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Early postnatal inhibition of serotonin synthesis results in long-term reductions of perseverative behaviors, but not aggression, in MAO A-deficient mice

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Abstract

Monoamine oxidase (MAO) A, the major enzyme catalyzing the oxidative degradation of serotonin (5-hydroxytryptamine, 5-HT), plays a key role in emotional regulation. In humans and mice, MAO-A deficiency results in high 5-HT levels, antisocial, aggressive, and perseverative behaviors. We previously showed that the elevation in brain 5-HT levels in MAO-A knockout (KO) mice is particularly marked during the first two weeks of postnatal life. Building on this finding, we hypothesized that the reduction of 5-HT levels during these early stages may lead to enduring attenuations of the aggression and other behavioral aberrances observed in MAO-A KO mice. To test this possibility, MAO-A KO mice were treated with daily injections of a 5-HT synthesis blocker, the tryptophan hydroxylase inhibitor p-chloro-phenylalanine (pCPA, 300 mg/kg/day, IP), from postnatal day 1 through 7. As expected, this regimen significantly reduced 5-HT forebrain levels in MAO-A KO pups. These neurochemical changes persisted throughout adulthood, and resulted in significant reductions in marble-burying behavior, as well as increases in spontaneous alternations within a T-maze. Conversely, pCPA-treated MAO-A KO mice did not exhibit significant changes in anxiety-like behaviors in a novel open-field and elevated plus-maze; furthermore, this regimen did not modify their social deficits, aggressive behaviors and impairments in tactile sensitivity. Treatment with pCPA from postnatal day 8 through 14 elicited similar, yet milder, behavioral effects on marble-burying behavior. These results suggest that early developmental enhancements in 5-HT levels have long-term effects on the modulation of behavioral flexibility associated with MAO-A deficiency.

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1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) plays a fundamental role in the orchestration of emotional reactivity and in the pathophysiology of numerous mental disorders, ranging from anxiety to pathological aggression (Baldwin and Rudge, 1995; Lucki, 1998; Olivier, 2004). One of the most important mechanisms for the regulation of 5-HT neurotransmission is its metabolism by oxidative deamination, primarily catalyzed by monoamine oxidase (MAO) A (Bortolato *et al.*, 2008; Bortolato *et al.*, 2009). This mitochondrial membrane-bound enzyme, which is encoded by a gene located on the X-chromosome in all vertebrate species (Bortolato *et al.*, 2008), also serves the degradation of other monoamine transmitters, such as norepinephrine (NE) and dopamine (DA) (Shih *et al.*, 1999; Bortolato *et al.*, 2010). The bulk of evidence indicates that low MAO-A brain activity may be a viable marker for the severity of antisocial traits (Alia-Klein *et al.*, 2008). In addition, several studies have documented that *MAOA* allelic variants associated with low catalytic activity predispose maltreated and abused boys to develop antisocial and aggressive traits (Caspi *et al.*, 2002; Kim-Cohen *et al.*, 2006; Fergusson *et al.*, 2011).

In humans, a nonsense mutation of the *MAOA* gene results in Brunner syndrome, an X-linked condition characterized by antisocial and disruptive traits, violent aggression in response to sudden stressors, repetitive behaviors and mild cognitive impairments (Brunner *et al.*, 1993a; Brunner *et al.*, 1993b). Consistently, MAO-A knockout (KO) mice display marked aggression, in association with other phenotypic alterations, including social and communication deficits, perseverative and neophobic-like responses, sensory disturbances, as well as disrupted barrel fields in the somatosensory cortex (Cases *et al.*, 1995; Kim *et al.*, 1997; Godar *et al.*, 2011; Bortolato *et al.*, 2012a). These aberrances are accompanied by elevated 5-HT brain concentrations, particularly during the first two postnatal weeks of life (Cases *et al.*, 1995).

Several findings suggest that the phenotypic abnormalities associated with MAO-A deficiency may depend on their high 5-HT brain levels during early postnatal stages: first, inhibition of 5-HT synthesis with the tryptophan hydroxylase inhibitor *p*-chloro-phenylalanine (pCPA) during the first postnatal weeks was shown to normalize barrel field morphogenesis (Cases *et al.*, 1996); second, the aberrant phenotypes in MAO-A KO mice are rescued by the reinstatement of this enzyme in the forebrain at birth (Chen *et al.*, 2007); third, perinatal administration of MAO-A blockers induces long-term aggressive responses and other behavioral changes (Whitaker-Azmitia *et al.*, 1994; Mejia *et al.*, 2002).

In contrast with this background, the long-term impact of early pCPA administration on the aggression and other behavioral impairments of adult MAO-A KO mice has not yet been examined. Thus, in the present study we examined whether pCPA administration in the first two postnatal weeks of life may affect aggression and other behavioral phenotypes of MAO-A KO mice, including social deficits, perseverative and alterations of anxiety-like responses. In addition, we verified whether this treatment may induce specific alterations of NMDA glutamate receptor subunit composition, in view of our recent discovery that alterations in this receptor are likely to contribute to the aggression in MAO-A KO mice (Bortolato *et al.*, 2012b).

2. Materials and methods

2.1 Subjects and treatment schedule

We used experimentally naïve male 129S6 MAO-A^{A863T} KO and wild-type (WT) mice, generated and genotyped as previously described (Scott *et al.*, 2008). Since MAO A is

located on the X-chromosome, mice were bred from WT males crossed with heterozygous MAO-A KO females and litters were culled to 6-8 pups. Animals were injected with either pCPA (300 mg/kg/day, i.p., q.d.; Sigma-Aldrich, St. Louis, MO, USA) or saline solution during the first or the second postnatal week (Fig.1). Intraperitoneal injections were performed at an injection volume of 10 ml/kg, using an injector attached to a micro-syringe. In addition, a group of mice was left untreated to control for the potentially stressful influence of early daily injections. In preliminary studies, we observed that the stress associated with early tattooing or permanent marking led to alterations of maternal behavior (including cannibalism) and/or enduring alterations of stress response. To avoid these potential confounds, all male offspring of each mother (both WT and MAO-A KO littermates) was subjected to the same treatment conditions. Litter effects were minimized by using animals from at least 8 litters for each treatment group for P1-P7 injections and 4 litters for each treatment group for P8-P14 injections. No more than 2 homogenotypic male littermates (randomly selected) in each group were used. Once weaned, male mice were group-housed with other conspecifics from other litters and treatment groups, and received *ad libitum* access to food and water, in facilities maintained at 22°C on a 12 h: 12 h light/dark cycle.

Mice were isolated on postnatal day 80 (P80) and tested between P90 and P120 (adulthood). This isolation regimen was selected to minimize the potentially detrimental behavioral outcomes of aggressive interactions with MAO-A KO male mice (which display spontaneous aggression towards cage mates); in addition, these conditions are optimal to elicit mild levels of aggressiveness in WT males during the resident-intruder test.

All *in vivo* experimental procedures were in compliance with the National Institute of Health guidelines and approved by the Animal Use Committees of the University of Southern California and University of Cagliari.

2.2 Neurochemical analyses

For biochemical analyses, male mice were deeply anesthetized with isoflurane and promptly decapitated. Brains were removed within approximately 30 s after decapitation, and forebrain regions were freshly dissected as described by Spijker (2011). Prefrontal cortices were obtained from adult brains by coronal sections (Spijker, 2011). Whole pup forebrains were harvested, to obtain sufficient biological material for further analyses.

2.2.1 HPLC determination of monoamine levels—Forebrain samples were kept in dry ice and rapidly homogenized with an ultrasonic tissue disrupter (Sonoplus HD60, Bandelin, Germany), in a solution containing 0.1 M trichloroacetic acid, 10 mM sodium acetate, and 0.1 mM EDTA; 1 μM isoproterenol was used as an internal standard. The homogenates were centrifuged, and the supernatants were used for high performance liquid chromatography (HPLC) analysis. The mobile phase was the same as the homogenization buffer with 7% methanol for detection of 5-HT. The mobile phase was filtered and deaerated, and the pump speed (Shimadzu LC-6A liquid chromatograph, Columbia, MD, USA) was 1.5 ml/min. The reverse-phase column used was a Rexchrom S50100-ODS C18 column with a length of 25 cm and an internal diameter of 4.6 mm. The compounds were measured at +0.7 V using a Shimadzu L-ECD-6A electrochemical detector.

2.2.2. Western blot analyses of NMDA receptor subunits—Prefrontal cortex samples were homogenized in ice cold buffer (50 mM HEPES, pH 7.4, 40 mM β-glycerol phosphate, 1mM sodium orthovanadate, 50mM Sodium Fluoride, 2mM EDTA, 20 μM ZnCl₂, 1% Sodium deoxycholate) and protease inhibitors. The homogenates were centrifuged at 75,000 × *g* for 30 min at 4°C and supernatants used for western blotting

assays. Equal amounts of protein from each sample (20 µg) were used in standard western-blot assays for NR1 (Cell Signaling, Danvers, MA, USA), NR2B and NR2A (NeuroMab, Davis, CA, USA). Blots were developed by chemiluminescence detection in a Kodak 4000MM imaging station. Bands were quantified in arbitrary units and normalized for protein concentrations using β-actin as loading control.

2.3. Behavioral testing

Environmental light and sound levels were maintained at 10 lux and 70 dB for all behavioral tests unless otherwise indicated. Testing was performed between 10 am and 3 pm with lights on at 6 am in the listed sequence below. Experiments on animals injected during the first and second postnatal weeks were performed and analyzed separately. All behaviors were video-recorded and analyzed by trained observers, who were blind to the genotype and treatment of the mice. To reduce carryover effects due to stress ensuing repeated testing, each animal was used for no more than 3 different tests (with a time interval of at least 1 week between two consecutive tests) and the order of testing was randomized (with the exception of social interaction and resident-intruder aggression tests, which were always performed at the end of behavioral batteries).

2.3.1. Open field—Analysis of open-field behaviors was performed to assess locomotor and exploratory responses, as well as anxiety-related reactivity. Testing was performed in a Plexiglas square grey arena (40 × 40 cm) surrounded by 4 black walls (40 cm high), based on a modified version of the protocol detailed elsewhere (Bortolato *et al*, 2011). On the floor, two zones of equivalent areas were defined: a central square quadrant and a concentric peripheral frame. Mice (n = 11-13 mice per group) were placed in the central zone and their behavior was monitored for 5 min using Ethovision behavioral tracking software (Noldus Instruments, Wageningen, The Netherlands). Behavioral measures included the total locomotor activity, time spent in the center, and the percent locomotor activity in the center (calculated as the distance traveled in the center over the total distance traveled).

2.3.2. Elevated plus-maze—The elevated plus-maze protocol was used to measure contextual anxiety and performed as previously described (Pellow *et al*, 1985; Bortolato *et al*, 2009). Briefly, the apparatus was comprised of two open arms and two closed arms in black Plexiglas with a light grey floor, which extended from a central platform. Mice (n= 11-13 per group) were placed individually on the central platform, facing an open arm, and allowed to explore the maze for 5 min. An arm entry was counted only when all four paws were inside the arm. Behavioral measures included the number of entries and time spent in each compartment of the maze, as well as the number of stretch-attend postures and head dips (previously defined in Godar *et al*, 2011).

2.3.3. Novel object exploration—Novel object exploration was tested to assess exploratory responses and neophobia. The assay was conducted in the home cage to maximize neophobic responses in MAO-A KO mice as previously detailed (Godar *et al*, 2011). Two novel black plastic cylinders (8-cm high; 3.5 cm diameter) were symmetrically placed in the cage. Mice (n = 8-10 per group) were allowed to freely explore the objects and environment for 15 min. Exploration was defined as sniffing or touching the object with the snout, and measured as the frequency and total time spent exploring both objects; climbing on the object was not considered exploration.

2.3.4. Marble burying—This paradigm was used to capture anxiety-driven perseveration. Testing was performed in Makrolon cages containing 5 cm of sawdust, as previously described (Bortolato *et al*, 2009). Mice (n = 11-13 per group) were individually placed in the cage and their behavior was monitored for 30 min. Mice were removed and 20 glass marbles

(1.5 cm in diameter) were placed on the sawdust at equal distances. The animal was then reintroduced into the cage and its behavior was monitored for 30 additional min. The number of buried marbles was counted; a marble was considered buried if at least two-thirds of its surface area was covered in sawdust.

2.3.5. Spontaneous alternations in the T-maze—To ascertain the potential effect of pCPA on perseverative responses, spontaneous alternations were tested in a T-maze, as previously described (Bortolato *et al*, 2012). Briefly, each session consisted of eight consecutive trials. In each trial, the mouse (n = 10-12 per group) was placed in the start compartment of a T-maze. After 15 s, the door was lifted and the mouse was left free to explore the two arms of the maze. As soon as the animal entered (with all four paws) one of the two alternative arms (left or right), the door of that compartment was closed for 15 s to confine the animal. The animal was removed between trials and the T-maze was quickly cleaned and dried to remove any olfactory cue that may condition the performance in the next trial. A trial was considered failed if the animal did not enter an arm within 120 s. Mice that recorded two failures were eliminated from testing. The percentage of arm alternations was analyzed for each mouse.

2.3.6. Social interaction—Mice were tested as described elsewhere (Bortolato *et al*, 2011). Mice (n = 9-10 per group) were placed into a neutral, unfamiliar Makrolon cage, with foreign age- and weight-matched male WT conspecifics (from separate litters). Testing sessions lasted 10 min and were scored for social behaviors, which consisted of the number and overall duration of social exploratory approaches towards the conspecific (defined as investigative sniffing of the facial and anogenital areas of the conspecific).

2.3.7. Resident-intruder aggression—Testing was conducted as previously described (Bortolato *et al*, 2011). To study aggression, animals were isolated at P80 for 14 days, a duration that we have previously identified as optimal to establish aggression in MAO-A KO males. Following a 30-min habituation period within the home cage in the experimental room, mice (n = 12-14 per group) were exposed to age- and weight-matched WT males (previously acclimated to the same room) from different litters, for 10 min. Measures included the number and duration of fighting bouts.

2.3.8. Sticky-tape test—The sticky-tape test was carried out as described elsewhere (Bortolato *et al*, 2012), to measure potential deficits in sensorimotor reflexes in MAO-A KO mice. Animals (n = 9-11 per group) were briefly restrained and a circular piece of tape (5 mm in diameter) was placed on the bottom of each forepaw. The time to remove each piece of tape was recorded.

2.3.9. Hot plate—To assess the impact of pCPA on pain sensitivity, mice were tested on a hot plate (IITC, Woodland Hills, CA, USA) as previously described (Bortolato *et al*, 2012). Mice (n = 11-13 per group) were placed on the hot plate and the latency to lick their paws was measured; a 20-s cut-off time was used to avoid potential tissue damage. The hot plate was maintained at 52.5°C, as this temperature was shown to elicit a significant delay in paw-licking latency in MAO-A KO mice (Bortolato *et al*, 2012).

2.4. Statistical analyses

Normality and homoscedasticity of data distribution were verified by using Kolmogorov-Smirnov and Bartlett's tests. Statistical analyses on parametric data were performed using two-way ANOVAs, followed by Newman-Keuls test for *post-hoc* comparisons. Significance threshold was set at 0.05. All statistical analyses were performed by STATISTICA 7 (Statsoft, Tulsa, OK).

3. Results

3.1. Neurochemical analyses

3.1.1. Forebrain 5-HT levels in 8-day old mice—We ascertained that pCPA administration resulted in the normalization of forebrain 5-HT levels in MAO-A KO pups at postnatal day 8. We found that this treatment in the first week led to a reduction in 5-HT concentrations across both genotypes (Table 1). Significant main effects of genotype [$F(1, 30) = 114.58$; $P < 0.001$, ANOVA] and treatment [$F(2, 30) = 49.10$; $P < 0.001$, ANOVA] were detected. In addition, a significant genotype \times treatment interaction was found [$F(2, 30) = 17.18$; $P < 0.001$, ANOVA]. *Post-hoc* analyses revealed that untreated and saline-injected MAO-A KO pups had higher 5-HT forebrain concentrations than their WT counterparts ($P < 0.001$ for both comparisons). In WT mice, pCPA administration significantly reduced 5-HT levels in comparison with both untreated ($P < 0.05$) and saline-injected ($P < 0.05$) counterparts. Similarly, pCPA treatment produced a marked decrement in forebrain 5-HT concentrations in MAO-A KO mice, as compared with both untreated and saline-treated ($P < 0.001$ for both comparisons) animals.

3.1.2. Prefrontal 5-HT levels in adult mice—Early pCPA treatment resulted in long-term reductions in 5-HT levels also in the prefrontal cortex of adult (90-day old) WT and MAO A-KO mice (Table 1). Indeed, ANOVA identified significant main effects of genotype [$F(1,42) = 20.81$; $P < 0.001$] and treatment [$F(2,42) = 53.30$; $P < 0.001$], as well as a significant genotype \times treatment interaction [$F(2,42) = 7.42$; $P < 0.01$]. *Post-hoc* comparisons revealed that this latter effect was due to significant differences between untreated WT and MAO A KO mice ($P < 0.01$), saline-treated WT and MAO A KO mice ($P < 0.001$), as well as pCPA-treated mice and their untreated or saline-injected counterparts ($P < 0.01$ for all comparisons; Newman-Keuls).

3.1.3. Prefrontal NMDA receptor (NMDAR) subunit expression in adult mice—To verify whether pCPA treatment may affect NMDAR subunit composition, we measured NR1, NR2A and NR2B levels in the prefrontal cortex of pCPA-treated WT and MAO-A KO mice, as compared with their saline-injected counterparts. As shown in Table 2, we found that NR1 [Main effect of genotype: $F(1,15) = 0.51$, NS] and NR2A [$F(1,15) = 0.51$, NS] subunits were not altered in MAO-A KO mice; conversely, NR2B subunits were significantly increased [$F(1,15) = 22.84$, $P < 0.001$] in MAO-A KO mice, as compared to WT counterparts. Notably, early postnatal pCPA administration did not affect the expression of any of these subunits [genotype \times treatment interactions: NR1: $F(1, 15) = 0.51$, NS; NR2A: $F(1, 15) = 0.06$, NS; NR2B: $F(1, 15) = 0.07$, NS].

3.2. Behavioral testing

3.2.1. Open field—The analysis of overall locomotor activity in a novel open field (Fig. 2A) revealed a significant main effect of genotype [$F(1, 53) = 12.82$; $P < 0.001$], but not treatment; furthermore, no significant genotype \times treatment interactions were found, signifying that the hypolocomotion of MAO-A KO mice was not affected by early pCPA or saline administration. The analysis of the time spent in the center quadrant of the open field (Fig. 2B) revealed significant main effects of both genotype [$F(1, 54) = 4.14$; $P < 0.05$] and treatment [$F(2, 54) = 5.53$; $P < 0.01$], but no interactions between the two factors. Similarly, the analysis of percent locomotor activity in the center (Fig. 2C) disclosed a statistical trend for a main effect of genotype [$F(1, 54) = 3.91$; $P < 0.06$] and a significant effect for treatment [$F(2, 54) = 3.82$; $P < 0.05$], but no genotype \times treatment interactions.

3.2.2. Elevated plus-maze—In parallel with our findings in the open field, MAO-A KO mice exhibited a reduced number of total entries (Fig. 3A) [Main effect of genotype: $F(1, 64)$

= 6.10; $P < 0.05$] in the elevated plus-maze. Although the analysis of the percent entries in the open (Fig.3B) and closed (Fig.3E) arms revealed significant genotype \times treatment interactions [%open entries: $F(2, 64) = 3.74$; $P < 0.05$; %closed entries: $F(2, 64) = 3.18$; $P < 0.05$], post-hoc comparisons did not identify any significant changes between any genotype \times treatment groups, but only a statistical trend ($P < 0.10$) for an increase in open entries by MAO-A KO mice. Early pCPA administration was found to significantly increase the time spent by MAO-A KO mice in the open arms (Fig.3C) [genotype \times treatment interaction: $F(2, 64) = 5.04$; $P < 0.01$]. This effect was found to depend on a significant difference ($P < 0.05$) between pCPA- and saline-treated MAO-A KO mice. Conversely, no significant difference was found between treatment groups of WT mice; furthermore, no differences were found between WT and MAO-A KO mice with respect to any treatment condition. Treatment with pCPA did not significantly affect the time spent in the central platform (Fig.3D) [$F(2, 64) = 0.12$; NS] or in the closed arms (Fig.3F) [$F(2, 64) = 2.06$; NS]. As previously described (Godar et al, 2011), MAO-A KO mice also exhibited reductions in stretch-attend postures (Fig. 3G) [$F(1,64)=8.78$; $P < 0.01$; Main effect of genotype] and head dips (Fig. 3H) [$F(1,64)=8.41$; $P < 0.01$; Main effect of genotype] compared to WT mice. Nevertheless, these effects were not significantly affected by pCPA treatment, as revealed by the lack of significant treatment \times genotype interaction for either parameter [stretch-attend postures: $F(2, 64) = 0.15$; NS; head dips: $F(2, 64) = 0.46$; NS]. Similar to the open field, MAO-A KO animals showed a significant reduction in the overall distance travelled in the elevated plus maze [genotype: $F(1, 64) = 6.10$; $P < 0.05$] (untreated WT: 1012.39 ± 81.35 cm; untreated MAO-A KO: 911.69 ± 66.48 cm; saline-treated WT: 1044.28 ± 59.88 cm; saline-treated MAO-A KO: 887.88 ± 39.26 cm; pCPA-treated WT 1057.1 ± 40.25 cm; pCPA-treated MAO-A KO: 948.73 ± 34.98 cm).

3.2.3. Novel object exploration—In comparison with WT mice, MAO-A KO littermates engaged in fewer exploratory approaches towards novel objects (Fig.4A) [genotype \times treatment interaction: $F(2,49) = 10.45$; $P < 0.001$]. Post-hoc comparisons identified a significant difference ($P < 0.001$) between untreated WT and MAO-A KO mice; notably, this deficit was rescued by early pCPA treatment ($P_s < 0.05$ for comparisons between pCPA-treated MAO-A KO and either non-treated or saline-injected MAO-A KO mice). Furthermore, both saline and pCPA treatment reduced the number of exploratory approaches in WT mice ($P < 0.05$ and $P < 0.01$ for comparisons between saline-treated WT and pCPA-treated WT vs untreated WT, respectively). All other comparisons between genotype \times treatment combinations did not reveal any significant difference. The analysis of the sniffing duration (Fig.4B) confirmed that, while MAO-A KO mice explored the novel objects for a significantly shorter time [genotype \times treatment: $F(2, 49) = 6.16$; $P < 0.01$; $P < 0.01$ for comparison between non-treated WT and MAO-A KO mice], this impairment was fully prevented by pCPA administration ($P < 0.001$). The analysis of the latencies to the first exploratory approach (Fig. 4C) revealed significant main effects of the genotype [$F(1,49) = 12.78$; $P < 0.001$] and treatment [$F(2,49) = 5.27$; $P < 0.01$], as well as a significant genotype \times treatment interaction [$F(2,49)=5.06$, $P < 0.05$]. Post-hoc comparisons revealed that untreated MAO-A mutants had a significantly longer latency to explore novel objects than WT counterparts ($P < 0.001$). This latency was reduced by pCPA ($P < 0.001$) and, to a lesser extent, by saline treatment ($P < 0.05$). Neither pCPA nor saline affected the same parameters in WT mice. No significant difference among groups was found in locomotor activity (data not shown).

3.2.4. Marble burying—As previously shown (Bortolato *et al.*, 2012a), MAO-A KO mice buried a greater number of marbles (Fig. 5A) [genotype \times treatment: $F(2, 57) = 8.75$; $P < 0.001$] than WT littermates. This alteration was prevented by pCPA pre-treatment ($P < 0.001$ and $P < 0.01$ for comparisons between pCPA-treated MAO-A KO vs non-treated

and saline-injected MAO-A KO mice, respectively). In addition, the analysis of digging duration showed a main effect of the genotype [$F(1, 56) = 8.66$; $P < 0.01$], but no significant genotype \times treatment interaction (data not shown).

3.2.5. Spontaneous alternations in the T-maze—In line with our previous findings (Bortolato *et al.*, 2012a), we confirmed that MAO-A KO mice engaged in a significantly lower percentage of spontaneous alternations (Fig. 5B) [genotype \times treatment: $F(2, 55) = 6.47$; $P < 0.01$; $P < 0.05$ for comparison between non-treated WT and MAO-A KO mice]. This effect, however, was rescued by early postnatal pCPA treatment ($P < 0.05$ for comparisons between pCPA-treated MAO-A KO and either non-treated or saline-injected MAO-A KO).

3.2.6. Social interaction and resident intruder task—MAO-A KO mice displayed a significant reduction in the frequency (Fig. 6A) [genotype: $F(1, 39) = 29.46$; $P < 0.001$] and duration (Fig. 6B) [genotype: $F(1, 40) = 27.34$; $P < 0.001$] of social exploration. In addition, we found a significant treatment effect for the number of social approaches [$F(2, 39) = 4.00$; $P < 0.05$]. No significant interactions between genotype \times treatment were found. The locomotor activity was comparable across experimental groups (data not shown).

In the resident-intruder paradigm, MAO-A KO mice exhibited an increased number of fighting bouts (Fig. 6C) [Main effect of genotype: $F(1, 50) = 49.50$; $P < 0.001$] and fighting duration (Fig. 6D) [Main effect of genotype: $F(1, 50) = 30.57$; $P < 0.001$]. Moreover, a significant treatment effect was detected for both parameters [bouts: treatment: $F(2, 50) = 4.62$; $P < 0.05$ and duration: treatment: $F(2, 50) = 4.00$; $P < 0.05$]. No significant differences in genotype \times treatment interactions were detected. All groups displayed equivalent locomotor activities (data not shown).

3.2.7. Sticky-tape and hot plate tests—As previously shown (Bortolato *et al.*, 2012a), MAO-A KO mice exhibited a significant increase in the latency to remove sticky tape from their forepaws (Fig. 7A) [Main effect of genotype: $F(1, 48) = 19.80$; $P < 0.001$]. In parallel, MAO-A-deficient mice displayed an enhanced latency to lick their paws in response to the exposure to a hot plate (Fig. 7B) [Main effect of genotype: $F(1, 60) = 6.02$; $P < 0.05$]. Notably, neither alteration was affected by pCPA treatment.

3.2.8. Effects of PCPA administration between P8 and P14—MAO-A mutants treated with pCPA during the second postnatal week exhibited a significant reduction in marble burying activity (Fig. 8A) [genotype \times treatment: $F(2, 32) = 4.10$; $P < 0.05$]. *Post-hoc* analyses revealed that, while non-treated MAO-A KO mice buried significantly more marbles than their non-treated WT counterparts, both saline ($P < 0.05$) and pCPA ($P < 0.01$) markedly reduced this behavior. MAO-A-deficient animals showed a significant reduction in exploratory approaches towards an unfamiliar object [main effect for genotype: $F(1, 31) = 13.77$; $P < 0.001$]. In addition, ANOVA detected a main effect of treatment [$F(2, 31) = 40.46$; $P < 0.001$] and a significant genotype \times treatment interaction [$F(2, 31) = 6.07$; $P < 0.01$] (Fig. 8B). Newman-Keuls test disclosed that untreated MAO-A KO mice exhibited a significantly lower number of approaches in comparison with their WT counterparts ($P < 0.001$). In addition, saline and pCPA treatment significantly reduced the same parameters in both WT ($P < 0.001$ for both comparisons) and MAO-A KO mice ($P < 0.05$). The analysis of the duration of novel-object exploration disclosed a main genotype effect [$F(1, 31) = 6.73$; $P < 0.05$], but no significant genotype \times treatment interactions [$F(1, 31) = 0.78$; NS] (Fig. 8C).

The analysis of resident-intruder aggression revealed that MAO-A KO mice displayed a significantly higher number of fighting episodes [Main effect of genotype: $F(1, 61) = 7.92$; $p < 0.01$], but this difference was not affected by treatment [genotype \times treatment: $F(2, 61) =$

0.82; NS] (Fig. 8D). Overall fighting duration was also significantly greater in MAO-A deficient animals [Main effect of genotype: $F(1,60) = 8.85$; $p < 0.01$]. In addition, a significant genotype \times treatment interaction was found [$F(2, 60) = 3.36$; $P < 0.05$]. *Post-hoc* analyses showed that, while non-treated MAO-A KO mice engaged in significantly longer fighting behaviors than their untreated WT counterparts ($P < 0.01$), pCPA pretreatment increased this parameter in WT ($P < 0.05$), but not in MAO-A KO mice.

4. Discussion

The results of the present study showed that neonatal subchronic administration of pCPA, the prototypical inhibitor of tryptophan hydroxylase, produced an enduring reduction of brain 5-HT forebrain levels in WT and MAO-A KO mice, which lasted until adulthood. These neurochemical changes led to a complex alteration of the abnormal phenotypes associated with MAO A deficiency. On one hand, counterbalancing the early elevation of 5-HT in MAO-A KO pups preserved the microcolumnar pattern in the barrel fields of the somatosensory cortex (Cases *et al.*, 1996), and led to a marked attenuation of their perseverative and restricted responses, such as marble burying and lack of spontaneous alternations in a T-maze (Bortolato *et al.*, 2011; Bortolato *et al.*, 2012a). On the other hand, pCPA treatment from postnatal day 1 through 7 did not rescue the overtly aggressive responses, social deficits and tactile alterations associated with MAO-A deficiency (Cases *et al.*, 1995; Scott *et al.*, 2008; Bortolato and Shih, 2011; Bortolato *et al.*, 2012a). In addition, this treatment had only limited effects on behavioral responses related to multiple facets of anxiety-like behavior. Indeed, in the open field, it did not result in significant changes in thigmotactic behavior. Furthermore, in the elevated plus maze pCPA reduced the total duration spent by MAO-A KO mice in the open arms, but produced only a statistical trend for a reduction (rather than a significant effect) of the number of open-arm entries and failed to ameliorate their reduction in stretch-attend postures and head dips. Finally, pCPA increased the number of exploratory approaches performed by MAO-A KO mice towards unfamiliar objects (Godar *et al.*, 2011); however, this amelioration was not accompanied by a selective effect of this drug (as compared to saline treatment) on either the overall exploratory duration or the latency to the first approach to the novel object.

Our findings also documented that pCPA treatment during the second week of postnatal life elicited a similar, albeit milder, attenuation of repetitive behaviors in MAO-A KO mice. Collectively, these data strongly suggest that, in mice, the first (and, to a lesser extent, the second) week of postnatal life is a critical stage for the role of MAO-A and 5-HT in brain and behavioral development. These results also complement previous evidence reported by our group and others, indicating that the abnormal phenotypes of MAO-A KO mice are influenced by early developmental neurochemical imbalances. In fact, the perturbations featured by these mutants were rescued by the genetic reinstatement of forebrain MAO-A from postnatal day 1 onwards (Chen *et al.*, 2007); in addition, pharmacological inhibition of MAO-A in perinatal periods was shown to elicit long-term behavioral sequelae in rodents (Boylan *et al.*, 2000; Mejia *et al.*, 2002).

We recently documented that MAO-A KO mice, in addition to their aggressive responses, display a spectrum of behavioral alterations reminiscent of the key pathognomonic deficits of autism-spectrum disorder (ASD), including social deficits, reduced communication and repetitive, restricted behaviors (Bortolato *et al.*, 2012a). An increased risk of social, communicative, cognitive and neuromorphological impairments in ASD were also found in subjects carrying polymorphic variants associated with low MAO-A catalytic activity (Yirmiya *et al.*, 2002; Cohen *et al.*, 2003; Davis *et al.*, 2008; Cohen *et al.*, 2011). Our data show that the perseverative and restricted behaviors in MAO-A KO mice may indeed be supported by the high levels of 5-HT in early postnatal stages. This finding is consistent

with the well-documented link between ASD and hyperserotonemia (Schain and Freedman, 1961; Ritvo *et al.*, 1970; Anderson *et al.*, 1987; Anderson *et al.*, 1990; Mulder *et al.*, 2004). In particular, it is worth noting that recent studies have raised the possibility that high 5-HT concentrations may result in stereotyped behavior and delays in age of social smile in ASD patients (Sacco *et al.*, 2010; for contrasting results see Kolevzon *et al.*, 2010).

Previous reports documented that perinatal and neonatal pCPA treatment rescued early neurological deficits and the dysmorphogenesis of the barrel cortex in MAO-A KO mice (Cases *et al.*, 1995; Cases *et al.*, 1996). It has been clarified that the critical period for MAO-A inhibition to result in impairments of the barrel cortex is limited to the first week of postnatal life (Vitalis *et al.*, 1998; Boylan *et al.*, 2000). While our data cannot substantiate a causal nexus between the restorative action of pCPA on corticogenesis and perseverative behaviors, it is worth noting that the barrel fields afford a well-characterized template of minicolumnar organization (Bruno *et al.*, 2003), whose abnormalities have been posited to underlie ASD pathophysiology (Casanova *et al.*, 2002; Casanova *et al.*, 2006). These premises support the possibility that 5-HT input in perinatal periods may govern the ontogenesis of brain circuits subserving behavioral plasticity; alterations of these processes may lead to impaired cortical development and specific aberrances of information processing.

Early pCPA treatment induced a permanent reduction of 5-HT levels in the prefrontal cortices of WT and MAO-A KO mice. The bases of this phenomenon, albeit still unknown, are likely reflect the modulatory influence of 5-HT on early neurogenesis, developmental cellular migration and cytoarchitecture (Lauder and Krebs, 1978; Daubert and Condron, 2010). In particular, recent evidence indicates that 5-HT autoregulates the morphology and innervation of its own system (Daubert and Condron, 2010); furthermore, the inhibition of 5-HT synthesis by pCPA at different developmental stages has been shown to affect cortical connectivity and development (Chen *et al.*, 1994; Vitalis *et al.*, 2007). Thus, it is possible that early treatment with the tryptophan hydroxylase inhibitor may cause persistent reductions and/or damages in 5-HTergic fibers in the prefrontal cortex.

Irrespective of the mechanisms accounting for the enduring reduction in prefrontal 5-HT content in pCPA-treated mice, this alteration may account for some of our behavioral findings, such as the lack of ameliorative effects of this regimen on the social deficits and aggression displayed by adult MAO-A KO mice. In fact, most studies have reported a consistent link between 5-HT hypofunction and impulsive aggression in vertebrates (Coccaro, 1989; Virkkunen and Linnoila 1993; Miczek *et al.*, 2001). Deficits in prefrontal 5-HT content are posited to be particular relevant for the control of aggression, insofar as they may lead to an insufficient inhibition of affective triggers of impulsive aggression in limbic areas (Amen *et al.*, 1996). Accordingly, our tests showed that early pCPA treatment selectively *increased* fighting duration in WT mice. Thus, it is possible that the persistent reduction of 5-HT in the prefrontal cortex may intrinsically lead to an intrinsic enhancement of aggression in adulthood, which could mask and override potential beneficial effects on the early developmental bases of impulsive hostility in MAO-A KO mice. Similar views may help explain the failure of early pCPA treatment to enhance social interaction and modify most anxiety-related responses in these mutants.

Alternatively, our behavioral results may subtend a phenomenological dissociation between aggression vulnerability and early elevation of 5-HT levels, which may challenge the possibility of a primary role for this neurotransmitter in the high aggressiveness of MAO-A KO mice. For example, it is possible that the high proclivity of MAO-A KO mice to engage in antagonistic behaviors may depend on high levels of other MAO-A substrates, such as DA and NE. Indeed, mice with null-allele mutations of DA transporter, NE transporter and

catechol-O-methyl-transferase (COMT), which feature high levels of catecholamines, have been shown to be significantly more aggressive than WT conspecifics (Gogos *et al.*, 1998; Haller *et al.*, 2002; Rodriguiz *et al.*, 2004; Volavka *et al.*, 2004; Guo *et al.*, 2007; Bedard *et al.*, 2010). Given that the elevation in catecholamine levels in MAO-A KO mice occurs mainly after the second postnatal week of life (Cases *et al.*, 1995), the potential contribution of DA and NE to aggression may occur upon periadolescent stages, underscoring the possibility of multiple critical developmental stages with respect to different neurochemical substrates and behavioral outcomes.

We recently reported that MAO-A KO mice display changes in NMDAR subunit composition in the prefrontal cortex. These alterations appear to play a critical role in the aggressive responses enacted by MAO-A KO mice, as these behaviors are suppressed by NMDAR antagonists (Bortolato *et al.*, 2012b). Notably, the present results show that early pCPA treatment did not alter the subunit expression of prefrontal NMDA receptors in MAO-A KO mice, further supporting a critical role of these ionotropic channels in the pathogenesis of aggression in these animals. We confirmed that the expression of NR2B, but not NR1, was increased in the composition of NMDA receptors in the prefrontal cortex of MAO-A KO mice. In contrast, no significant enhancement of NR2A was found, possibly signifying a lesser involvement of this subunit in the behavioral aberrances in MAO-A KO mice. Recent findings have documented that NR2B subunits in the prefrontal cortex are required to regulate synaptic plasticity and working memory (Cui *et al.*, 2011; Wang *et al.*, 2013), by interacting with calcium/calmodulin-dependent protein kinase II (CaMKII) (Strack *et al.*, 2000; Fink and Meyer, 2002).

Our behavioral assessments revealed that early saline treatment produced a number of specific changes in both WT and MAO-A KO mice in comparison with their non-treated counterparts. For example, we found that this treatment reduced the number of exploratory approaches directed towards novel objects in WT mice. These data suggest that the stress caused by daily punctures during the first postnatal week may result in enduring enhancements in neophobia or reduction in novelty-seeking behavior, and are in alignment with ample evidence indicating a link between early childhood traumas and the development of anxiety-related symptoms in adult life (Heim and Nemeroff, 2001).

From a translational perspective, our findings temper the possibility that the early inhibition of 5-HT synthesis may be a viable approach to prevent the aggressive and antisocial traits associated with low brain MAO-A activity (Alia-Klein *et al.*, 2008). Nevertheless, our results cannot rule out that these behavioral problems may be efficiently countered by more specific approaches aimed at the reduction of the early impact of 5-HT within select brain regions and/or receptor subtypes. Ample evidence has shown that aggression is associated with different changes in 5-HTergic activity across multiple areas within the aggression circuitry (Summers *et al.*, 2005). Moreover, different 5-HT receptor families mediate opposite modulatory effects on distinctive aspects of aggression (Takahashi *et al.*, 2011; Bortolato *et al.*, 2013); for example, while the genetic and pharmacological inactivation of 5-HT_{1B} receptors increases the levels of aggression (Saudou *et al.*, 1994), 5-HT_{2A} receptor antagonists exert anti-aggressive properties (Shih *et al.*, 1999). Thus, the global reduction of 5-HT synthesis induced by pCPA may have actually reversed some imbalances related to aggression in MAO-A KO mice, but these effects may be masked by alterations related to impaired signaling of other 5-HT receptors or in specific subcortical regions. Notably, the dysmorphogenic effect of excessive 5-HT on the barrel fields in MAO-A KO mice has been shown to depend the overactivation of 5-HT_{1B} receptors (Vitalis *et al.*, 1998; Salichon *et al.*, 2001). Further studies will be necessary to evaluate the specific contribution of different 5-HT receptor subtypes and brain regions to the aggressive phenotype of MAO-A KO mice.

5. Conclusions

The results of the present study indicate that alterations in perseverative behaviors associated with MAO-A deficiency are supported by high 5-HT levels in early developmental stages. These findings support the existence of a sensitive period during which 5-HT neurotransmission is likely to influence the formation of critical brain circuits that govern the expression of anxiety-related and repetitive behaviors. Future research is needed to unravel the complex set of neurobiological bases supporting aggressive features and other behavioral aberrances caused by MAO-A deficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- Monoamine oxidase (MAO) A catalyzes the degradation of serotonin (5-HT).
- MAO A knockout (KO) mice display high 5-HT levels, aggression and perseverative behaviors.
- MAO A KO pups were treated with the 5-HT synthesis inhibitor *p*-chloro-phenylalanine (pCPA).
- Early pCPA treatment reduced perseveration, but not aggression in MAO A KO mice

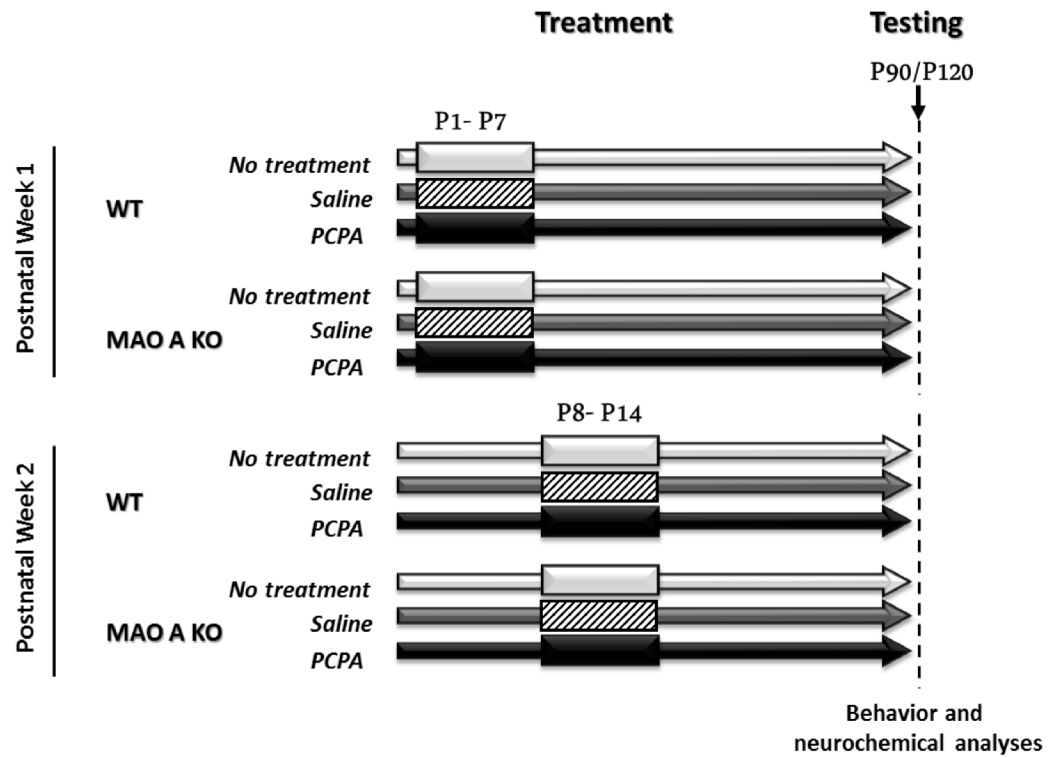


Figure 1. Schematic representation of the experimental design for pCPA administration during the first two postnatal weeks. Behavioral analyses were performed between postnatal day 90 and postnatal day 120 (corresponding to adulthood).

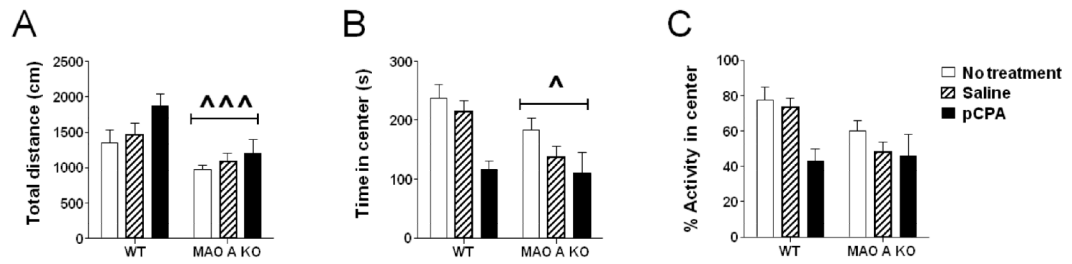


Figure 2.

Effects of pCPA treatment from postnatal day 1 through 7 on open-field behaviors in adult wild type (WT) and MAO-A KO mice. Values are represented as mean \pm SEM. ^P<0.05; ^^P<0.001 compared to WT mice (main effect of genotype). Main effects of treatment are not indicated. For more details, see text.

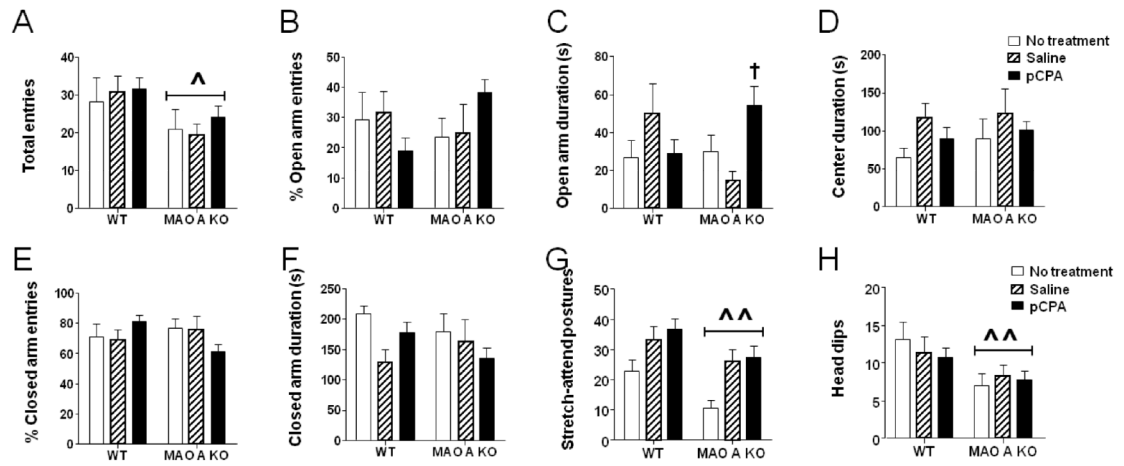


Figure 3.

Effects of pCPA treatment from postnatal day 1 through 7 on elevated-plus maze behaviors in adult wild type (WT) and MAO-A KO mice. Values are represented as mean \pm SEM.

$^{\wedge}$ P<0.05; $^{\wedge\wedge}$ P<0.001 compared to WT mice (main effect of genotype). † P<0.05 compared to MAO-A KO mice treated with saline. For more details, see text.

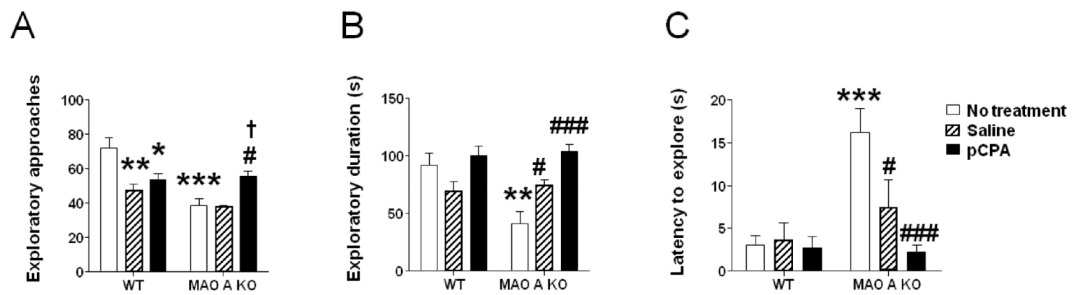


Figure 4.

Effects of pCPA treatment from postnatal day 1 through 7 on the proclivity of adult wild type (WT) and MAO-A KO mice to explore a novel object in their home cage. Values are represented as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared to untreated WT mice. # $P < 0.05$; ### $P < 0.001$, compared to untreated MAO-A-deficient mice; † $P < 0.05$, compared to saline-treated MAO-A KO mice. For more details, see text.

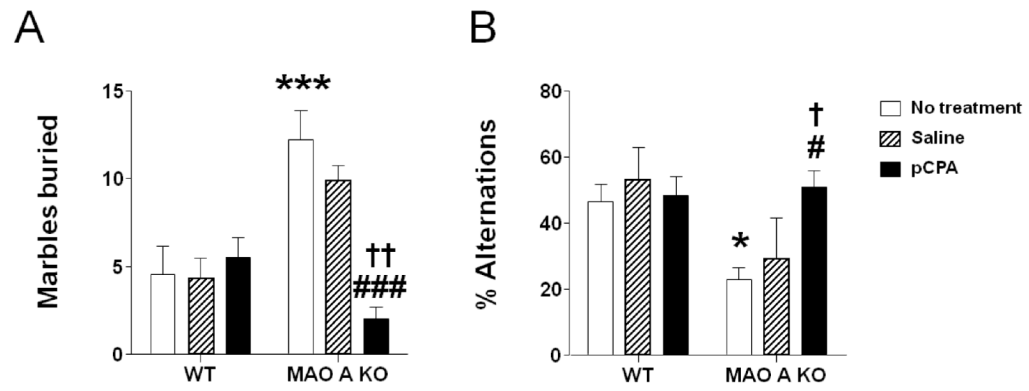


Figure 5.

Effects of pCPA treatment from postnatal day 1 through 7 on A) marble-burying behavior and B) spontaneous alternations in a T-maze of adult wild type (WT) and MAO-A KO mice. Values are represented as mean \pm SEM. * $P < 0.05$; *** $P < 0.001$, compared to untreated WT mice. # $P < 0.05$; ### $P < 0.001$, compared to untreated MAO-A-deficient mice; † $P < 0.05$; †† $P < 0.01$ compared to saline-treated MAO-A KO mice. For more details, see text.

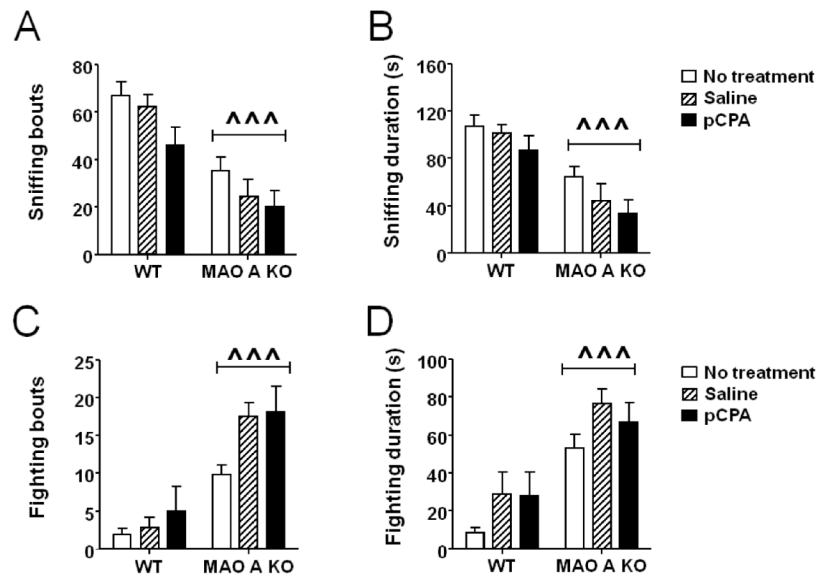


Figure 6. Effects of pCPA treatment from postnatal day 1 through 7 on A-B) social interaction and C-D) resident-intruder aggression in adult wild type (WT) and MAO-A KO mice. Values are represented as mean \pm SEM. $^{***}P < 0.001$ compared to WT mice (main effect of genotype). For more details, see text.

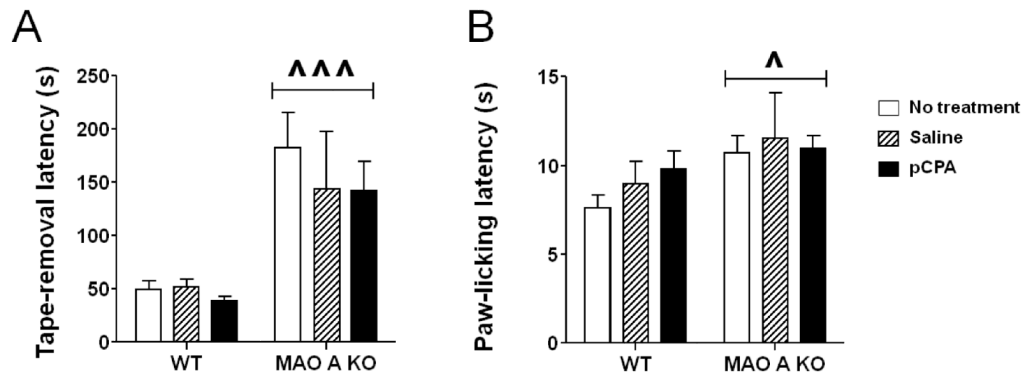


Figure 7.

Effects of pCPA treatment from postnatal day 1 through 7 on the latencies to A) remove a sticky tape from the paws and B) lick paws in the hot plate test of adult wild type (WT) and MAO-A KO mice. Values are represented as mean \pm SEM. ^P<0.05; ^^^P<0.001 compared to WT mice (main effect of genotype). For more details, see text.

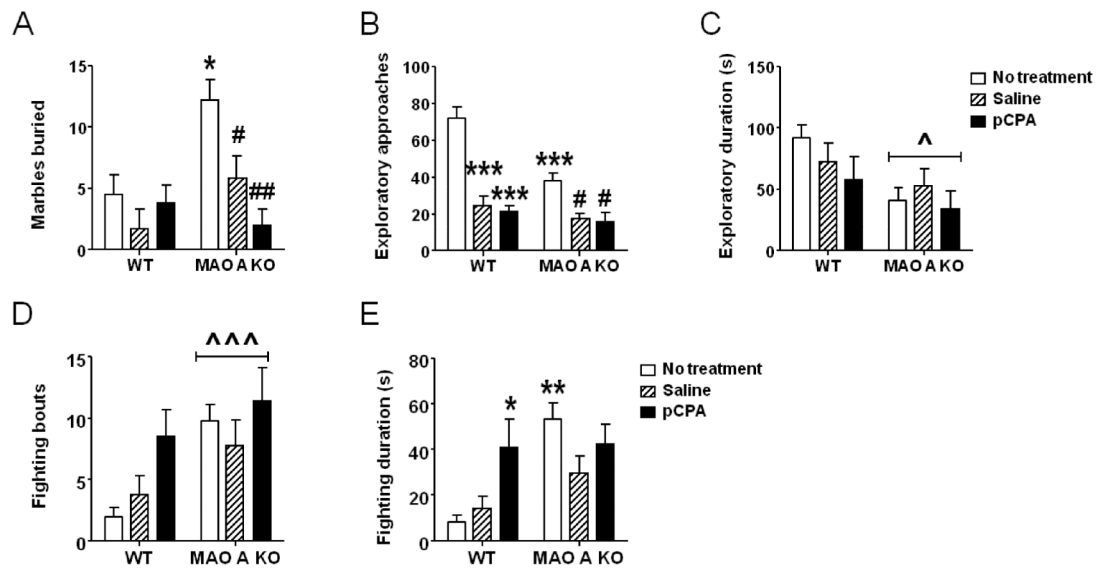


Figure 8.

Effects of pCPA treatment from postnatal day 8 through 14 on A) marble-burying behavior, B-C) novel-object exploration; and D-E) resident-intruder responses of adult wild type (WT) and MAO-A KO mice. Values are represented as mean \pm SEM. $^{\wedge}P < 0.05$; $^{\wedge\wedge\wedge}P < 0.001$ compared to WT mice (main effect of genotype); $*P < 0.05$; $***P < 0.001$, compared to untreated WT mice. $\#P < 0.05$; $\#\#P < 0.01$, compared to untreated MAO-A-deficient mice. Main effects of treatment are not indicated. For more details, see text.

Table 1

Effects of postnatal pCPA treatment from postnatal day 1 through 8 on 5-HT forebrain concentrations in 8-day old (P8) and adult (90-day old; P90) mice. Values are displayed as mean \pm SEM.

Table 1		P8 – FOREBRAIN (pg/mg of tissue)	P90 - PREFRONTAL CORTEX (pg/mg of tissue)
WT	Untreated	300.02 \pm 27.48	393.44 \pm 45.05
	Saline	384.92 \pm 38.80	316.21 \pm 40.08
	pCPA	163.32 \pm 29.04* \diamond	125.11 \pm 15.74*** \diamond
MAO A KO	Untreated	944.45 \pm 58.51***	590.68 \pm 54.17**
	Saline	1012.87 \pm 35.36 \diamond \diamond \diamond	594.61 \pm 52.03 \diamond \diamond \diamond
	pCPA	266.88 \pm 21.17### \dagger \dagger \dagger	101.59 \pm 17.23### \dagger \dagger \dagger

Main effects are not indicated. For further details, see text.

* P<0.05;

** P<0.01;

*** P<0.001, compared to untreated WT mice.

\diamond P<0.05;

\diamond P<0.01;

\diamond \diamond P<0.001, compared to saline-treated WT mice.

P<0.001 compared to untreated MAO A KO mice.

\dagger \dagger \dagger P<0.001 compared to saline-treated MAOA KO mice.

Table 2

Effects of postnatal pCPA treatment from postnatal day 1 through 7 on NMDA receptor subunit expression in the prefrontal cortex of adult WT and MAO-A KO mice.

Table 2		NR1	NR2A	NR2B
WT	Saline	1.062 ± 0.116	1.066 ± 0.154	1.018 ± 0.243
	pCPA	1.052 ± 0.136	0.980 ± 0.178	1.116 ± 0.168
MAO A KO	Saline	0.992 ± 0.129	1.083 ± 0.203	1.538 ± 0.222 ^{^^}
	pCPA	1.062 ± 0.105	0.958 ± 0.113	1.578 ± 0.249 ^{^^}

Values represented as mean ± SEM.

^{^^}P<0.001, compared to WT mice (main effect of genotype). For further details, see text.