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Developmental Exposure to Low Level Ambient Ultrafine Particle Air Pollution and Cognitive Dysfunction

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Abstract

Developmental exposures to ambient ultrafine particles (UFPs) can produce multiple neuropathological and neurochemical changes that might contribute to persistent alterations in cognitive-type functions. The objective of the current study was to test the hypothesis that developmental UFP exposure produced impairments in learning, memory and impulsive-like behaviors and to determine whether these were selective and thus independent of deficits in other behavioral domains such as motor activity or motivation. Performance on measures of learning (repeated learning), memory (novel object recognition, NOR), impulsive-like behavior (differential reinforcement of low rate (DRL), schedule of reward and delay of reward (DOR)), motor activity (locomotor behavior) and motivation (progressive ratio schedule) were examined in adult mice that had been exposed to concentrated (10–20x) ambient ultrafine particles (CAPS) averaging approximately 45 ug/m³ particle mass concentrations from postnatal day (PND) 4–7 and 10–13 for 4 hr/day. Given the number of behavioral tests, animals were tested in different groups. Results showed male-specific alterations in learning and memory functions (repeated learning, NOR and DRL) specifically during transitions in reinforcement contingencies (changes in rules governing behavior) that did not appear to be related to alterations in locomotor function or motivation. Females did not exhibit cognitive-like deficits at these exposure concentrations, but displayed behaviors consistent with altered motivation, including increases in response rates during repeated learning, significantly increased latencies to respond on the delay of reward paradigm, and reductions in the progressive ratio break point. Consistent with our prior findings, male-specific learning and memory-related deficits were seen and occurred even at relatively low level developmental UFP exposures, while females show alterations in motivational behaviors but not final performance. These findings add to the evidence suggesting the need to regulate UFP levels.

Keywords

ultrafine particles; air pollution; learning; repeated learning; locomotor activity; progressive ratio; delay of reward; differential reinforcement of low rate; motivation

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INTRODUCTION

Air pollution, a complex mixture of particles, gases, trace metals, and adsorbed organic contaminants, has been reported to be the 7th leading global cause of mortality [1]. Its adverse effects are considered to derive from its ability to produce inflammation and oxidative stress, as has been most extensively examined in cardiopulmonary systems [2, 3]. Air pollution particulate sizes range from coarse ($<10\mu\text{m}$) to fine ($<2.5\mu\text{m}$) to ultrafine (UFPs; $<0.1\mu\text{m}$). The UFP fraction achieves orders of magnitude higher particle count concentrations and surface area than do larger particle sizes, which allows for greater adsorption of other toxic air pollutants such as oxidant gases (e.g., O_3 , NO_x), organic compounds, and transition metals per unit mass. UFPs are deposited in pulmonary alveolar regions of lung from where they can access pulmonary interstitium after traversing the alveolocapillary barrier. UFPs can then cross endothelial cells into blood circulation from where they can subsequently impact multiple organs [4], leading to serious health consequences. UFPs can also deposit in the nasal cavity and from there can be translocated to brain [5]. Given their inflammatory properties, UFPs are generally considered among the most reactive elements of air pollution [6, 7].

Increasingly, it is being recognized that air pollution targets other organs and systems of the body, including the central nervous system (CNS). Of particular concern is the developing brain that must undergo a precisely timed sequence of cell proliferation, differentiation, migration, and maturation. Disruption of these ontogenetic sequences can lead to neuropathological and morphological changes in brain that can adversely impact behavioral function. Numerous epidemiological studies have reported associations of various measures of air pollution with diagnosis of autism spectrum disorder, a heterogeneous disorder diagnosed on the basis of behavioral deficits [8, 9] including social deficits and perseveration. Increased odds ratios for autism diagnosis have been associated with traffic-related air pollution [8, 11], diesel exhaust and particles [12], and air toxics [9]. Maternal air pollution exposure increased autism risk based on data from across the U.S [23], with stronger associations for boys, consistent with the sex bias of autism cases. A study of 49,073 Taiwanese children of both sexes reported that air pollution exposure in the preceding 1–4 years increased autism diagnosis risk [10].

Multiple studies now also link air pollution exposure with cognitive deficits and reductions in IQ [11]. Such studies have included a prospective cohort showing a decline in the trajectory of cognitive growth in relation to levels of $\text{PM}_{2.5}$ in school children over a one-year period [12]. Another study links air pollution with diagnosis of attention deficit disorder [13], a disorder that can also impact executive and cognitive functions.

In our prior studies of developmental exposures of mice to concentrated ambient ultrafine particles (CAPS), we have observed behavioral, neurochemical and neuropathological deficits particularly in male mice [14–17]. These included deficits in a waiting for reward behavioral paradigm [14] that were suggestive of impulsivity, another facet of behavior that could contribute to learning, memory or attention deficits. The current study sought to extend those prior efforts to specifically evaluate cognitive functions following developmental exposure to concentrated ambient UFP exposure during the early postnatal

period, considered equivalent to human third trimester of pregnancy [18–20] using measures of learning and memory (repeated learning and performance and novel object recognition, respectively), and impulsive-like behaviors (delay of reward and a differential reinforcement of low rates of response schedule). To determine the extent to which any potential learning, memory or impulsivity deficits in those paradigms might instead reflect altered motor function or altered motivation levels, measures of locomotor activity and progressive ratio performance were likewise included [21, 22]. As we were able to expose mice to ambient CAPS concentrations in the current study that were only half of the concentration of our prior exposure concentrations [14–16], the current study also permitted assessment of behavioral deficits at levels of exposure lower than those in which CNS effects were previously observed and thus information on ‘dose-response’.

METHODS AND MATERIALS

Animals and Ultrafine Particle Exposure

Male and female C57Bl6/J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and allowed to acclimate in the housing room for 1 week prior to breeding. Male and females were group housed prior to breeding. Estrous cycles of females were synchronized via pheromone-induced ovulation by placing females in cages with dirty bedding from males [23]. Monogamous pairs of mice were then bred for 3 days, males were removed and dams remained singly housed with litters until weaning. All animals utilized for this study were born in the same cohort between 8pm to 6am the following day to minimize ontogenetic variability and harmonize animal age. Mouse pups were exposed to concentrated (10–20 fold) ambient ultrafine particles (CAPS) using the Harvard University Concentrated Ambient Particle (HUCAPS) described below. As with human air pollution exposures, this includes day-to-day variability in particle mass concentrations as well as various adhering components. The gas-phase components of the ambient aerosol are present, but are not concentrated by the HUCAPS system.

Mice were removed from the dams and randomly assigned to exposures to CAPS or HEPA-filtered room air providing 99.99% effective Hepa filtration. To preclude litter specific effects, only a single pup, per time point, per sex, per litter, per squad was used in the study and pups were randomly assigned to respective treatment groups. Exposures lasted for 4 hours/day between 1000 – 1400 hr and were carried out at postnatal days (PND) 4–7 and 10–13 in compartmentalized whole-body inhalation exposure chambers using the HUCAPS System fitted with a size selective inlet which contains a high volume (5000 liters/min) UFP concentrator to concentrate ambient particles generated from a nearby, highly trafficked road [16]. These exposure periods (PND4–7 and 10–13) were initially chosen based on their equivalence to human 3rd trimester [18–20] that represents a period of marked neuro- and gliogenesis [24] that were used in our prior studies demonstrating neuropathological and behavioral consequences of CAPS [15, 16, 25], thus allowing direct comparisons to prior studies. These exposure levels are consistent with those reported for U.S. cities such as Raleigh, NC and Pittsburgh, PA [26]. Filtered air and CAPS- treated mice experienced similar experimental manipulations. Controls were exposed to HEPA-filtered air in an

adjacent chamber. Both CAPS and control mouse chambers were maintained at 77–79°F and 35–40% humidity.

Upon weaning at postnatal day 25, offspring were pair-housed by sex and treatment conditions (2–3/cage) under a 12hr reversed light/dark cycle and temperature maintained at 72°F. Behavioral testing commenced at approximately 35 days of age for initial locomotor testing (see Table 1). At approximately 60 days of age, all mice were reduced to 90% of their ad libitum weight to provide motivation for food-rewarded behavioral testing and maintained at those weights for the duration of the experiment. Because of the number of behavioral tests in this study, mice were randomly allocated to three different groups with the following order of behavioral testing and age at testing for each as shown in Table 1: *Group 1*: social behavior as juveniles, locomotor activity, novel object recognition (NOR), social preference testing, differential reinforcement of low rate (DRL) schedule of reinforcement, progressive ratio (PR) schedule and elevated plus maze (EPM); *Group 2*: locomotor activity, multiple schedule of repeated learning and performance, conditioned place preference (CPP) and the PR schedule; *Group 3*: delay of reward (DOR), locomotor activity, CPP, and PR. Order of testing within groups and assignment of different behavioral paradigms to different groups was based on several initial considerations. First, operant testing, given its requirement for lever pressing, had to await sufficient maturity and size before testing could begin (usually at PND60), so that paradigms without such requirements were scheduled prior to that time. Order of testing within each group was structured so as to prevent, to the fullest extent possible, any carry-over effects of behavioral history, particularly for tests of learning and/or memory functions. For all 3 groups, significant early handling was involved that should also have minimized stress associated with behavioral testing. Social/anxiety behavioral performance outcomes will be reported elsewhere. The time of day of behavioral testing was consistent across the testing period. Final sample sizes were n=10/12 per group/sex in each of the three groups.

All mice used in this study were treated humanely and with regard for alleviation of suffering and all procedures were conducted with approval of the Institutional Animal Care and Use Committees at the University of Rochester.

Behavioral Paradigms

Air pollution has been linked to autism spectrum disorder [27] as well as to deficits in attention [28, 29] and in various cognitive and academic outcomes [12, 30, 31]. Autism diagnosis is based on behavioral domains that include deficits in communication and in social/emotional interactions as well as repetitive or stereotyped movement, but can also include intellectual impairments. Attention deficits have included inability to sustain attention, impulsivity and hyperactivity. Thus, a broad set of behavioral functions were included for assessment and to be able to separate motivational and/or motor deficits from changes in learning, memory and impulsive-like functions. Novel object recognition is routinely used as a measure of memory functions [32], but can be confounded by alterations in motor activity levels. For that reason, locomotor activity was used to assess general ambulatory function of mice. The multiple schedule of repeated learning and performance has been used across species from mice to humans. It measures learning per se in the

repeated learning component while the performance component controls for non-specific behavioral deficits [33–36]. It also allows measurement of learning repeatedly across time. Both the DRL (differential reinforcement of low rates) schedule and the delay of reward procedures have been used to measure impulsive-like behaviors [37]. Progressive ratio was used to determine motivation, consistent with a significant literature of its use in evaluating reinforce efficacy [38].

Locomotor Activity—Locomotor activity was assessed in automated chambers equipped with 48-channel infrared photo beams (Med Associates, Inc, St Albans, Vermont). Photo beam breaks were recorded every 5 minutes for 60 min to assess horizontal vertical, and ambulatory movements. Ambulatory counts were defined as the number of beam breaks while in ambulatory movement, while ambulatory episodes are defined by a minimum number of ambulatory counts (at least 3 successive photo beams). The total ambulatory time is the time in ambulatory movement status, while distance represents the Euclidean distance of all ambulatory episodes. Together they can be used to determine speed. Vertical activity was defined as movement that broke photo beams placed in the z-axis. Stereotypical/Horizontal movement was defined as number of photo beam breaks in a 2×2 beam box that were non-ambulatory. Stereotypic time and stereotypic counts measure activity within a defined space in the arena. Resting time was defined as time spent with no new photo beam breaks.

NOR—NOR testing consisted of two phases as we previously reported [16] and was conducted in an open Plexiglas arena (dimensions: 30.5 cm $\times$ 30.5 cm $\times$ 30.5 cm). In the first session, mice were placed for 10 min in the test environment that contained two objects, enabling individual assessments of response to novelty, decreased exploratory behavior or neophobia. During that time, side preference, exploration time, and patterns of exploration among treatment groups were assessed. The second session of the NOR paradigm assessed short-term memory, premised on an animal's awareness of novelty and its memory of already familiar objects. In the second session, occurring 24 h after session 1, mice were returned for 5 min to the arena, in which a novel object now replaced one of the previous two objects. Placement of the familiar vs. novel object was counterbalanced across treatments and subjects to preclude bias. All sessions were videotaped and scored using Noldus Observer by a reviewer blinded to treatment group. Exploration was defined as a mouse oriented toward the object with head first entry into a pre-marked 2 cm circle surrounding the object. A recognition index was calculated based on the number of bouts and time per approach to the novel object compared to the familiar object (bouts or time per approach to the novel object/(bouts or time per approach to the novel object + bouts or time per approach to the familiar object). Time per approach was calculated by the average time spent per bout of investigation for either the novel or familiar object.

Multiple Schedule of Repeated Learning (RL) and Performance (P)—Mice were first autoshaped to press the levers, as previously described [41], in operant chambers (Med Associates, Model ENV-307W) housed in sound-attenuating cabinets equipped with fans for ventilation and white noise generation. Three response levers were located horizontally across the back wall of the chamber, with a pellet dispenser for reinforcer delivery on the

front (opposite) wall. Following shaping of responding on all three levers, a two response RL program was implemented for a total of 6 sessions. This was followed by a three response RL program for a total of 6 sessions. Subsequently, a multiple schedule of RL and P was imposed.

Two Response RL: The two response RL paradigm required mice to complete a sequence of two responses to earn each food delivery, with the correct sequence changed with each successive behavioral test session. One session was carried out for each of the following two lever sequences: CL (center left lever), CR (center right lever), LC, RL, LR and RC, for a total of 6 behavioral test sessions, with sequences presented in random order. Sequences with repeated responses on one lever, e.g., LL, were not used so as to prevent perseverative lever pressing. Incorrect responses produced a 2 second time out period that was reset by any additional responses during the time out. Incorrect responses also required the subject to start the sequence over again.

Three response RL: The three response RL paradigm required mice to complete a sequence of three responses for each food delivery, with the correct sequence changed with each successive behavioral test session. Three lever sequences included RLC, LCR, LRC, CLR, CRL and RCL, and one behavioral test was carried out for each sequence; sequences were presented in random order across sessions. Sequences with repeated responses on one lever, e.g., LLR, were not used so as to prevent perseverative behavior. Other contingencies were identical to those in the two response RL paradigm.

Multiple Schedule of RL and P: Following the three response RL paradigm, a multiple schedule that included two components, RL and P, was imposed. On this multiple schedule, each behavioral test session was comprised of one presentation each of the RL and P components, and the sessions in phases 1 and 2 always began with the P component. The RL component was identical to the three-response training program described above. The P component also required mice to complete a sequence of 3 responses for each reward delivery, but in this component the sequence of three responses remained constant across sessions. Other contingencies were identical to those described for the three response RL paradigm. The offset of the lights above the levers was the discriminative stimulus associated with the P component, while onset of the lever lights served as the discriminative stimulus associated with the RL component. Based on the accuracy data from three response RL, the sequence LCR was chosen because of the high associated accuracy levels to serve as the correct sequence for the P component. The remaining 5 three response sequences (LRC, CLR, CRL, RLC and RCL) were used in the RL component. The components shifted from P to RL or ended after the RL with the acquisition of 25 reinforcers or the elapse of 30 min, whichever occurred first. The multiple schedule format allows a determination of the specificity of any observed learning deficits, as intact motor, sensory and motivation are required for both the RL and P components, but learning per se is only required in the RL component [21, 22]. Thus, selective learning impairments are manifest as decrements in accuracy in the RL, but not the P component.

During Phase 1 of the multiple RL and P schedule, a total of 6 presentations of each of the 5 RL sequences, presented in random order within each block occurred, for a total of 30

sessions. In Phase 2, only two of these sequences, specifically RLC and RCL, were used. In each case the RL sequence was re-used over 5 successive behavioral test sessions for a total of 10 sessions. During Phase 3, each of these two sequences were again used in two successive sessions, but in this case the order of components and their corresponding discriminative stimuli were changed, such that the RL component was presented first with stimuli normally signaling the P component, followed by the P component with stimuli normally signaling the RL component.

Measures of behavioral performance included percent accuracy (correct responses/total responses minus time out responses), rate of response (total responses/total time), responses per completed sequence (total correct responses/total reward deliveries), total number of reinforcers earned, and percent of errors that were perseverative (repeated response on a lever) and that were skipping errors (errors that involved skipping forward or backward in a sequence).

Delay of Reward (DOR)—The delay of reward paradigm, also known as delayed (temporal) discounting or self-control paradigm, provides a choice between a short delay followed by a small reward, or a long delay followed by a larger reward [42]. After initial lever press autoshaping, mice were trained to chain respond center-left and center-right for reward until a criterion of 30 reinforcer deliveries had been met. Subsequently, the DOR paradigm was implemented. Each session consisted of the presentation of 3 blocks of 10 trials, with the left lever designated as the long delay lever and the right lever as the short delay lever [43]. Each block began with two forced choice trials, one forcing a long delay choice after the center lever response initiated the trial, and the other forcing a short delay choice, with a probability = 0.5 of long or short on the first forced choice trial. The 2 forced choice trials within each block were thereafter followed by 8 choice trials. In choice trials, a center lever response initiated the trial, shut off the center lever light, and illuminated the left and right lever lights. Responses on the right (short delay) lever extinguished both the right and left lever lights, and produced a flash of the right lever lights and the immediate delivery (0.1 s, but hereafter referred to as 0s) of a single food pellet; this was followed by a 70 s inter-trial interval before the center lever light was again illuminated providing the opportunity to initiate the next trial. Responses on the left (long delay) lever extinguished both the right and left lever lights, and after a specified delay period, produced a brief flash of the left lever lights and delivery of 3 food pellets. This was followed by an inter-trial interval of 70 s minus the delay value, after which the center lever lights were re-illuminated again providing the opportunity to initiate the next trial. Center lever responses at any other point initiated a 2-s timeout with all lights off that was reset by any additional center lever responses. Failure to initiate a trial with a center lever response within 10 s of the opportunity was recorded as either a forced or choice trial omission response and re-started the 10-s opportunity clock. Responses on the right or left lever prior to an initiation response on the center lever likewise resulted in a 2-s timeout with all lights off that was re-set by any additional center lever responses. Sessions ended after completion of the 3rd block (30 total trials) or 45 min, whichever occurred first. Long delay lever values were initially set at 0s, and then increased to 5, 10 and 15 sec across sessions before returning to 0 sec. Measures of performance included correct responses on the center lever (trial initiations) and, percent

large reward (long delay) choice, latency to center lever responses and latency to choice responses as well as total omission responses (failure to respond in the 10-sec window to initiate a trial).

Differential Reinforcement of Low Rate (DRL) Schedule—The DRL schedule reinforces responses that exceed a designated interresponse time [44]. Autoshaping of lever press responding was initially carried out, then followed by implementation of the DRL schedule of reinforcement. The schedule required a specified length of time to elapse before a response could occur to produce food reward; responses occurring prior to the end of this time value reset the timer. The initial DRL value was set at 6 seconds and was subsequently increased to 12, 18, 24, 30, 36 and 42 seconds across sessions. Behavioral test sessions were 30 min in length. Measures of performance included total responses, total reinforcers earned, numbers of responses and response rates on the active and inactive levers, premature responses and responses/reinforcer.

Progressive Ratio (PR) Schedule—A progressive ratio schedule was used to further evaluate motivation. On the progressive ratio schedule, a fixed number of responses on the active (correct) lever were required for each food delivery. The ratio of responses for each reward increased after each reward delivery [45, 46]. The initial ratio was set at a requirement of 1, with successive increases of 1 (to 8), followed by increases of 2 (10–24), then increases of 4 (28–52) and subsequently by increases of 8 (64 and above). The session ended when 5 min without any response occurred and the ratio at which this occurred was defined as the break point. Measures of progressive ratio performance included response rate (total responses/total time) and the ratio value at the break point.

Statistical Analyses

Overall analyses were initially conducted with sex as a factor. On nearly all endpoints, data confirmed either main effects of sex or sex x time interactions, for paradigms involving repeated measures. For that reason and given that our prior studies have consistently shown sex differences in behavioral and neuropathological effects of this CAPS exposure [15–17], behavioral performances across sessions were analyzed using repeated measures ANOVA and carried out separately by sex. For measures of behavioral performance that did not include a time (sessions) component, ANOVAs were carried out again by sex. In all cases, post-hoc assessments were carried out based on interactions for the overall ANOVAs, using a Fisher's least significant differences tests.

RESULTS

Exposure Characterization

Details of the exposures are presented in Figure 1. As it shows, particle mass concentrations were quite low, and generally ranged from 40 to 60 $\mu\text{g}/\text{m}^3$, averaging approximately 45 $\mu\text{g}/\text{m}^3$ across days. Particle count concentrations showed a wide range, consistent with intra- and inter-day real-world variability, and averaged between 17,215 to 83,378 particles/ cm^3 . In addition, particle sizes were, as expected, generally below 100 nm (not shown), as in our prior studies [15].

Learning and Memory Behavioral Paradigms

Repeated Learning

Two Response RL: Figure 2 presents measures of accuracy, responses required to complete a sequence, total number of reinforcers earned and overall response rate for the initial two response sequence RL paradigm for both males and females from group 1 (Table 1). As in our prior studies, performance on the paradigm was highly sequence- and sex-dependent [35, 47, 48]. Subsequent ANOVAs by sex revealed a near significant effect of CAPS treatment on overall accuracy in males (main effect of treatment; $F(1,22)=4.15$, $p=0.054$). As can be seen, this was primarily due to small reductions in levels of accuracy for sequences CL, CR, LC and RL, of which only sequence CL was statistically significant in post-hoc tests relative to control males ($F(1,22)=8.45$, $p=0.008$). No effects were found in females.

Following the two response RL paradigm, there were no deficits observed in learning accuracy for the three response RL, nor on the multiple schedule of RL and P. However, CAPS-exposed females showed higher response rates than female controls during the RL but not the P component of the multiple schedule (Figure 3; $F(1,22)=4.06$, $p=0.056$) that ranged from 25–45% above control. In addition, females exposed to CAPS showed evidence of increased total numbers of perseverative errors for 3 of the 6 sequences relative to female controls (data not shown: LRC: $F(1,22)=3.75$, $p=0.066$; CLR: $F(1,22)=10.2$, $p=0.004$; RCL: $F(1,22)=5.7$, $p=0.026$). The increases in total numbers of perseverative errors likely reflect the higher response rates of CAPS-exposed females relative to female controls and thus did not contribute to changes in accuracy.

Novel Object Recognition—Male-specific deficits were found in NOR performance that consisted of significant increases during session 2 in the number of non-novel bouts by approximately 48% ($F(1,19)=4.74$, $p=0.042$; Figure 4) in group 1 (Table 1) CAPS-treated males relative to control males. Correspondingly, there was a significant reduction of approximately 20% in the novel index recognition score ($F(1,19)=4.74$, $p=0.042$) of CAPS-treated males relative to control males. Similar but non-significant trends occurred with time per approach measures. No effects were found in session 2 in females, nor did either males or females show any systematic CAPS-related differences in behavior during session 1 in rate of contacts, total contacts, or mean time per approach.

Impulsivity Behavioral Paradigms

DRL Schedule—CAPS-related changes in group 1 (Table 1) DRL performance were seen primarily at transition points when the required DRL value was increased (e.g., last session at 6 sec to first session at 12 sec, etc.) and were predominant in males. For example (Figure 5), CAPS-treated males earned significantly fewer reinforcers than control males with the transition from a 6 to a 12 sec DRL schedule (CAPS x time: $F(1,22)=5.14$, $p=0.034$), with this trend persisting during the transition from a 12 sec to an 18 sec DRL value (CAPS, $F(1,22)=3.1$, $p=0.092$). In addition, CAPS-treated males showed a greater increase than control males in total number of responses, including premature responses in the transition from a 6 sec to a 12 sec DRL (CAPS x time: $F(1,22)=4.77$, $p=0.04$ and $F(1,22)=4.5$, $p=0.046$, respectively). This included a greater increase in active lever responses, as well as increased active lever response rates (Figure 6; CAPS x time: $F(1,22)=5.88$, $p=0.024$, and

($F(1,22)=4.44$, $p=0.047$, respectively). These increases resulted in a significantly greater number of responses required for each reinforcer earned in CAPS-treated males than in control males during the transition from DRL 6 to DRL 12 sec (Figure 5; CAPS, $F(1,22)=6.8$, $p=0.016$; CAPS x time, $F(1,21)=5.35$, $p=0.031$).

In contrast, significant transition effects in females were limited to a significantly greater increase in inactive lever responses and inactive lever response rates during the transition from DRL 6 to DRL 12 sec (Figure 6; CAPS x Time, $F(1,20)=4.89$, $p=0.039$ and $F(1,20)=4.89$, $p=0.04$, respectively) relative to control females.

DOR Performance—Deficits in DOR performance in group 3 (Table 1) were observed to a greater extent in CAPS-treated females than in males (Figures 7 and 8). Specifically, CAPS-treated females showed a decrement in correct center lever responses (Figure 7) at DOR values of 0 ($F(1,19)=5.43$, $p=0.03$) and 5 second long delay values (CAPS x time: $F(18,342)=2.9$, $p<0.0001$) relative to control females, with similar but non-significant trends evidenced at 10 and 15 sec DOR values. CAPS-treated females also showed (Figure 8) significantly longer latencies not only to initiate trials (center response latency; 0 sec: $F(1,19)=4.57$, $p=0.046$; 10 seconds: $F(1,9)=3.48$, $p=0.078$), but to make a choice response once the trial was initiated (choice response latency; 0 sec: $F(1,19)=5.41$, $p=0.031$; 5 sec: $F(1,19)=3.98$, $p=0.061$; 10 sec: $F(1,19)=4.45$, $p=0.485$; 15 sec: $F(1,19)=8.27$, $p=0.0097$) than did female controls, being equivalent to control values only at the return of the DOR value to 0 seconds in the final sessions. Additionally, CAPS-treated females showed a significantly or marginally significantly greater number of trials in which response omission occurred (Figure 8: failure to make a choice response within the 10 sec window (total omission responses; 0 sec: $F(1,19)=4.07$, $p=0.058$; 5 sec: CAPS x time, $F(19,361)=1.98$, $p=0.009$; 10 sec: $F(1,19)=3.77$, $p=0.067$; 15 sec: $F(1,19)=6.67$, $p=0.0182$) as compared to control females. Because of these omissions, session length was also significantly increased (Figure 7) by CAPS in CAPS-treated females at DRO values of 0 sec ($F(1,18)=4.54$, $p=0.047$), marginally at 5 sec ($F(1,18)=3.08$, $p=0.096$), and significantly at both 10 and 15 sec values ($F(1,18)=4.41$, $p=0.050$ and $F(1,18)=7.23$, $p=0.015$, respectively) relative to control females. This however, did not influence the number of responses required for each reinforce earned.

In contrast, CAPS-treated males showed small but significantly higher percentages of long delay large reward choices later in the course of testing (percent large reward choice; 10 sec: $F(1,22)=9.14$, $p=0.006$; 15 sec: $F(1,22)=4.73$, $p=0.0406$; final 0 sec: $F(1,22)=3.84$, $p=0.063$) than control males and a lower number of total omission responses than control males early in the course of testing (0 sec: CAPS x time, $F(4,19)=3.06$, $p=0.042$).

Progressive Ratio Performance—Progressive ratio performance did not produce any CAPS-related differences in total responses, total reinforcers, response rates or in the break point in either groups 2 or 3 (Table 1; data not shown), but was altered by CAPS in group 1 (Table 1) that had undergone behavioral testing on the NOR and DRL paradigms, and this CAPS effect was observed primarily in females (Figure 9). CAPS-treated females showed reductions in total numbers of responses ($F(1,21)=12.66$, $p=0.002$), both on the active lever ($F(1,21)=10.0$, $p=0.0048$) and inactive levers ($F(1,21)=13.82$, $p=0.0014$), and earned

significantly fewer reinforcers ($F(1,21)=11.3$, $p=0.003$) than did control females. In addition, the mean final PR value (break point) of CAPS-treated females (158.4) was 32% lower (229.3) than that of female filtered air controls ($F(1,21)=11.3$, $p=0.003$). CAPS-treated males exhibited a significantly lower number of inactive lever responses than control males ($F(1,22)=5.34$, $p=0.031$), but otherwise performance did not differ from filtered air controls.

Locomotor Activity—Locomotor activity was influenced by CAPS treatment both early in life (group 1, Table 1; tested at 34 days of age) and in later adulthood (group 3, Table 3, tested at 7.5 mos of age) in males. Locomotor alterations in response to CAPS treatment were male-specific when mice were juveniles (Figure 10 **from group 1**) and primarily related to modest reductions in measures of ambulatory behavior across blocks of the session (ambulatory counts: CAPS x time, $F(11,12)=2.93$, $p=0.039$; ambulatory episodes: CAPS x time, $F(11,12)=4.57$, $p=0.007$; ambulatory distance: CAPS x time, $F(11,12)=4.26$, $p=0.0097$; ambulatory time: CAPS x time, $F(11,12)=2.97$, $p=0.037$) in CAPS-treated males as compared to control males. As can be seen, these were intermittent periods of the 60 min session in which CAPS-treatment reduced ambulatory behavior. No effects were found in females at 34 days of age.

CAPS-related changes in locomotor activity were also seen at later adulthood (Figure 11 **from group 3**, Table 1). At this later time period, however, increases rather than reductions were seen in ambulatory time (CAPS x time: $F(8,15)=3.67$, $p=0.014$), ambulatory distance (CAPS x time, $F(8,15)=3.58$, $p=0.016$), and ambulatory counts (CAPS by time, $F(8,15)=2.71$, $p=0.046$) in CAPS-treated males as compared to control males. These increases were accompanied by a corresponding reduction in resting time (CAPS x time, $F(8,15)=2.67$, $p=0.048$). While a significant CAPS x time interaction was found for jump time in females, no significant differences were found for any specific block during post hoc testing. Both stereotypic time ($F(1,19)=4.41$, $p=0.049$) and stereotypic counts ($F(1,19)=4.45$, $p=0.048$) were significantly reduced by CAPS in females as compared to control females.

DISCUSSION

The objective of the current study was to ascertain the potential cognitive-type deficits (e.g., learning, memory) and impulsive-like behaviors that could result from developmental exposures to UFP in a mouse model [15–17, 25]. The fact that UFP exposures in the current study were significantly lower than in our previous studies [14–16, 25, 49] allowed a determination of behavioral consequences of a lower exposure concentration in this case. Considered collectively, the findings demonstrate that even at these relatively low UFP exposure concentrations, a preferential impact of developmental CAPS exposure on learning and memory functions in males was observed, as well as what might be conceived as potential motivational shifts in females.

Learning impairments were seen in males in the form of small decrements in acquisition of learning of two lever RL response chains; as we have previously observed, such decrements are sequence specific [35, 47, 50]. CAPS-treated males also showed more robust deficits in novel object recognition, a paradigm used to evaluate short-term memory than did male controls. Notably, most of these findings related to points of transitions in contingencies of

reinforcement, i.e., changes in the rules governing reward, rather than persistent deficits. With respect to repeated learning, the two lever RL of specific sequences paradigm was the first exposure of subjects to RL after lever press training that simply required a change to a different lever for each reinforcer delivery, suggesting deficits in periods of early acquisition. While effects on RL were transient in that they were not observed when three lever RL or the multiple schedule of RL and P were subsequently imposed, they are consistent with a transition deficit that did not appear to reflect alterations in response rate or motivation.

This type of transition effect was also seen in DRL performance of CAPS-treated males relative to control males. Specifically, sustained deficits were not found in DRL performance across sessions at any given DRL value, but again were seen in the transition from the final session at a lower DRL value to the first session at a higher DRL value. As with repeated learning, CAPS-related DRL deficits were particularly prominent in the first such transition, i.e., from DRL 6 sec to DRL 12 sec, where treated males ‘over-responded’ on the active lever relative to male controls, further delaying reward and ultimately earning fewer reinforcers and having to expend more responses per each reinforcer earned. In addition to a type of learning deficit, such behavior could reflect increases in locomotor activity per se or impulsive-like behavior. Both such explanations seem unlikely, however, as deficits were transition-specific rather than observed consistently.

Similarly, the deficit in recognition memory on the NOR paradigm seen in CAPS-treated males relative to control males reflects a transition point in the paradigm when changes in one component of the environmental stimuli present are imposed. Here, males spent a greater amount of time with the non-novel stimulus. While such an effect is typically interpreted as a memory deficit, it can indicate other behavioral deficits, such as heightened fear of novelty, lower levels of activity overall, etc. However, the lack of differences in numbers and timing of contacts with objects during the training session indicates that the NOR deficit in session 2 was likely not a function of decreased familiarity or interaction with the objects. Thus, the reduction appears to be more consistent with a short-term memory impairment. These findings are consistent with other reports of deficits in NOR following diesel exhaust with secondary organic aerosols for one or three months in mice [51]. In addition, they are consistent with deficits in NOR observed in our prior study [16] following developmental UFP exposures. That study incurred a higher particle exposure concentration (~40,000 to 496,000 particles/cm³) and deficits were seen under those conditions in both sexes. Collectively, the current and prior study [16] suggest both a ‘dose-response’ in males for impaired memory, with approximately 50% reductions in NOR recognition memory seen at the higher particle exposure concentration and an approximately 20% reduction at the lower particle exposure concentrations experienced here. In addition, they are consistent with a greater sensitivity of males to CAPS effects, since effects in females were only seen at higher exposure concentrations, findings again consistent with our prior studies [11, 15–17]. However, the latter finding requires replication as the NOR, with a 24 hr interim period may have been too difficult for females, as indicated by a novel index score in Session 2 that did not suggest greater attention to the novel object in control females.

Behavioral alterations in CAPS-treated females relative to control females were seen primarily during the DOR paradigm. These were not reductions in the percentage of long delay large reward lever choices, as the delay value increased, such as would be expected with an impulsive-like phenotype. Rather, they appear to be primarily motivational, in that CAPS-treated females delayed initiation of trials, and once initiated, they also delayed making a choice between the long delay and short delay levers. This increased the number of omission responses, i.e. the time period during which initiations and choices had to be made. Since the session ended only after completion of the third block of trials, however, it did not ultimately influence the number of reinforcers earned, as CAPS-females sustained a longer session length than control females. That this deficit may be motivational is also suggested by the reductions in the final ratio value on the progressive ratio schedule that were observed in one group of CAPS-treated females. However, a lower PR breakpoint did not occur in the other two groups, that had previously undergone testing on different sets of behavioral paradigms. These differential PR breakpoints between behavioral groups raises interesting questions as to whether CAPS-related behavioral impairments interact with differences in behavioral history [52, 53]. Contrary to suggestions of reduced motivation in the DOR and PR paradigms, however, females exhibited higher response rates during the RL component of the multiple schedule, suggesting higher levels of motivation.

The findings from this study in males are in accord with learning and memory deficits that have been reported in association with air pollution in many, although not all, studies in humans and animal models [54–62]. For example, more rapid cognitive (learning, memory and attention) decline was reported in elderly U.S. women exposed from 7–14 years to PM_{2.5–10} and PM_{2.5} [63]. Similarly, in a London cohort, levels of PM were associated with a decline in learning, memory and reasoning over a 5 year period [64]. In another prospective study, residential proximity to a major roadway impaired verbal learning and memory, psychomotor speed, language and executive functioning in Boston community-dwelling seniors [65]. In a national sample of older U.S. adults, PM_{2.5} was associated with a 1.5 times higher error rate in a cognitive assessment task [66]. Higher black carbon levels in a prospective cohort study were shown to be predictive of decreased cognitive function, particularly verbal and nonverbal intelligence and memory function, in Boston children [67]. In a study of subcutaneous exposures of mice to 200 ug/kg body weight diesel exhaust particle suspensions on gestational days 6, 9, 12, 15 and 18, adult male offspring (females not tested) showed deficits in acquisition of water maze performance, as indicated by longer latencies to find the escape platform; however, significant effects were only found on one day, i.e. day 7 of the 9 days of behavioral testing [68]. Four week old male mice exposed to concentrated ambient PM_{2.5} (94.3 ug/m³) or filtered air for 6 hr per day, 5 days per week for approximately 10 months demonstrated increased latencies to find the escape hole in a Barnes maze, although this effect was only statistically significant for day 2 of the 4 days of assessment; numbers of errors on the maze (wrong escape hole attempts) were also increased by PM_{2.5} during session 2 of training [69].

A preferential vulnerability of males to CAPS-related transition learning impairments is consistent with prior neurochemical and neuropathological findings from this model [15–17]. At the higher UFP exposure levels that occurred during the human third trimester equivalent [18–20] in our prior studies, and at a time of marked neuro- and gliogenesis [24],

CAPS produced persistent and male-specific ventriculomegaly and microglial activation, greater reductions in size of the corpus callosum, hypomyelination of the corpus callosum and frontal cortex excitatory-inhibitory imbalance.

Multiple questions arise from these studies with respect to developmental exposures to air pollution and subsequent learning and memory and impulsivity deficits seen in males. One such question concerns the mechanisms of such impairments. They could derive from more subclinical levels of many of the types of changes seen in male brains at the higher UFP exposure levels [14–16]. As is well known, reductions in myelination and decreased corpus callosum size, for example, signify hemispheric disconnectivity that can adversely impact learning [70–74]. Microglial inflammation and its consequences can likewise alter learning and memory functions [75–79]. Alterations in glutamate status are likewise related to aspects of learning and memory [80–82].

An additional question relates to the component(s) of air pollution responsible for such deficits. As of yet, answers to this question are lacking. Human studies have used multiple different surrogates of air pollution, many of which are highly correlated, making distinctions between responsible components difficult to ascertain [64–67]. Animal studies, which have been limited to date, have included exposures to diesel engine exhaust or to PM_{2.5} [68, 69]; in some of these studies, moreover, exposures are not to ambient sources or even via inhalation which increases the difficulty of ascertaining their relevance to ambient conditions. In the current study, the ambient UFP exposures would include both metal and organic contaminants that could both play a role. Also unclear is whether the core carbon particle itself contributes to neurochemical and/or neuropathology related to cognition. Also to be considered is the fact that different components of such a mixture may underlie different behavioral impairments.

A third critical question relates to critical periods of exposure during development and the effects of air pollution on cognitive functions, a question that has not been systematically addressed. The current studies expose mice during the early postnatal period, a time considered equivalent to human third trimester [18–20]. Other studies however, have focused on gestation [83], or carried out exposures during adulthood [84]. It seems highly likely that exposures during different periods of development will produce different types of deficits in cognitive functions, contingent upon the developmental period of exposure, such that a single critical window does not exist.

In summary, exposures to low-levels of concentrated ambient ultrafine particles in mice during the third trimester equivalent produced male-preferential deficits in cognitive functions that were seen primarily during transitions in response requirements, while females display behaviors consistent with complex alterations in motivational aspects of performance. Such findings may be relevant to regulatory aspects of air pollution. Current air pollution regulations are focused on levels of PM₁₀ and PM_{2.5}, but not on UFPs. While technologically challenging, such regulation may be warranted, particularly when evidence has shown that regulation of PM particle sizes does not necessarily control or correlated with UFP levels [85–87].

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Highlights

- Developmental exposure to low concentration CAPS male-specific cognitive impairments
- Learning impairments in males emerged when rules of reward changed
- Male impairments did not appear to be attributable to non-cognitive behavioral changes
- Low level CAPS produced alterations in motivation-based behaviors in females
- Females did not show impairments in cognitive performance.

Figure 1. Characterization of exposures to ambient ultrafine particles
 Top plot shows particle size (nm) and mass concentrations ($\mu\text{g}/\text{m}^3$) over the exposures from PND4–7 and 10–13. Bottom plot shows particle count number mean values and range for the same time period.

Figure 2. Two lever repeated learning paradigm

Measures of RL accuracy, number of responses necessary to complete a sequence, total reinforcers earned and overall rate of female (top row) and male (bottom row) filtered air and CAPS-treated mice for each of the 6 sequences used during two lever learning arranged in order of their testing across sessions. Data from group 1 (see Table 1). Each bar depicts the group mean $\pm$ S.E. values.

Figure 3. Overall response rates on the multiple RL and P baseline

Group mean $\pm$ S.E. overall response rates on the RL (left) and P components (right) of the multiple RL and P schedule for females (top row) and males (bottom row). Data from group 1 (see Table 1). CAPS indicates near significant main effect of treatment.

Figure 4. Performance on the Novel Object Recognition Task (NOR)

Measures of total non-novel bouts, novel bouts and a total novel bouts index, as well as corresponding measures of the time to approach of these bouts in the NOR paradigm for female and male mice exposed to filtered air or CAPS as indicated. Data from group 1 (see Table 1). Each bar depicts the group mean $\pm$ S.E. values. * indicates significant difference from corresponding sex control group.

Figure 5. Performance on the Differential Reinforcement of Low Rates Schedule of Reinforcement (DRL)

Measures of the number of total reinforcers earned, total responses emitted, premature responses (i.e., before the end of the designated DRL value) and numbers of responses required to earn each reinforce on the DRL schedule. Data are shown for transition sessions, i.e., last session of DRL 6 sec to first session of DRL 12 sec, DRL 12 to DRL 18 and DRL 19 to DRL 24 of female (top row) and male (bottom row) filtered air and CAPS-treated mice. Each data point depicts the group mean $\pm$ S.E. value. Data from group 1 (see Table 1). CAPS x time: significant interaction of CAPS by session in RMANOVA.

Figure 6. Performance on the Differential Reinforcement of Low Rates Schedule of Reinforcement (DRL)

Measures of the total number of active lever responses, inactive lever responses, active lever response rate and inactive lever response rate on the DRL schedule. Data are shown for transition sessions, i.e., last session of DRL 6 sec to first session of DRL 12 sec, DRL 12 to DRL 18 and DRL 19 to DRL 24 of female (top row) and male (bottom row) filtered air and CAPS-treated mice. Data from group 1 (see Table 1). Each data point depicts the group mean $\pm$ S.E. value. CAPS x time: significant interaction of CAPS by session in RMANOVA.

Figure 7. Performance on the Delay of Reward Baseline

Measures of correct center lever (trial initiation) responses, percent choice of long delay large reward value lever, total session length and number of responses per reinforcer earned of female and male filtered air and CAPS-treated mice across 5 session blocks at the indicated DOR values used. Data from group 3 (see Table 1). Each data point depicts the group mean $\pm$ S.E. values. CAPS: significant main effect of CAPS in RMANOVA for indicated DOR value; ~CAPS: marginally significant main effect of CAPS in RMANOVA for indicated DOR value.

Figure 8. Performance on the Delay of Reward Baseline

Measures of latency to initiate a trial (center response latency), latency to choose the long vs. short delay lever following trial initiation (choice response latency) and total omissions (numbers of choice response opportunities that exceeded the 10 sec time limit) of female and male filtered air and CAPS-treated mice across 5 session blocks at the indicated DOR values used. Data from group 3 (see Table 1). Each data point depicts the group mean $\pm$ S.E. values. CAPS: significant main effect of CAPS in RMANOVA for indicated DOR value; ~CAPS: marginally significant main effect of CAPS in RMANOVA for indicated DOR value.

Figure 9. Assessment of Progressive Ratio Performance (PR)

Measures of total responses, total numbers of responses on the active lever, total numbers of responses on the active lever, percent of total responses on the active lever, total reinforcers earned and the final ratio value prior to the break-point on the progressive ratio schedule of reward of female and male filtered air and CAPS-treated mice. Data from group 1 (see Table 1). Each data point depicts the group mean $\pm$ S.E. values. * indicates significant difference from corresponding sex control group.

Figure 10. Assessment of Locomotor Activity

Measures of ambulatory counts, ambulatory episodes, ambulatory distance and ambulatory time during session 2 of the 3 day test of locomotor activity of female and male filtered air and CAPS-treated mice at postnatal day 34 across 5 min blocks of the 60 min session. Data from group 1 (see Table 1). Each data point depicts the group mean $\pm$ S.E. values for the indicated session. CAPS x time: significant interaction of CAPS by session in RMANOVA.

Females

Males

Figure 11. Assessment of Locomotor Activity

Measures of jump time, stereotypic time and stereotypic counts in females and of ambulatory time, ambulatory distance, ambulatory counts, and resting time of males during session 2 of the 3 day test of locomotor activity of female and male filtered air and CAPS-treated mice at 7.5 mos of age across 5 min blocks of the 60 min session. Data from group 3 (see Table 1). Each data point depicts the group mean $\pm$ S.E. values for the indicated session. CAPS: main effect of CAPS in RMANOVA; CAPS x time: significant interaction of CAPS by session in RMANOVA. * indicates significant difference from corresponding sex control group data point at that block.

Table 1
Sequential Order of Behavioral Testing, Paradigms Used and Age of testing for Each Squad

<i>Squad 1</i>		<i>Squad 2</i>		<i>Squad 3</i>	
Behavioral Assay	Age (mos)	Behavioral Assay	Age (mos)	Behavioral Assay	Age (mos)
Locomotor Activity	1	Locomotor Activity	3.5	Locomotor Activity	7
Novel Object Recognition	1.5	Repeated Learning	4.5	Conditioned Place Preference	8
DRL Schedule	3.5	Progressive Ratio Schedule	7	Progressive Ratio Schedule	8
Progressive Ratio Schedule	6.5	Conditioned Place Preference	8	Delay of Reward Schedule	9
Elevated Plus Maze	7				