cognitive deficits, leading to contemporary models of ADHD that emphasize the role of two or more independent processes (Johnson et al., 2009; Kuntsi et al., 2010). Twin studies find partially overlapping etiological influences on the two core symptom domains of inattention and hyperactivity-impulsivity (McLoughlin et al., 2007). Overall these findings indicate that ADHD is a heterogeneous condition with distinct etiological influences conferring risk to different behavioural and neuropsychological components of the disorder.

Cognitive performance impairments are seen on tasks measuring response inhibition and sustained attention such as the continuous performance test (Johnson, *et al* 2009; Willcutt, *et al* 2005). Compared to healthy controls individuals with ADHD make more errors of omission (index of sustained attention) and commission (index of response inhibition), and have slower and more variable response times thought to reflect impairments in arousal or cognitive-energetic processes (Epstein, *et al* 2003; Epstein, *et al* 2001; Klein, *et al* 2006; Uebel, *et al* 2010; Andreou, *et al* 2007; Johnson et al., 2009). Cognitive performance deficits have also been observed in choice impulsivity measured as the tendency to choose small rewards sooner than larger rewards later (Marco, *et al* 2009; Paloyelis, *et al* 2009). The ADHD combined subtype has been linked to the tendency to discount rewards more steeply, although evidence to date is limited and somewhat inconsistent (Barkley, *et al* 2001; Scheres, *et al* 2006; Paloyelis et al., 2009).

Comparable aspects of cognitive performance can be measured in animals. Reaction time mean and variability, accuracy errors, omission errors and anticipatory responses, thought to reflect processes related to attention and impulsivity, can be assessed with the 5-Choice Serial Reaction Time Test (5-CSRTT); choice impulsivity can be assessed in delay-discounting paradigms (Winstanley, *et al* 2006). These aspects of cognitive function have yet to be studied in animals prenatally exposed to nicotine, although other experimental measures have

been investigated. Identified effects include intolerance to hypoxia (Slotkin, *et al* 1995), hyperactivity (Tizabi, *et al* 1997; Pauly, *et al* 2004), cognitive impairments (choice accuracy in spatial spontaneous alteration: Levin, *et al* 1993; acquisition and retention of the avoidance behavior: Vaglenova, *et al* 2008; radial-arm maze choice accuracy: Sorenson *et al* 1991), increased anxiety (Vaglenova, *et al* 2004) and delayed development and maturation (Peters and Ngan, 1982; Murrin, *et al* 1987; Schneider, *et al* 2010). However, these findings are not entirely consistent since some studies found no decrement in avoidance behavior and spatial learning (Bertolini, *et al* 1982; Paulson, *et al* 1993), as well as hypoactivity (Lesage, *et al* 2006). Prenatal nicotine exposure has also been found to produce alterations in the development of neurochemical markers for DA in offspring (Fung, 1989; Ribary and Lichtensteiger, 1989; Muneoka, *et al* 1999).

In the current study we evaluate in an animal model whether prenatal nicotine exposure influences cognitive functions related to ADHD in adult life. In addition, maturational and developmental data were collected and activity level in a novel environment measured in adults. Because of the strong *a priori* hypothesis of altered dopamine regulation in ADHD, we also determined mRNA expression for markers of DA function in frontal cortex and striatum, regions known to be involved in ADHD (Durston et al., 2010).

Materials and Methods

Subjects

Both male (N=25) and female (N=67) Lister hooded rats (Harlan Olac, Bicester, UK), were used. They were housed individually (except during mating) and had *ad libitum* access to food and drinking fluids (tap water or nicotine solutions). Females (224-303 g at the beginning of the study) were weighed 3 times during the week preceding the start of the experiment. The average weight was calculated for each rat. Fifty-six females were divided

into two groups (NIC exposure, n=19, foster mothers, n=37) balanced according to their body weight. Nineteen of the foster mothers were randomly chosen for use as a control group for comparisons of pregnancy and litter characteristics. An additional group of females (n=11) was used to assess nicotine blood levels in pregnant animals. National and institutional guidelines for housing and treatment were followed. Animals were maintained in a temperature-controlled environment (21 ± 1°C) at 50% humidity and on a 12-h light/dark cycle.

Drug

Nicotine bitartrate (Sigma, USA) was dissolved in the drinking water at varying concentrations. Nicotine-containing water was adjusted to the pH of drinking water (pH 7) with 0.001 N NaOH. Doses are presented as those of nicotine base.

Nicotine consumption and nicotine blood level

The procedure was based on the methods of Schneider *et al* (2010) with some modifications. In brief, 19 females were habituated to increasing concentrations of nicotine solution (0.02, 0.04, 0.06 mg/ml) in tap water as the only source of fluid for 3 weeks before mating. The final concentration used was 0.06 mg/ml. Females drinking less than 10 ml of nicotine solution per day had supplementary access to water. Nicotine treatment was terminated on the day that pups were delivered. Female used as foster mothers (n=37) continued to receive tap water. The females (n=11) used to evaluate nicotine blood levels during the second week of pregnancy were exposed to nicotine in an identical manner and nicotine concentrations were determined using tail vein blood and gas chromatography.

Mating

Females were controlled according to their estrous cycle. Females in proestrus and estrous were mated during the dark phase of the day at the beginning of the fourth week of nicotine

exposure. Nicotine solution was not withheld before mating. The day on which a vaginal plug or spermatozoa were found in the vaginal smear was defined as gestational day 0.

Pregnancy

Pregnant females from the nicotine and control groups were weighed twice weekly. A 0.06 mg/ml nicotine solution was used throughout pregnancy and its consumption was assessed daily. Rats drinking less than 10 ml of nicotine solution on any particular day were given access to tap water for 3 min. Food consumption was evaluated 3 times a week.

Birth measures

All dams were checked twice daily (before 8 a.m. and after 4.30 p.m.) starting a few days before delivery. Deliveries completed by 8 a.m. were assigned to postnatal day 1 (PND1). Pups born later that day were assigned to PND1 on the following morning. Litters were examined on PND1 for obvious morphological anomalies (e.g., missing digits, facial malformations, etc.), sexed by relative ano-genital distance and, in the case of litters with more than 8 offspring, culled randomly to 8 pups with equal numbers of males and females per litter whenever possible. Both nicotine-exposed and control litters were cross-fostered to non-exposed foster mothers within 24 h after birth and the pups were evaluated throughout the lactation period in terms of reflex development and neuromuscular maturation. Tests were selected from standard neurobehavioral developmental test batteries (Adams, 1986).

Developmental milestones

Fourteen control litters (53 males) and 8 NIC-exposed litters (20 males) were used to assess development and maturation in offspring. The dam was first removed from the home cage and specific tests measuring reflex development, motor coordination, and muscle strength were applied to the offspring. All testing was conducted between 9.00 a.m. and 4.00 p.m.

To assess righting reflex each pup was given two successive trials per day from PND 2 to 5 and the time from being placed in a supine position until it righted itself onto all four feet was recorded. The cut-off time was 30 s. Surface righting reflects the development of labyrinthine and body righting mechanisms as well as vestibular function and motor development.

Negative geotaxis was observed daily from PND7 to PND10; pups were timed for completing a 180º turn within 30 s when placed in a head-down position on a 25º inclined wooden surface. Rats were given two consecutive trials per day and the mean was calculated. Negative geotaxis reflects vestibular function, motor development and activity.

Forelimb grip strength was assessed on PND 17. A steel wire (20 cm long, about 0.3 cm thick) was supported between two poles of wood 25 cm above the table covered with soft towels. The latency to fall off the wire grasped by both forepaws was measured with a maximum time of 20 s and is a measure of muscle strength.

Maturational milestones

Pups from each litter were weighed on PND 1, 5, 10, 15, and 20. The emergence of physical maturation landmarks were noted, including pinnae detachment (PND 3), incisor eruption (PND 7-10), fur appearance (PND 9), and eye opening (PND 12). Eyes were recorded as open only when both eyes were open.

Tests in adulthood

Tests in adulthood were conducted on groups of 10 (NIC) to 12 (Con) animals coming from 8 (NIC) and 12 (Con) litters.

Locomotor activity

The number of cage crosses was assessed in two-month old animals during a 60 min test session in photocell activity cages measuring 30 × 30 × 30 cm (Schneider, *et al* 2010). The animals had no previous exposure to the cages.

5-Choice Serial Reaction Time Test

Aluminum operant conditioning chambers (Cenes Ltd., Cambridge, UK) were illuminated by house lights and housed in ventilated enclosures. The curved rear wall of each chamber contained 5 square holes. At the entrance of each hole, a photocell monitored interruptions of an infrared-light beam and at the rear there was a green light-emitting diode. A tray for delivering food pellets was located in the opposite wall, equidistant from each aperture,.

The training phases of the experiments were based on procedures described elsewhere (Hahn, *et al* 2002). Twenty-two adult rats (NIC = 10, Con = 12) aged 3 months were assessed in the 5-CSRTT. They were housed singly one week before starting the 5-CSRTT. The mean weight of each animal was calculated as the average of the 3 weights from that week. The start point for each individual rat on the growth curve was identified and the body weight of each rat was reduced to 85% of its free-feeding weight by restricting the amount of food given during the following week. The experiment started on the fourth day of food restriction. Training was initiated by habituation to the chamber and magazine training, followed by attentional training beginning with response holes illuminated for 10 s (stimulus duration, SD), followed by the introduction of progressively more demanding task parameters (Table 1). In the final stage of training, a stimulus light in a randomly chosen hole was illuminated for 1 s. If a subject nose-poked into a hole while it was illuminated or within 5 s after the light had terminated (limited hold), a 45 mg food pellet (BioServ, Frenchtown, NJ, USA) was delivered into the food tray and a correct response was registered.

A response into any other hole during that time was recorded as an incorrect response and resulted in a 5 s time-out during which the house light was extinguished. A failure to respond before the end of the limited hold was registered as an omission error and had no programmed consequences until animals reached step 3 of the procedure, when a time-out of 5 s duration was introduced (Table 1).

The next trial was initiated immediately after a correct response was made or at the end of the time-out that followed an incorrect response. The mean duration of the inter-trial interval (ITI) was 5 s; individual ITI varied randomly within the range 0.625–9.375 s. Responses during inter-trial intervals were recorded as anticipatory responses and resulted in a time-out of 3 s duration starting from step 3 of the procedure (responses during the time-outs were not counted as anticipatory responses). All training and test sessions lasted for 30 min. Rats were advanced into consecutive experimental stages when their accuracy (% correct responses) reached 70% and number of omissions was no higher than 25%.

Several performance measures were recorded: percentage of correct responses (accuracy) = 100 x (correct responses / (correct + incorrect responses) as a measure of spatial attention; percentage of omission errors (omissions) = 100 x (omission errors / stimuli presented), reflecting attention but also influenced by the general rate of responding; latency of correct responses = the mean time between stimulus onset and a nose-poke in the correct hole; latency of incorrect responses = the mean time between stimulus onset and a nose-poke in an incorrect hole; anticipatory responses as percentage of trials = 100 x total number of responses in ITIs / number of trials, as a measure of impulsive responding; reinforcers earned, equal to absolute number of correct responses in a session, as a measure of overall success of task performance. A measure of the variability of correct response times was introduced. Sessions were divided into 3 periods of 10 minutes for each of which the mean latency was

recorded. The measure of variability was the standard deviation of the mean latencies for the 3 ten-minute periods.

Delay-Discounting Paradigm

Standard experimental chambers (Campden Instruments, London, UK) were contained in sound-insulated, ventilated enclosures. The chambers were fitted with two retractable levers separated by a recess in which 45 mg pellets of food could be presented. White noise was present at all times to mask external sounds. The experiments were controlled by programs written with the Arachnid system (Paul Fray, Cambridge, UK) running under RISC OS on Acorn computers.

A separate group of 22 adult rats (NIC = 10, Con = 12) aged 3 months were assessed in the delay-discounting test. They were habituated to experimental chambers during two 30 min sessions with reward pellets being delivered every 30 s. Training was conducted over 3 phases and was based on previously described experimental procedures (Winstanley, *et al* 2004). In the first phase, rats were trained to press the left or right levers on alternate sessions to receive a 45 mg food pellet (BioServ, Frenchtown, NJ, USA). Each 30 min session consisted of 60 trials. Subjects were trained for 4 sessions until all earned at least 50 rewards per session. In the second phase rats were trained in 45 min sessions divided into 3 blocks. During the first 2 blocks, 50 trials each, only one lever, either right or left, was presented. During the third block two forced trials (only one lever presented) were followed by 48 free choice trials (two levers presented). The second phase lasted for 6 days until all animals had reached 0% of omissions on two consecutive days. During the third phase each rat had one lever designated as the 'immediate' delivery lever (one pellet) and one lever as the 'delay' delivery lever (5 pellets), with a delay of 2 s. Each session consisted of 24 trials, divided into 3 blocks of 8 trials, with trials spaced apart by 100 s. Each 8-trial block began with 2 'forced' trials in which either the left or the right lever was presented in random order for every pair of

trials, followed by 6 'choice' trials in which both levers were presented. Levers assignments were counterbalanced across groups. The third phase lasted for 4 days until all animals had reached 90% preference for delayed larger reward on two consecutive days.

The main delay-discounting procedure was identical to the one used in the third phase of training except the delay to the larger reward was increased daily according to the sequence 2, 6, 18, 36, 48, 54, 60, 66 s. Choice ratios (delay-lever presses/total lever presses) were calculated for each rat at each delay using the choice trial responses (i.e. excluding single lever trials) summed across the 3 consecutive blocks.

Gene Expression studies

Sample Preparation: Ten rats aged 5 months from control (n=10) and NIC (n=10) groups were killed by decapitation and brains were immediately dissected. The striatum and a 2 mm slice from the frontal cortex were removed, snap frozen on dry ice and stored at –80^{o}C until RNA extraction. RNA was extracted using Qiagen AllPrep RNA/DNA minikits (Qiagen, UK). During the extraction procedure, RNA columns were treated with RNase-free DNase1 to eliminate genomic DNA contamination. Purity of RNA samples was assessed via the 260/280-wavelength ratio using a NanoDrop spectrophotometer. All ratios were of acceptable quality (RNA range; 1.88 – 2.38).

Quantitative Measurement of Gene Expression using qRT-PCR: Housekeeping gene (HK) selection was performed using geNorm kits (PrimerDesign, Southampton, UK). The stability of eleven commonly used housekeeping genes was assessed using 500 ng total RNA from 4 samples of each group. Samples were first strand reverse transcribed in 20 µl reactions using oligoT priming and Moloney Murine Leukaemia Virus (MMLV) reverse transcription (PrimerDesign, Southampton, UK). SYBR green chemistry was used to quantify HK mRNA following the manufacturer's guidelines for cycling conditions, with all samples run in

duplicate (www.primerdesign.co.uk). GeNorm, a Visual Basic application tool in Excel, was used to statistically model the stability of the HK genes for accurate normalization of target genes (Vandesompele, et al 2002). The geNorm output provides the user with the two most stably expressed HK genes, along with stability values for all genes analyzed. We chose three HK genes for normalization of target genes in each tissue; Cyc1, Mdh1 and Ywhaz for striatum and Cyc1, Mdh1 and Gapdh for frontal cortex.

Primers and PerfectProbe technology (PrimerDesign, Southampton, UK) were used to quantify 5 target genes; Th, Nr4a2, Slc6a3, Drd4 and Drd5, along with the 3 HK genes for each tissue. Primer sequences for target genes are given in Table 2. Total RNA was first strand cDNA synthesized in, 20 μl reactions using oligoT priming and MMLV reverse transcription; qRT-PCR reactions were performed in triplicate.

Statistical analysis

Behavioural data were analyzed using one- or two-factor ANOVA followed by Bonferroni modified Least Significant Difference test (LSD) for *post-hoc* analysis. For maturational and developmental data, litter (only males) was used as the unit for statistical analysis. Thus the data subjected to statistical analyses were means for entire litters rather than results for individual animals within litters. The 5-CSRTT percentage data for accuracy and omissions were arc-sine transformed, and latency data were log transformed (Hahn, *et al* 2002). Spearman's rank correlation test was used to correlate measures obtained in the 5-CSRTT. For those variables assessed multiple times, age (PND) and day of training were used as repeated measures.

Gene expression results (qRT-PCR data) were compared using a Mann-Whitney test. The Grubbs method was applied to identify outliers from triplicate samples (Burns, *et al* 2005) after which arithmetic means were taken across replicates and the comparative Ct method

(ΔΔCt) applied (Livak and Schmittgen, 2001). Animals were excluded from the analysis of all genes if they showed expression values that were >2 standard deviations from the mean in a given group for at least two genes (one animal from control and two animals from NIC group). All tests of significance were performed at alpha=0.05 using Unistat 5.6 (Unistat Ltd, London, UK). All data are presented as mean ± S.E.M. if not otherwise stated.

Results

Nicotine exposure before and during pregnancy

Three weeks of pre-exposure to increasing doses of nicotine as the only source of water resulted in decreased body weight before mating ($F(1,25)=15.1$, $p<0.001$). During the last week of habituation, when the final concentration of nicotine solution was used, both solution ($F(1,25)=109.2$, $p<0.001$) and food consumption ($F(1,25)=5.29$, $p<0.05$) per kg body weight were decreased in the nicotine exposed group. Lower body weight (255.8±4.7 vs. 297.7±4.0; $F(1,25)=46.4$, $p<0.001$) and decreased solution consumption (69.6±1.4 vs. 146.8±2.5 ml/kg; $F(1,25)=71.7$, $p<0.001$), but not decreased food consumption (65.1+0.8 vs. 67.1+0.9 g/kg; $F(1,25)=1.59$, NS), were also observed in pregnant animals exposed to nicotine.

Nicotine blood levels

The mean plasma nicotine blood level during the second week of pregnancy was 96.0±31.9 ng/ml (mean ± SD). There was no difference in mean nicotine solution consumption/kg body weight/day between the groups of nicotine-exposed pregnant females used for nicotine blood tests or for offspring delivery (67.9±8.9 vs. 69.6±1.4 ml/kg, corresponding to 4.07±0.05 vs. 4.17±0.08 mg/kg of nicotine, respectively).

Litter characteristics

There was no difference between control litters and those prenatally exposed to nicotine in any of the measures used: the number of live litters (11 cf. 14), the percentage of live litters (57.9 cf. 73.7), number of animals per litter (5.9±0.6 cf. 5.7±0.8), the numbers of females and males per litter (2.1±0.3 vs. 3.0±0.5 and 2.9±0.6 vs. 2.0±0.4, respectively), and numbers of dead or malformed animals (1.36±0.5 vs. 0.73±0.6).

Postnatal growth and maturation

Prenatal nicotine exposure had no effect on the body weight gain of the offspring ($F(4,80)=1.51$, NS) but birth weights were lower in exposed animals (Fig. 1A; $F(1,20)=24.8$, $p<0.001$). The other maturational measures used in the present study (pinnae detachment, fur appearance, incisor eruption, and eye opening) did not differ between the groups.

Neurobehavioral development

The ontogeny of the righting reflex was delayed in animals prenatally exposed to nicotine (Fig. 1B; $F(1,20)=40.3$, $p<0.001$). Rats in both groups showed decreased latencies to right themselves onto all four feet from a supine position over the consecutive sessions ($F(3,60)=27.2$, $p<0.001$). There was no group × PND interaction.

Similarly, the ontogeny of negative geotaxis was significantly delayed in rats prenatally exposed to nicotine (Fig. 1C; ($F(1,20)=5.92$, $p<0.03$). Both groups decreased the latencies to turn 180° over the consecutive sessions ($F(3,60)=15.8$, $p<0.001$). There was no group and PND interaction.

Rats prenatally exposed to nicotine showed also decreased grip strength on PND 17 (Fig. 1D; $F(1,20)=9.24$, $p<0.01$).

Locomotor activity in adulthood

There was no difference between nicotine exposed and control animals in the number of cage crosses during a 60 min session (57.4±8.8 vs. 59.9±9.3, respectively).

5-Choice Serial Reaction Time Task

There was no difference between control- and nicotine-exposed animals during acquisition of the task when the duration of the visual stimuli was either 10 s or 5 s. However, at the final stage when a 1 s stimulus duration was used, the performance of rats prenatally exposed to nicotine was compromised (Fig. 2). Under this condition adult rats prenatally exposed to nicotine exhibited: decreased accuracy ($F(1,20)=6.25$, $p<0.03$; Fig. 2A); smaller numbers of reinforcers earned ($F(1,20)=6.11$, $p<0.03$; Fig. 2C) and an increased percentage of anticipatory responses ($F(1,20)=22$, $p<0.0001$; Fig. 2D). There was also a trend towards increased omission errors ($F(1,20)=3.02$, $p<0.1$) and a significant group × day interaction ($F(11,220)=1.90$, $p<0.05$; Fig. 2B); the numbers of omission errors were increased during the first two days after introduction of the 1 s stimulus duration and on day 5. There was no group x day interaction for anticipatory responses ($F(11,220)=1.81$, $p=0.06$), accuracy ($F(11,220)=1.59$, $p=0.1$) and the number of reinforcers earned ($F(11,220)=1.54$, $p=0.1$), and there was no between group difference in speed of responding either for correct or for incorrect responses ($F(1,11)=3.31$, $p=0.1$ and $F(1,11)=0.04$, $p=0.8$, respectively). There was a significant effect of day for all variables shown in Fig. 2 (smallest $F(11,220)=5.38$, $p<0.001$) that was attributable to a progressive improvement of performance over the 12 days for accuracy, numbers of reinforcers and anticipations; only the pattern of omission errors did not show an orderly relationship over days.

Rats prenatally exposed to nicotine showed signs of an increased variability of response times for correct responses (group: $F(1,20)=3.49$, $p<0.07$; group x day interaction: $F(11,220)=2.0$,

$p<0.03$; Fig 3) with significantly increased variability on days 3 and 10. The variability of response times for correct responses was negatively correlated with accuracy ($r=-0.52$, $p<0.001$) and positively correlated with anticipation rate ($r=0.42$, $p<0.001$).

Delay discounting test

Both nicotine-exposed and control animals chose the large reward on almost every trial when the delay to the large reward was 2 s (Fig. 4). As the delay to the large reward increased, the preference of both groups of rats shifted towards the smaller but more immediate reward (delay: $F(7,18)=43.1$, $p<0.001$); however, there was no significant effect of nicotine exposure on choice behavior at the different delays (group: $F(1,18)=1.29$, NS; delay × group: $F(7,129)=0.73$, NS).

Gene expression

There was a significant increase in the expression of DRD5 mRNA in striatum of animals prenatally exposed to nicotine ($U=8$, $p<0.006$). There were no further differences between the two groups for any genes in either tissue (Fig. 5A and B).

Discussion

Here we present the first experimental evidence of a link between prenatal nicotine exposure and cognitive performance deficits on the 5-CSRTT in adult rats. Following gestational exposure to nicotine, the offspring were found not only to have lower birth weight and delayed sensorimotor development, but also to be impaired during adulthood with respect to several measures of performance of the 5-CSRTT. In contrast, nicotine exposure had no effect on adult rats' locomotor activity in a novel environment or on impulsive choice in the delay-discounting test.

Nicotine exposure and litter characteristics

The daily nicotine consumption of the pregnant mothers of 4.61±0.54 mg/kg resulted in nicotine blood levels of 96±31.9 ng/ml, which is at the upper end of the dose range for heavy smokers (Benowitz, *et al* 2009). In line with previous animal studies (e.g. Murrin, *et al* 1987; Schneider, *et al* 2010), females exposed to a nicotine solution as the only source of fluid during pregnancy showed decreased body weight gain and lower solution and food consumption, although the latter was not significant in the present study. The implications of the reduced weights of the nicotine-exposed mothers and decreased food and water consumption need further investigation. Prenatal exposure to nicotine had no effect on the number of live litters, litter size, numbers of males and females per litter or the number of malformed or dead offspring, suggesting only mild teratogenicity of the nicotine dose regimen used in the present study.

Developmental changes

Birth weight was decreased by prenatal exposure to nicotine, although there was no difference in weight gain during development (Fig. 1A). This was expected and is similar to the results of human studies (Eskenazi, *et al* 1995). The offspring of animals exposed to nicotine *in utero* consistently show lower birth weights (Paulson, *et al* 1993; Peters and Ngan, 1982; Schneider, *et al* 2010); and in humans the direct impact of prental nicotine exposure on birth weight remains after controlling for maternal genetic influences (Thapar, *et al* 2009). The long-term significance of lower birth weight is still unclear but studies in humans have found associations between low birth weight and long-term cognitive deficits (Hack, 2006; Gianni, *et al* 2007) and behavioral disorders including ADHD (Winzer-Serhan, 2008). Recent evidence from monozygotic twin pairs shows that low birth weight confers a direct risk of ADHD that is independent of genetic effects (Greven et al., 2010).

Other maturational measures used in the present experiment (pinnae detachment, fur appearance, incisor eruption and eye lid opening) were spared in offspring prenatally exposed to nicotine. In contrast, developmental measures were all compromised. Significant delay of the righting reflex and negative geotaxis, as well as a shorter latency to fall in the grip strength test were observed in rats prenatally exposed to nicotine, suggesting impairment of motor coordination and muscle strength (Fig. 1). Our results are in line with previous studies showing deficits in righting reflex and negative geotaxis in rats and mice exposed to similar doses of nicotine (Peters and Ngan, 1982; Ajarem and Ahmad, 1998; Schneider, *et al* 2010). The delay in attaining these skills is probably due to damage or poor development of the motor and vestibular systems of the brain, but this needs further study.

Deficits in tests of attention and impulsivity

Previous studies have demonstrated deficits in learning and memory in adult rats prenatally exposed to nicotine (Vaglenova, *et al* 2008; Levin, *et al* 1993), whereas the present report investigates possible impairments in attention, impulsive responding, variability of reaction times and delay discounting using the 5-CSRTT and delay discounting tasks.

The development of the 5-CSRTT for rats was initially stimulated by the need to understand, at a preclinical level, the nature of the deficits shown by children with ADHD and the effects of psychostimulant drugs such as methylphenidate (Robbins, 2002). The task is modeled after Leonard's five-choice serial reaction task used to study human attentional processes and is considered to have similarities with the continuous performance test of attention (Robbins, 2002). When stimulus duration in the 5-CSRTT is as short as 1 s the procedure is regarded as a means for assessing sustained attention rather than simply discriminated responding.

In the prenatal exposed nicotine group, we observed a trend ($p<0.1$) in the rate of omission errors in the 1s stimulus condition, which improved during the course of the 12 days of

testing, with a significant group by day interaction ($p<0.05$). The observed impairment therefore reflects a delayed ability to learn a task with a high attentional load, which could reflect a deficit of attentional processing or more general learning difficulties. However the group x day interaction was significant only for omission errors and only in the 1s stimulus condition, suggesting that the learning difficulty was restricted to a task condition that demanded high levels of sustained attention. This interpretation should be balanced by the possibility that the study might be underpowered to detect significant day by group interactions for the other variables, which would then indicate a more general learning difficulty.

In considering whether the pattern of increased omission errors in the 5-CSRTT is comparable to findings in ADHD, the study design with repeated daily measures needs to be taken into account. To the authors' knowledge no comparable studies have been performed in ADHD with repeated daily measures, so it is not known whether performance in children and adults with ADHD would improve and catch up with the performance of healthy controls.

The other significant impairments that emerged in the 1 s stimulus condition, which did not show significant group x day interactions, included decreased accuracy, increased anticipatory responses, smaller number of earned rewards and response time variability. Accuracy in the task is thought to represent processes related to sustained attention, while anticipatory responses during the intra-trial periods are thought to reflect a form of impulsive responding. Neither of these measures has been widely adopted in ADHD research so it is difficult to make direct comparisons. Accuracy is rarely included in ADHD studies because there are marked ceiling effects in equivalent human tasks such as the fast task (Andreou et al., 2007), with both cases and controls showing very low rates of accuracy errors. In contrast, anticipatory responses have been evaluated in a few studies and are found to be

significantly increased in children with ADHD compared to healthy controls (Bedard et al., 2003; Wada et al., 2000).

Response time variability (RTV) in the rats correlated negatively with accuracy scores and positively with anticipatory responses, suggesting that a general deficit might underlie the pattern of findings that links RTV to changes in attention and anticipatory responses. However the change in RTV in the rat model may not reflect the same processes that lead to increased RTV in human disorders such as ADHD. Firstly, increased RTV in humans with ADHD occurs under slow unrewarded conditions and tend to normalize under rewarded conditions (Andreou, *et al* 2007; Uebel et al., 2010), whereas responses in the 5-CSRTT are rewarded. Secondly, the measure of RTV used in this study is the standard deviation of data averaged across three, ten-minute periods, which is different from the trial by trial variability associated with ADHD (Klein et al 2006).

In the delay discounting task, which measures a specific aspect of choice impulsivity, there was no difference detected between nicotine-exposed and controls rats. Human research suggests an association between ADHD and performance on delay discounting tasks in children, although this is not consistently found in all children with ADHD (Marco, *et al* 2009; Paloyelis, *et al* 2009) and has not been studied in adults with ADHD. The discrepancy in our findings between impulsive responding indexed by anticipatory responses in the 5-CSRTT and the delay-discounting test is not unexpected, because these measure entirely different aspects of impulsivity, consistent with the non-unitary nature of impulsive behavior in humans (Evenden, 1999; Moeller, *et al* 2001; McDonald, *et al* 2003; Patton, *et al* 1995) and animals (see Winstanley, *et al* 2006 for review).

In utero nicotine exposure has also been associated with 'hyperactivity' in humans as measured by a combined parental rating of restlessness, being fidgety, unable to settle and easily distracted (Kotimaa, *et al* 2003), but no studies have used actigraph data. Overactivity

in ADHD has been shown to be more pronounced under constant (habituated) and unstimulating conditions and to normalise in novel or stimulating environments (Antrop et al., 2000; Sagvolden et al., 1998), suggesting that the best rodent model of activity in ADHD would be increased home cage activity but reduced or normal activity in novel environments (Mill et al., 2002). In the present study we evaluated activity during a single activity test session, reflecting exploratory activity in a novel environment. Furthermore, the lack of effect of prenatal exposure to nicotine on exploratory locomotor activity in adult rats contrasts with some previous reports (Tizabi, *et al* 1997; Pauly, *et al* 2004; Ajarem and Ahmad, 1998), but agrees with others (LeSage, *et al* 2006; Romero and Chen, 2004). We did however observe increased locomotor activity after repeated testing of adolescent rats exposed prenatally to nicotine (Schneider, *et al* 2009), which accords better with the human literature on ADHD.

Gene Expression analysis

The most probable direct effects of prenatal nicotine exposure would be on nicotinic acetylcholine (ACh) systems (Slotkin, 2004) but given the close anatomic association of the ACh and the DA systems, it is likely to have secondary effects on the DA system (Shea and Steiner, 2008). Here we focused on the DA system because dysregulation of DA signaling has been clearly implicated in processes leading to deficits of attention and impulsive responding. Animal studies indicate that prenatal exposure to nicotine has lasting effects on behaviors regulated by dopamine, including locomotor activity, stereotypy and drug self-administration (Tizabi, *et al* 1997; Ajarem and Ahmad, 1998; Levin, *et al* 2006; Paz, *et al* 2007; Franke, *et al* 2008). The present study looked for long-lasting effects of prenatal nicotine exposure on quantitative expression of the DA-related genes NR4A2, TH, DAT1, DRD4 and DRD5; that index DA regulatory function or have been reported to be associated with ADHD in genetic association studies (Waldman and Gizer, 2006; Gizer et al., 2009). We investigated gene expression in the rat striatum and frontal cortex because cortico-striatal

pathways have been strongly implicated in ADHD (Castellanos, 2001) as well as attention and impulsive decision making processes (Muir, *et al* 1996; Rogers, *et al* 2001; Cardinal, 2006; Winstanley, *et al* 2006).

There was little evidence for expression differences between the two groups for any of the genes studied in either tissue, although there was a small increase in DRD5 mRNA expression in the striatum of nicotine exposed animals. Whether such a small difference is capable of influencing behavior remains an open question. Nevertheless, human studies suggest that DRD5 might be an important gene for ADHD with evidence for the association of a specific genetic marker close to the DRD5 gene providing some of the strongest evidence for association with ADHD in children (OR=1.34, 95% CI 1.21 - 1.50, $p=8 \times 10^{-8}$) in a meta-analysis of nine independent studies (Li, *et al* 2006). Furthermore, the allele-specific association was recently replicated in a sample of adult patients with ADHD (Johansson, *et al* 2008). Interestingly, the DRD5 repeat polymorphism was reported to be associated with lower performance scores on the TOVA continuous performance test in ADHD patients and their parents (Manor, et al 2004).

Study limitations

The present study has two main limitations. First, the possible teratogenic effects of prenatal exposure to nicotine cannot be clearly distinguished from the potential effects of dehydration and stress in the rodents given nicotine. For example, restriction of water intake during pregnancy induces marked alterations in maternal-fetal fluid homeostasis and reduces birth weights in newborns (Ross and Desai, 2005). Direct tests on the behavioral effects of gestational dehydration in rats do not seem to have been published and an impact on the cognitive performance measures used in this study cannot be excluded. The nicotine-exposed offspring were also low in birth weight, and low birth weight has been associated with several

neuropsychological disorders including ADHD (Casper, 2004). Further studies are therefore needed to control for these potential confounds.

Secondly, although it was clear that performance of the 5-CSRTT was impaired on several parameters, long-term persistence of effects was not demonstrated and the nature of the impairments therefore remains uncertain. As task performance was not stable when impairments were seen, these effects may have involved learning processes that are not specific to attentional tasks.

Conclusions

The findings indicate a direct impact of the prenatal environment on important aspects of cognition and inhibitory control later in life. The precise mechanisms by which such long term impacts on behavior arise remain unknown, but are likely to involve epigenetic changes induced by exposure to the environmental factors (Mill and Petronis, 2008). The preclinical data presented here challenges the conclusion that the observed association between ADHD and maternal smoking in pregnancy is mediated entirely by genetic effects (Thapar, *et al* 2009; D'Onofrio, *et al* 2008), by showing that direct experimental manipulation of the prenatal environment, under conditions where genetic variance is controlled by the use of the same rat strain in the experimental and control samples, leads to cognitive changes that could contribute to components of the ADHD phenotype; including impulsive responding and an increase in errors during tasks with a high attentional load. Further research is required to control for potential confounding factors yet these data indicate the importance of the prenatal environment for aspects of inattentive and impulsive behavior in adulthood.

Disclosure/Conflict of Interest

The authors have no Conflict of Interest with respect to the present manuscript.

Tomasz Schneider has no financial interests to disclose.

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Reference List

Adams J (1986): Methods in behavioral teratology. Plenum Press, York. pp 67-97.

Ajarem JS, Ahmad M (1998). Prenatal nicotine exposure modifies behavior of mice through early development. *Pharmacol Biochem Behav* **59**: 313-318.

Andreou P, Neale BM, Chen W, Christiansen H, Gabriels I, Heise A, *et al* (2007). Reaction time performance in ADHD: improvement under fast-incentive condition and familial effects. *Psychol Med* **37**: 1703-1715.

Antrop I, Roeyers H, Van Oost P, *et al* (2000). Stimulation seeking and hyperactivity in children with ADHD. Attention Deficit Hyperactivity Disorder. *J Child Psychol Psychiatry* **41**: 225-231.

Asherson P, Brookes K, Franke B, Chen W, Gill M, Ebstein RP, *et al* (2007). Confirmation that a specific haplotype of the dopamine transporter gene is associated with combined-type ADHD. *Am J Psychiatry* **164**: 674-677.

Barkley RA, Edwards G, Laneri M, Fletcher K, Metevia L (2001). Executive functioning, temporal discounting, and sense of time in adolescents with attention deficit hyperactivity disorder (ADHD) and oppositional defiant disorder (ODD). *J Abnorm Child Psychol* **29**: 541-556.

Bedard, AC, Ickowicz A, Logan GD, et al (2003). Selective inhibition in children with attention-deficit hyperactivity disorder off and on stimulant medication. *J Abnorm Child Psychol* **31**: 315-327.

Benowitz NL, Hukkanen J, Jacob P, III (2009). Nicotine chemistry, metabolism, kinetics and biomarkers. *Handb Exp Pharmacol*: 29-60.

Bertolini A, Bernardi M, Genedani S (1982). Effects of prenatal exposure to cigarette smoke and nicotine on pregnancy, offspring development and avoidance behavior in rats. *Neurobehav Toxicol Teratol* **4**: 545-548.

Burns MJ, Nixon GJ, Foy CA, Harris N (2005). Standardisation of data from real-time quantitative PCR methods - evaluation of outliers and comparison of calibration curves. *BMC Biotechnol* **5**: 31.

Cardinal RN (2006). Neural systems implicated in delayed and probabilistic reinforcement. *Neural Netw*, 19: 1277-1301.

Casper RC (2004). Nutrients, neurodevelopment, and mood. *Curr Psychiatry Rep* **6**: 425-429.

Castellanos FX (2001). Neural substrates of attention-deficit hyperactivity disorder. *Adv Neurol* **85**:, 197-206.

Cornelius MD, Day NL (2009). Developmental consequences of prenatal tobacco exposure. *Curr Opin Neurol* **22**: 121-125.

D'Onofrio BM, Van Hulle CA, Waldman ID, Rodgers JL, Harden KP, Rathouz PJ, *et al* (2008). Smoking during pregnancy and offspring externalizing problems: an exploration of genetic and environmental confounds. *Dev Psychopathol*, 20: 139-164.

Durston S, Belle JV, Zeeuw PD (2010). Differentiating Frontostriatal and Fronto-Cerebellar Circuits in Attention-Deficit/Hyperactivity Disorder. *Biol Psychiatry,* Oct 19. [Epub ahead of print].

Dwyer JB, Broide RS, Leslie FM (2008). Nicotine and brain development. *Birth Defects Res C Embryo Today* **84**: 30-44.

Epstein JN, Erkanli A, Conners CK, Klaric J, Costello JE, Angold A (2003). Relations between Continuous Performance Test performance measures and ADHD behaviors. *J Abnorm Child Psychol* **31**: 543-554.

Epstein JN, Johnson DE, Varia IM, Conners CK (2001). Neuropsychological assessment of response inhibition in adults with ADHD. *J Clin Exp Neuropsychol* **23**: 362-371.

Ernst M, Moolchan ET, Robinson ML (2001). Behavioral and neural consequences of prenatal exposure to nicotine. *J Am Acad Child Adolesc Psychiatry* **40**: 630-641.

Eskenazi B, Prehn AW, Christianson RE (1995). Passive and active maternal smoking as measured by serum cotinine: the effect on birthweight. *Am J Public Health* **85**: 395-398.

Evenden JL (1999). Varieties of impulsivity. *Psychopharmacology (Berl)* **146**: 348-361.

Faraone SV, Biederman J, Mick E (2006). The age-dependent decline of attention deficit hyperactivity disorder: a meta-analysis of follow-up studies. *Psychol Med* **36**: 159-165.

Faraone SV, Perlis RH, Doyle AE, Smoller JW, Goralnick JJ, Holmgren MA*, et al* (2005). Molecular genetics of attention-deficit/hyperactivity disorder. *Biol Psychiatry* **57**: 1313-1323.

Franke RM, Park M, Belluzzi JD, Leslie FM (2008). Prenatal nicotine exposure changes natural and drug-induced reinforcement in adolescent male rats. *Eur J Neurosci* **27**: 2952-2961.

Fung YK (1989). Postnatal effects of maternal nicotine exposure on the striatal dopaminergic system in rats. *J Pharm Pharmacol* **41**: 576-578.

Fung YK, Lau YS (1989). Effects of prenatal nicotine exposure on rat striatal dopaminergic and nicotinic systems. *Pharmacol Biochem Behav* **33**: 1-6.

Gianni ML, Picciolini O, Vegni C, Gardon L, Fumagalli M, Mosca F (2007). Twelve-month neurofunctional assessment and cognitive performance at 36 months of age in extremely low birth weight infants. *Pediatrics* **120**: 1012-1019.

Gizer IR, Ficks C, Waldman ID (2009). Candidate gene studies of ADHD: a meta-analytic review. *Hum Genet* **126**: 51-90.

Greven U., Ronald A., Rodriguez A (2010). Non-shared environmental effects of birth weight on ADHD symptoms persist into early adolescence. A 10-year longitudinal twin study. *Longitudinal and Life Course Studies* **3** (supplement): 332.

Hack M (2006). Young adult outcomes of very-low-birth-weight children. *Semin Fetal Neonatal Med* **11**: 127-137.

Hahn B, Shoaib M, Stolerman IP (2002). Nicotine-induced enhancement of attention in the five-choice serial reaction time task: the influence of task demands. *Psychopharmacology (Berl)* **162**: 129-137.

Jauniaux E, Burton GJ (2007). Morphological and biological effects of maternal exposure to tobacco smoke on the feto-placental unit. *Early Hum Dev* **83**: 699-706.

Johansson S, Halleland H, Halmoy A, Jacobsen KK, Landaas ET, Dramsdahl M, *et al* (2008). Genetic analyses of dopamine related genes in adult ADHD patients suggest an association with the DRD5-microsatellite repeat, but not with DRD4 or SLC6A3 VNTRs. *Am J Med Genet B Neuropsychiatr Genet* **147B**: 1470-1475.

Johnson KA, Wiersema JR, Kuntsi J (2009). What would Karl Popper say? Are current psychological theories of ADHD falsifiable? *Behav Brain Funct* **5**: 15.

Klein C, Wendling K, Huettner P, Ruder H, Peper M (2006). Intra-subject variability in attention-deficit hyperactivity disorder. *Biol Psychiatry* **60**: 1088-1097.

Kotimaa AJ, Moilanen I, Taanila A, Ebeling H, Smalley SL, McGough JJ, *et al* (2003). Maternal smoking and hyperactivity in 8-year-old children. *J Am Acad Child Adolesc Psychiatry* **42**: 826-833.

Kuntsi J, McLoughlin G, Asherson P (2006). Attention deficit hyperactivity disorder. *Neuromolecular Med* **8**: 461-484.

Kuntsi J, Wood AC, Rijsdijk F, Johnson KA, Andreou P, Albrecht B, *et al* (2010). Separation of cognitive impairments in attention deficit hyperactivity disorder into two familial factors. *Arch Gen Psychiatry*. (in press).

Lesage MG, Gustaf E, Dufek MB, Pentel PR (2006). Effects of maternal intravenous nicotine administration on locomotor behavior in pre-weanling rats. *Pharmacol Biochem Behav* **85**: 575-583.

LeSage MG, Gustaf E, Dufek MB, Pentel PR (2006). Effects of maternal intravenous nicotine administration on locomotor behavior in pre-weanling rats. *Pharmacol Biochem Behav* **85**: 575-583.

Levin ED, Briggs SJ, Christopher NC, Rose JE (1993). Prenatal nicotine exposure and cognitive performance in rats. *Neurotoxicol Teratol* **15**: 251-260.

Levin ED, Lawrence S, Petro A, Horton K, Seidler FJ, Slotkin TA (2006). Increased nicotine self-administration following prenatal exposure in female rats. *Pharmacol Biochem Behav* **85**: 669-674.

Li D, Sham PC, Owen MJ, He L (2006). Meta-analysis shows significant association between dopamine system genes and attention deficit hyperactivity disorder (ADHD). *Hum Mol Genet* **15**: 2276-2284.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**: 402-408.

Manor I, Corbex M, Eisenberg J, Gritsenkso I, Bachner-Melman R, Tyano S, *et al* (2004). Association of the dopamine D5 receptor with attention deficit hyperactivity disorder (ADHD) and scores on a continuous performance test (TOVA). *Am J Med Genet B Neuropsychiatr Genet* **127B**: 73-77.

Marco R, Miranda A, Schlotz W, Melia A, Mulligan A, Muller U, *et al* (2009). Delay and reward choice in ADHD: an experimental test of the role of delay aversion. *Neuropsychology* **23**: 367-380.

McDonald J, Schleifer L, Richards JB, de WH (2003). Effects of THC on behavioral measures of impulsivity in humans. *Neuropsychopharmacology* **28**: 1356-1365.

Mill J, Petronis A (2008). Pre- and peri-natal environmental risks for attention-deficit hyperactivity disorder (ADHD): the potential role of epigenetic processes in mediating susceptibility. *J Child Psychol Psychiatry* **49**: 1020-1030.

Misener VL, Luca P, Azeke O, Crosbie J, Waldman I, Tannock R, *et al* (2004). Linkage of the dopamine receptor D1 gene to attention-deficit/hyperactivity disorder. *Mol Psychiatry* **9**: 500-509.

Moeller FG, Barratt ES, Dougherty DM, Schmitz JM, Swann AC (2001). Psychiatric aspects of impulsivity. *Am J Psychiatry* **158**: 1783-1793.

Muir JL, Everitt BJ, Robbins TW (1996). The cerebral cortex of the rat and visual attentional function: dissociable effects of mediofrontal, cingulate, anterior dorsolateral, and parietal cortex lesions on a five-choice serial reaction time task. *Cereb Cortex* **6**: 470-481.

Muneoka K, Nakatsu T, Fuji J, Ogawa T, Takigawa M (1999). Prenatal administration of nicotine results in dopaminergic alterations in the neocortex. *Neurotoxicol Teratol* **21**: 603-609.

Muneoka K, Ogawa T, Kamei K, Muraoka S, Tomiyoshi R, Mimura Y, *et al* (1997). Prenatal nicotine exposure affects the development of the central serotonergic system as well as the dopaminergic system in rat offspring: involvement of route of drug administrations. *Brain Res Dev Brain Res* **102**: 117-126.

Murrin LC, Ferrer JR, Zeng WY, Haley NJ (1987). Nicotine administration to rats: methodological considerations. *Life Sci* **40**: 1699-1708.

Paloyelis Y, Asherson P, Kuntsi J (2009). Are ADHD symptoms associated with delay aversion or choice impulsivity? A general population study. *J Am Acad Child Adolesc Psychiatry* **48**: 837-846.

Patton JH, Stanford MS, Barratt ES (1995). Factor structure of the Barratt impulsiveness scale. *J Clin Psychol* **51**: 768-774.

Paulson RB, Shanfeld J, Vorhees CV, Sweazy A, Gagni S, Smith AR, *et al* (1993). Behavioral effects of prenatally administered smokeless tobacco on rat offspring. *Neurotoxicol Teratol* **15**: 183-192.

Pauly JR, Slotkin TA (2008). Maternal tobacco smoking, nicotine replacement and neurobehavioural development. *Acta Paediatr* **97**: 1331-1337.

Pauly JR, Sparks JA, Hauser KF, Pauly TH (2004). In utero nicotine exposure causes persistent, gender-dependant changes in locomotor activity and sensitivity to nicotine in C57Bl/6 mice. *Int J Dev Neurosci* **22**: 329-337.

Paz R, Barsness B, Martenson T, Tanner D, Allan AM (2007). Behavioral teratogenicity induced by nonforced maternal nicotine consumption. *Neuropsychopharmacology* **32**: 693-699.

Peters MA, Ngan LL (1982). The effects of totigestational exposure to nicotine on pre- and postnatal development in the rat. *Arch Int Pharmacodyn Ther* **257**: 155-167.

Polanczyk G, de Lima MS, Horta BL, Biederman J, Rohde LA (2007). The worldwide prevalence of ADHD: a systematic review and metaregression analysis. *Am J Psychiatry* **164**: 942-948.

Ribary U, Lichtensteiger W (1989). Effects of acute and chronic prenatal nicotine treatment on central catecholamine systems of male and female rat fetuses and offspring. *J Pharmacol Exp Ther* **248**: 786-792.

Robbins TW (2002). The 5-choice serial reaction time task: behavioural pharmacology and functional neurochemistry. *Psychopharmacology (Berl)* **163**: 362-380.

Rogers RD, Baunez C, Everitt BJ, Robbins TW (2001). Lesions of the medial and lateral striatum in the rat produce differential deficits in attentional performance. *Behav Neurosci* **115**: 799-811.

Romero RD, Chen WJ (2004). Gender-related response in open-field activity following developmental nicotine exposure in rats. *Pharmacol Biochem Behav* **78**: 675-681.

Ross MG, Desai M (2005). Gestational programming: population survival effects of drought and famine during pregnancy. *Am J Physiol Regul Integr Comp Physiol* **288**: R25-R33.

Scheres A, Dijkstra M, Ainslie E, Balkan J, Reynolds B, Sonuga-Barke E*, et al* (2006). Temporal and probabilistic discounting of rewards in children and adolescents: effects of age and ADHD symptoms. *Neuropsychologia* **44**:, 2092-2103.

Sagvolden T, Aase H, Zeiner P, *et al* (1998). Altered reinforcement mechanisms in attention-deficit/hyperactivity disorder. *Behav Brain Res* **94**: 61-71.

Schneider T, Bizarro L, Asherson PJN, Stolerman IP (2010). Gestational exposure to nicotine in drinking water: teratogenic effects and methodological issues. *Behav Pharmacol*. **21**: 206-216.

Schneider T, Bizarro L, Asherson PJN, Stolerman IP (2009). Impulsivity, hyperactivity, enhanced "risk-taking" behaviour and excessive nicotine consumption in adolescent rats prenatally exposed to nicotine. *Behav Pharmacol*, **20**: S21.

Shea AK, Steiner M (2008). Cigarette smoking during pregnancy. *Nicotine Tob Res* **10**: 267-278.

Slotkin TA (2004). Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. *Toxicol Appl Pharmacol*, 19**8**: 132-151.

Slotkin TA, Lappi SE, McCook EC, Lorber BA, Seidler FJ (1995). Loss of neonatal hypoxia tolerance after prenatal nicotine exposure: implications for sudden infant death syndrome. *Brain Res Bull* **38**: 69-75.

Smith KM, Bauer L, Fischer M, Barkley R, Navia BA (2005). Identification and characterization of human NR4A2 polymorphisms in attention deficit hyperactivity disorder. *Am J Med Genet B Neuropsychiatr Genet* **133B**: 57-63.Sorenson CA, Raskin LA, Suh Y (1991). The effects of prenatal nicotine on radial-arm maze performance in rats. *Pharmacol Biochem Behav* **40**: 991-993.

Thapar A, O'Donovan M, Owen MJ (2005). The genetics of attention deficit hyperactivity disorder. *Hum Mol Genet* **14 Spec No. 2**: R275-R282.

Thapar A, Rice F, Hay D, Boivin J, Langley K, van den Bree M, *et al* (2009). Prenatal smoking might not cause attention-deficit/hyperactivity disorder: evidence from a novel design. *Biol Psychiatry* **66**: 722-727.

Tizabi Y, Popke EJ, Rahman MA, Nespor SM, Grunberg NE (1997). Hyperactivity induced by prenatal nicotine exposure is associated with an increase in cortical nicotinic receptors. *Pharmacol Biochem Behav* **58**: 141-146.

Uebel H, Albrecht B, Asherson P, Borger NA, Butler L, Chen W, *et al* (2010). Performance variability, impulsivity errors and the impact of incentives as gender-independent endophenotypes for ADHD. *J Child Psychol Psychiatry* **51**: 210-218.

Vaglenova J, Birru S, Pandiella NM, Breese CR (2004). An assessment of the long-term developmental and behavioral teratogenicity of prenatal nicotine exposure. *Behav Brain Res* **150**: 159-170.

Vaglenova J, Parameshwaran K, Suppiramaniam V, Breese CR, Pandiella N, Birru S (2008). Long-lasting teratogenic effects of nicotine on cognition: gender specificity and role of AMPA receptor function. *Neurobiol Learn Mem* **90**: 527-536.

Wada N, Yamashita Y, Matsuishi T, *et al* (2000). The test of variables of attention (TOVA) is useful in the diagnosis of Japanese male children with attention deficit hyperactivity disorder. *Brain Dev* **22**: 378-382.

Waldman ID, Gizer IR (2006). The genetics of attention deficit hyperactivity disorder. *Clin Psychol Rev* **26**: 396-432.

Williams, NM, Zaharieva I, Martin A, *et al* (2010). Rare chromosomal deletions and duplications in attention-deficit hyperactivity disorder: a genome-wide analysis. *Lancet* Sep 29. [Epub ahead of print]

Willcutt EG, Doyle AE, Nigg JT, Faraone SV, Pennington BF (2005). Validity of the executive function theory of attention-deficit/hyperactivity disorder: a meta-analytic review. *Biol Psychiatry* **57**: 1336-1346.

Winstanley CA, Dalley JW, Theobald DE, Robbins TW (2004). Fractionating impulsivity: contrasting effects of central 5-HT depletion on different measures of impulsive behavior. *Neuropsychopharmacology* **29**: 1331-1343.

Winstanley CA, Eagle DM, Robbins TW (2006). Behavioral models of impulsivity in relation to ADHD: translation between clinical and preclinical studies. *Clin Psychol Rev* **26**: 379-395.

Winzer-Serhan UH (2008). Long-term consequences of maternal smoking and developmental chronic nicotine exposure. *Front Biosci* **13**: 636-649.

Table 1. Consecutive steps during 5-CSRTT training

Step	Stimulus duration (s)	Limited hold (s)	Mean inter-trial interval (s)	Incorrect time-out (s)	Anticipatory time-out (s)	Number of sessions
1	10	10	5	0	0	9
2	5	5	5	0	0	4
3	5	5	5	5	3	4
4	1	3	5	5	3	12

Table 2. Primers used for amplification of five target genes. Primers were designed and supplied by PrimerDesign, UK.

Gene	Sense Primer (5'→ 3')	Anti-sense Primer (5'→ 3')	*Product length (bp)*
Th	CCCTACCAAGATCAAACCTACC	CTGGATACGAGAGGCATAGTTC	96
NR4A2	CTTCACAACTTCCACCACCAGAACTA	GGGGCGACTGCTTAAAGGA	103
DAT1	TCCAGTTACAATAAGTTCACCAATAA	CGACGAAGCCAGAGGAGAA	94
Drd4	TATGTCAACAGTGCCCTCAAC	AGACATCAGCGGTTCTTTCAG	110
Drd5	GGGAGAGGAGGAGGAGGAG	GGGGTGAGAGGTGAGATTTTG	144

Figure legends

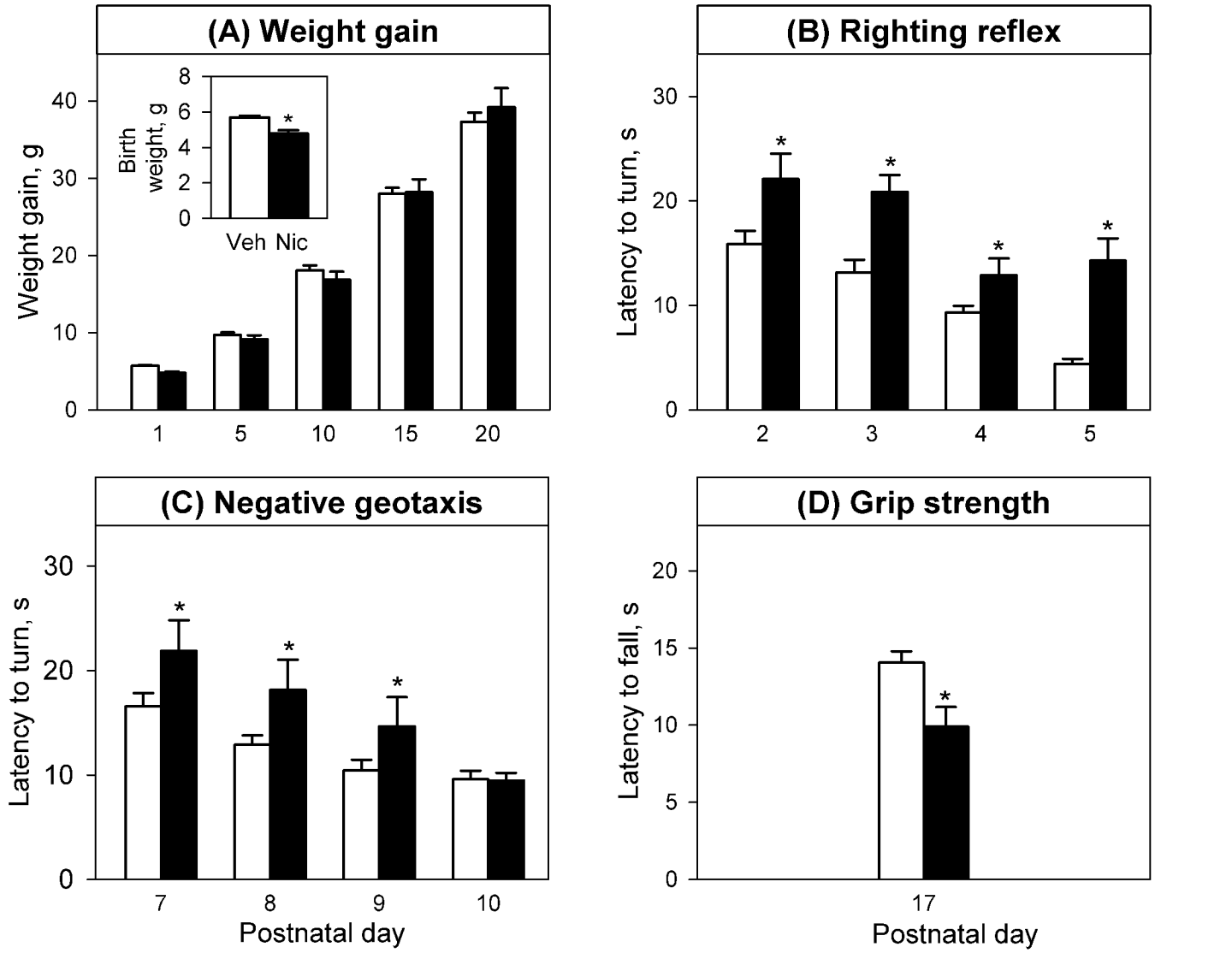
Figure 1. Decreased birth weight (A), impairment of motor co-ordination (B and C) and muscle strength (D) in male rats prenatally exposed to nicotine. Data are shown as means ± SEM (white bars, controls, n=14; black bars, nicotine-exposed, n=8,). Litter was used as a unit for analysis. *, $p<0.05$ from *post hoc* tests of between-group effects by least significance difference.

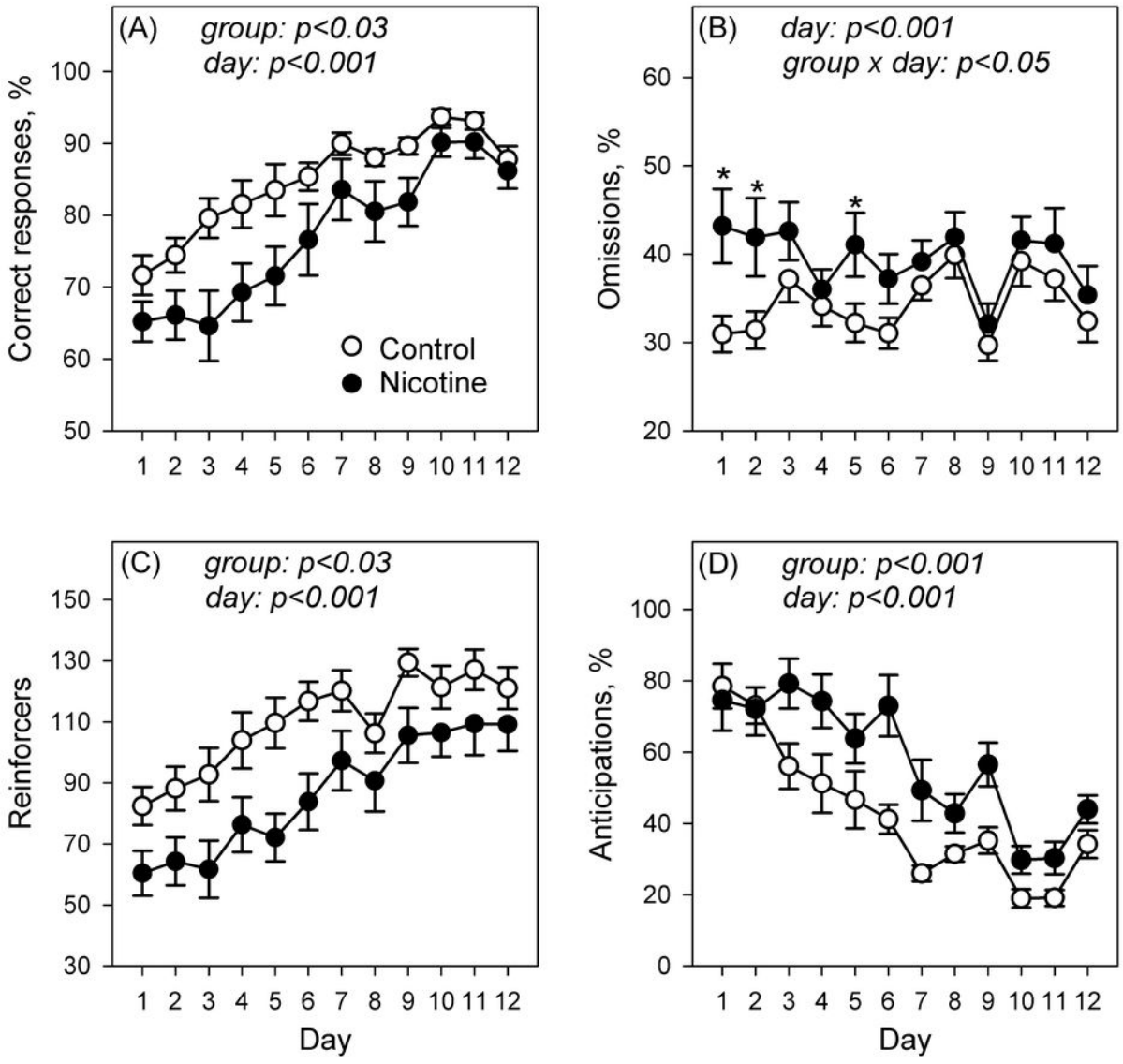
Figure 2. Impairments in attentional performance in the 5-CSRTT in adult control rats (n=12) and in nicotine-exposed rats (n=10). Data are shown for percentage correct responses (A), number of anticipations, percentage omission errors (B), number of reinforcers earned (C) and percentage numbers of anticipatory responses (D), for 12 days when a 1 s stimulus duration was used (means ± SEM).

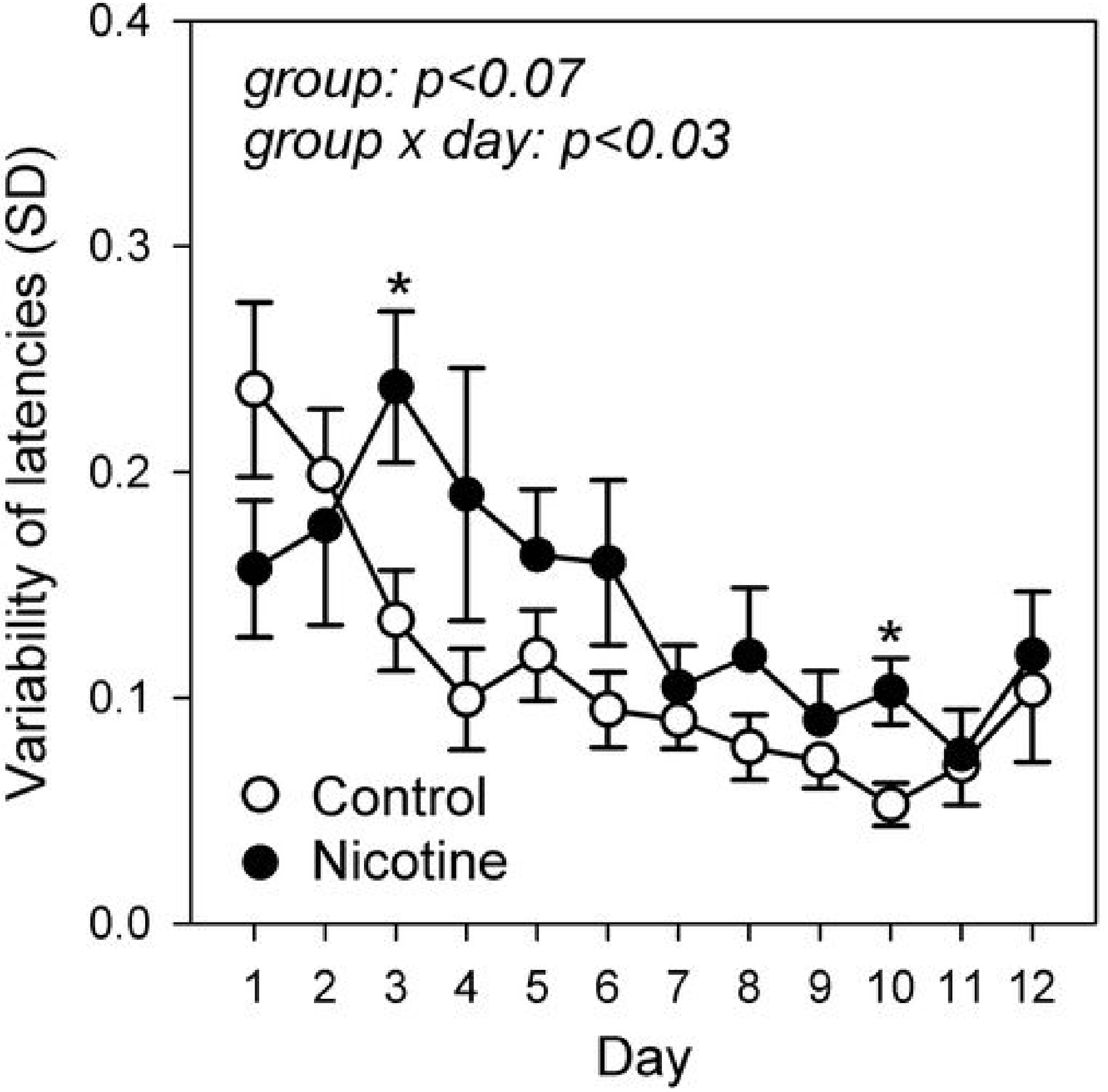
Figure 3. Increased intra-individual variability (standard deviation, SD) of response times for correct responses in the 5-CSRTT in adult control and nicotine exposed rats. The SD of latency for correct responses are shown as means ± SEM. Other details as for fig. 2.

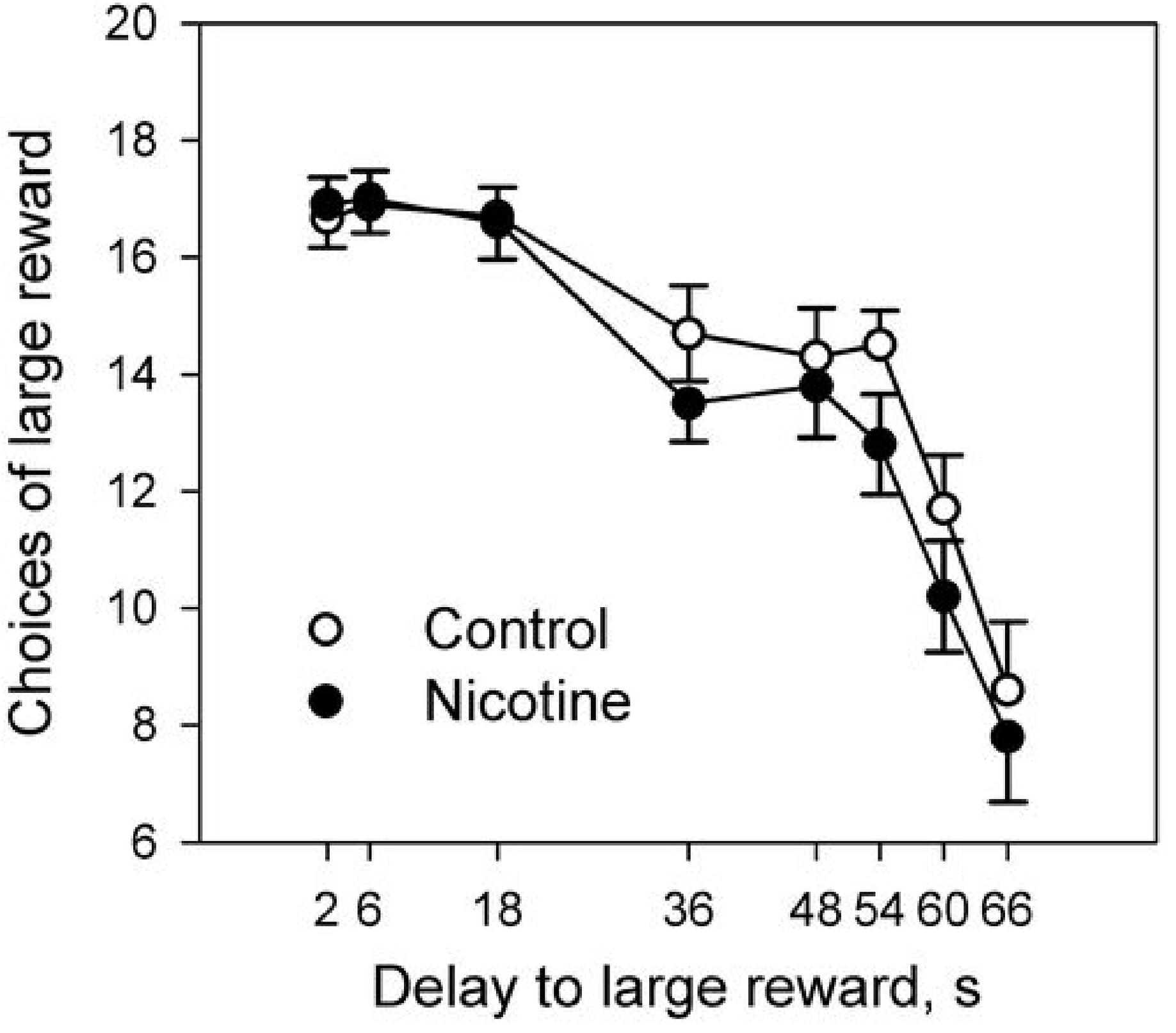
Figure 4. Lack of effect of gestational exposure to nicotine on delay-discounting (controls, n=12; nicotine-exposed, n= 0). Data are shown as means ± SEM.

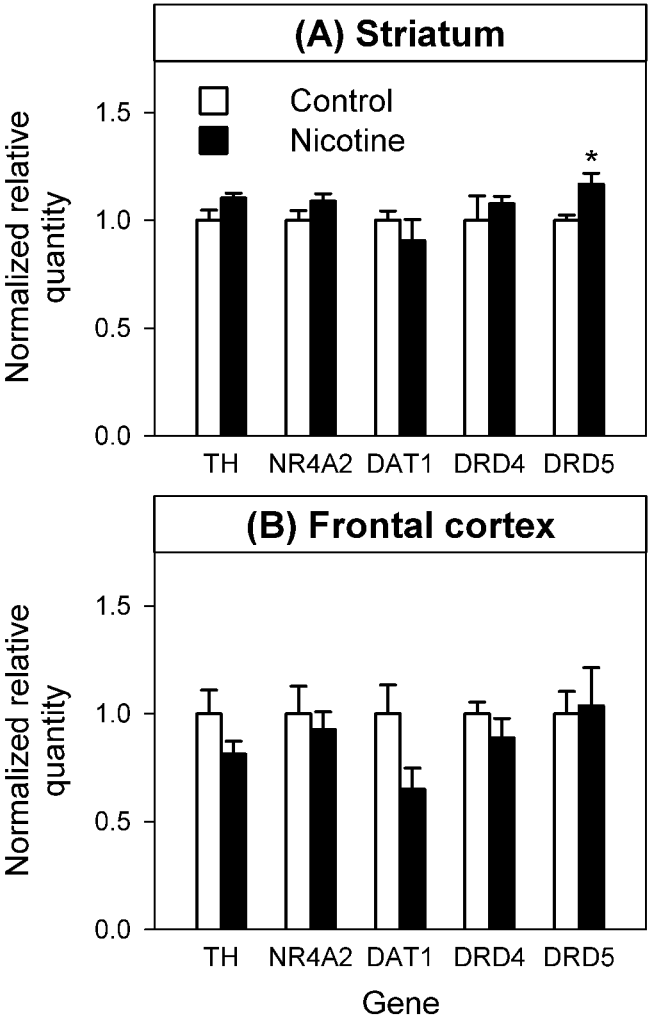
Figure 5. Effects of prenatal exposure to nicotine on the expression of dopamine-related genes of adult rats; striatum (A), prefrontal cortex (B). Data obtained by RT-PCR are shown as means ± SEM for control (n=9; white bars) and nicotine-exposed (n=8, black bars) animals. Mann-Whitney U test results significant at least at $P<0.05$ are marked as *.


(A) Weight gain
Weight gain, g
0
10
20
30
40
1
5
10
15
20
Birth weight, g
0
2
4
6
8
Veh
Nic
*
(B) Righting reflex
Latency to turn, s
0
10
20
30
2
3
4
5
*
(C) Negative geotaxis
Latency to turn, s
0
10
20
30
7
8
9
10
Postnatal day
*
(D) Grip strength
Latency to fall, s
0
5
10
15
20
17
Postnatal day
*


(A)
group: p<0.03
day: p<0.001
Correct responses, %
Control
Nicotine
(B)
day: p<0.001
group x day: p<0.05
Omissions, %
(C)
group: p<0.03
day: p<0.001
Reinforcers
Day
(D)
group: p<0.001
day: p<0.001
Anticipations, %
Day


group: p<0.07
group x day: p<0.03
Variability of latencies (SD)
0.4
0.3
0.2
0.1
0.0
*
*
Control
Nicotine
1
2
3
4
5
6
7
8
9
10
11
12
Day


20
18
16
14
12
10
8
6
Choices of large reward
Control
Nicotine
2 6
18
36
48 54 60 66
Delay to large reward, s


(A) Striatum
Control
Nicotine
Normalized relative quantity
1.5
1.0
0.5
0.0
*
TH
NR4A2
DAT1
DRD4
DRD5
(B) Frontal cortex
Normalized relative quantity
1.5
1.0
0.5
0.0
TH
NR4A2
DAT1
DRD4
DRD5
Gene