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Dopamine and serotonin signaling during two sensitive developmental periods differentially impact adult aggressive and affective behaviors in mice

Qinghui Yu, PhD^{1,2}, Cátia M. Teixeira, PhD^{1,4}, Darshini Mahadevia^{1,4}, Yung-Yu Huang³, Daniel Balsam^{1,4}, J John Mann, MD³, Jay A Gingrich, MD, PhD^{1,4,*}, and Mark S. Ansorge, PhD^{1,4}

¹Divisions of Developmental Neuroscience, Department of Psychiatry, Columbia University, New York

²Department of Biological Sciences, Columbia University, New York

³Department of Molecular Imaging and Neuropathology, New York State Psychiatric Institute and Department of Psychiatry, Columbia University, New York

⁴Sackler Institute for Developmental Psychobiology, Columbia University and the New York State Psychiatric Institute, New York

Abstract

Pharmacologic blockade of monoamine oxidase A (MAOA) or serotonin transporter (5-HTT) has antidepressant and anxiolytic efficacy in adulthood. Yet, genetically conferred MAOA or 5-HTT hypo-activity is associated with altered aggression and increased anxiety/depression. Here we test the hypothesis that increased monoamine signaling during development causes these paradoxical aggressive and affective phenotypes. We find that pharmacologic MAOA blockade during early postnatal development (P2-P21) but not during peri-adolescence (P22-41) increases anxiety- and depression-like behavior in adult (> P90) mice, mimicking the effect of P2-21 5-HTT inhibition. Moreover, MAOA blockade during peri-adolescence, but not P2-21 or P182-201, increases adult aggressive behavior, and 5-HTT blockade from P22-P41 reduced adult aggression. Blockade of the dopamine transporter, but not the norepinephrine transporter, during P22-41 also increases adult aggressive behavior. Thus, P2-21 is a sensitive period during which 5-HT modulates adult anxiety/depression-like behavior, and P22-41 is a sensitive period during which DA and 5-HT bidirectionally modulate adult aggression. Permanently altered DAergic function as a consequence of increased P22-P41 monoamine signaling might underlie altered aggression. In support of this hypothesis, we find altered aggression correlating positively with locomotor response to amphetamine challenge in adulthood. Proving that altered DA function and aggression are

*To whom correspondence should be addressed: Sackler Professor of Clinical Developmental Psychobiology in the Dept. of Psychiatry, Director, Sackler Institute for Developmental Psychobiology, Division of Developmental Neuroscience, Columbia University and the NYSPI, 1051 Riverside Drive, room 4911A New York, NY 10032, jag46@columbia.edu, 212-543-6083. Supplementary information is available at Molecular Psychiatry's website.

Supplementary information.

Figure S1 (jpg file format) contains data on the behavioral response to amphetamine after peri-adolescent MAOA and 5-HTT blockade.

Conflict of interest. All authors declare that there are NO competing financial interests in relation to the work described.

causally linked, we demonstrate that optogenetic activation of VTA DAergic neurons increases aggression. It therefore appears that genetic and pharmacologic factors impacting dopamine and serotonin signaling during sensitive developmental periods can modulate adult monoaminergic function and thereby alter risk for aggressive and emotional dysfunction.

Keywords

Serotonin; Dopamine; Development; Aggression; Anxiety; Depression; Mouse

Introduction

Most neuropsychiatric disorders have developmental origins, and an emerging model postulates that such developmental vulnerability is often restricted to sensitive periods¹⁻³. The concept of sensitive developmental periods for the indelible modulation of complex behaviors is similar to that described for sensory systems (e.g. visual cortex, ocular dominance plasticity)^{4,5}, but affected behaviors, modulating factors, and underlying mechanisms are much less well understood. Furthering our knowledge of sensitive periods that determine the developmental trajectory of complex behaviors is a necessary step towards improving prevention and treatment approaches for neuropsychiatric disorders. Therefore, we here investigate how genetic and environmental risk factors act during sensitive periods of brain development to alter adult behavior and thereby potentially confer vulnerability to neuropsychiatric disorders.

Monoamine oxidase A (MAOA) inactivates bioamines, including serotonin (5-HT), norepinephrine (NE), dopamine (DA), and trace amines through oxidative deamination. Pharmacologic MAOA blockade is an effective treatment for depression and anxiety disorders in adults⁶. Unlike the salutary effects of pharmacologic MAOA inhibition, constitutive mutations of *maoa* function result in a syndrome characterized by antisocial/aggressive behavior in human males⁷. Moreover, low expressing *maoa* variants are also associated with aggression and anxiety traits in rhesus macaques and humans⁸⁻¹⁰. Consistent with higher primates, mice with genetic inactivation of *maoa* exhibit heightened levels of aggression and neophobia¹¹⁻¹³. The divergent effects of genetic (lifelong) mutations versus pharmacologic inhibition (during adulthood) lead us to hypothesize that lower MAOA function during sensitive periods of brain maturation increases the likelihood of aggressive and anxiety traits later in life.

The role of monoamine signaling in brain development has been firmly established. During the embryonic and early postnatal period, monoamines act as trophic factors, modulating neurodevelopmental processes, such as cell division, migration, and differentiation¹⁴⁻¹⁶. In mice, genetic *maoa* ablation increases brain tissue concentrations of 5-HT, NE, and DA during development and early adulthood¹¹. The most prominent teratogenic consequence of murine *maoa* ablation, the disruption of barrel fields in the somatosensory cortex, has been linked to increased 5-HT signaling during perinatal development¹⁷⁻¹⁹. After closure of this sensitive window relating to the establishment of somatosensory cortex organization, increased 5-HT signaling during early postnatal development increases anxiety/depression-

like behavior including neophobia in adult mice^{20, 21}. Thus, we hypothesized first that increased 5-HT signaling during early postnatal development causes neophobia in *maoa* deficient mice. Secondly, we predicted that the origins of aggressive behaviors are also developmental and postnatal, because re-expressing *maoa* from P1 onwards restores normal aggressive behavior in conditional *maoa* knock-out mice²². To test both hypotheses, we compared the effects of developmental MAOA inhibition and monoamine transporter blockade on adult behavior and monoamine signaling in mice.

Materials and methods

Subjects

Mice (129SvEv/Tac) were bred at Columbia Psychiatry, New York State Psychiatric Institute. Mice used for experiments were born to litters containing 4-6 pups. Mice were separated by sex and weaned into groups of 5 mice per cage at P22. Mice were treated during postnatal development (P2-21, as previously described^{20, 21}) or periadolescence (P22-41). One additional control group was treated during adulthood (P182-201). Treatment consisted of the following intraperitoneal (i.p.) drug injections: vehicle (VEH, 0.9% NaCl, 5 ml/kg), fluoxetine (FLX, 10 mg/kg, 5 ml/kg, 5-HTT blocker), clorgyline (CLO, 20 mg/kg, MAOA inhibitor), desipramine (DMI, 20 mg/kg, NET blocker), or GBR12909 (GBR, 20 mg/kg, DAT blocker). The dose for each treatment was chosen based on previous experiments: FLX at 10 mg/kg/day and DMI at 20 mg/kg/day produce serum and brain drug and metabolite levels that are comparable to those with therapeutic efficacy in humans^{20, 21}; GBR at 20 mg/kg is a standard dose to achieve functional DAT blockade in mice²³; and a CLO dose of 20 mg/kg/day produces partial disruption of MAOA-dependent barrel field formation (to achieve full disruption of barrel field formation, CLO has to be administered every 8h)¹⁹. Treatment assignment was randomized in each cage. Injections were administered daily between 3 pm and 5 pm. Animals were maintained on a 12-hour light-dark cycle (lights on at 8 am) and provided with food and water *ad libitum*. Animal testing was conducted in accordance with the *Principles of Laboratory Animal Care* National Institute of Health (NIH) guidelines and the institutional animal committee guidelines.

Optogenetics

In order to selectively express channelrhodopsin2 (ChR2) in dopaminergic cells we crossed a Dopamine-transporter Cre-driver-line (DAT^{IRESCre}²⁴), with a ROSA26-floxedSTOP ChR2-eYFP line (ai32 line²⁵). Under isofluorane surgery, optical fiber ferrules (Ceramic ferrules (OD 1.2 mm): Precision fiber products; Optical fiber (OD 0.2mm): Thor labs), were implanted over the VTA (AP:-3.5; ML:0.5; DV:-4.2). After recovery, mice were subjected to the isolation-induced aggression test as described below, but pairing DAT^{IRESCre};ai32 mice with single mutant controls. During each encounter only one mouse of the pair was stimulated using blue light pulses (473 nm, 20 Hz, 10 ms pulse duration, 8-10mW) originating from a laser source. Each mouse of the pair was stimulated in consecutive days, counterbalancing the stimulation-order by genotype. After behavioral tests were completed, mice were euthanized, and genotypes and fiber placements were confirmed.

Behavioral testing

For neophobia/anxiety-related behavioral testing, male and female mice were investigated and all animals were exposed to the same series of behavioral paradigms starting at 3 months of age. The tests were administered in the following order: open field, novelty suppressed feeding, and shock escape with a minimum of 7 days between each test. Aggression and amphetamine response was tested in behavioral naïve independent male cohorts starting at 5 months of age for the P2-21 and P22-41 groups and at 10 months of age for the P182-201 group. All behavioral testing took place during the light cycle between 12:00 pm and 7:00 pm. To eliminate odor cues, each apparatus was thoroughly cleaned after each animal.

Open field test—Exploration and reactivity to a novel environment was assessed using the open field test as previously described²⁶. In brief, square Plexiglas activity chambers equipped with infrared detectors to track animal movement in the horizontal and vertical planes were used as the novel environment. The conflicting innate tendencies to avoid bright light and open spaces but explore novel environments influence locomotor behavior. Mice were placed into the center of the open field and activity was recorded for 30 minutes. Testing took place under bright ambient light conditions. Total distance, total ambulatory time, and vertical activity were measured.

Novelty-suppressed feeding test—The novelty-suppressed feeding test is a behavior conflict paradigm that is sensitive to chronic but not acute antidepressant treatment as well as acute benzodiazepine treatment. The test was performed as described previously²⁰: the testing apparatus consisted of a plastic box (50 x 50 x 20 cm). The floor was covered with 2 cm of wooden bedding. Twenty-four hours before behavioral testing, animals were deprived of food in the home cage. At the time of testing, two food pellets were placed on a round filter paper (12 cm diameter) positioned in the center of the box. Animals were placed in a corner of the box and the latency to approach the pellet and begin feeding was recorded (maximum time, 10 min). Each mouse was weighed before food deprivation and before testing to determine the percentage of body weight lost.

Shock escape test—Shock escape latency is the primary dependent measure affected by uncontrollable stress and chronic antidepressant treatment. The shock escape test was performed in a 2-chambered Plexiglas shuttle box with the two chambers separated by an automated guillotine door as previously described²⁶. Each apparatus is equipped with infrared detectors to track animal movement and located within a sound-attenuated chamber. At the beginning of each trial, the door was raised and a mild scrambled foot shock (0.2 mA; 10 s duration) was delivered to the subject through the floor bars. The end of a trial was signaled by the closing of the guillotine door and was triggered either by a transition to the opposite chamber or after 10 s. Transition latencies were recorded. If the subject failed to make a transition during the 10s duration of the foot shock, a maximum latency of 10 s was recorded. A session consisted of 30 trials separated by a 30 s inter-trial interval. Locomotor activity was assessed during the pre-shock habituation phase with the guillotine door open.

Isolation-induced aggression test—We used a rectangular cage that was divided in half by a perforated partition made of clear plastic. A pair of mice with the same treatment was placed in the cage, one in each compartment. The mouse in one compartment is able to see, hear and smell the other mouse through the holes in the plastic divider, but physical interaction is blocked. Mice were housed for at least 10 days before experiment was performed. On the test day, dividers were taken out and mice were allowed to freely interact for 10 min. All behaviors were videotaped. The time spent mounting, tail rattling, and biting was scored.

Amphetamine response—Adult mice were injected with amphetamine (3 – 5 mg/kg, i.p.) or VEH and locomotor activity was assessed in open field apparatus (see above).

High Pressure Liquid Chromatography (HPLC)

HPLC was carried out on several brain regions as previously described²⁷. Concentrations of biogenic amines and metabolites were measured using reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection. The dissected brain samples in a 1.5-ml microfuge tube were homogenized in 0.5-1.0 ml of 0.4 M perchloric acid with an Ultra Cell Disruptor (Microson, NY). The homogenate was centrifuged for 5 minutes at 14,000 g and 4°C. 50 µl of typically four-fold diluted aliquot of the supernatant was injected over the HPLC system. The HPLC system, equipped with Waters Millennium software, consists of a Waters 515 HPLC pump, a Waters 717 Autosampler, a Varian Microsorb 100-5 C18 reverse-phase column (DYNAMAX 150x4.6 mm) attached with a Guard column (4.6 mm) and an ESA Coulochem electrochemical detector (Model 5100A) with a guard cell (Model 5020) and a dual analytical cell (Model 5011A). The electrochemical detector was set at potential of +0.05 V for the first cell and +0.5 V for the second cell. The mobile phase contained 0.75 mM sodium phosphate (pH 3.1), 1.4 mM 1-Octanesulfonic acid, 10 µM sodium EDTA and 8% acetonitrile. The mobile phase was filtered through a Millipore 0.22 µm filter (Type GV) and degassed in vacuo. The flow rate was maintained at 0.8 ml/min. A chromatography software package (Waters Millennium) was used for data acquisition and analysis. Values are calculated based on peak area and compared to standard solutions. The inter- and intra-assay coefficients of variation of the assay were each less than 5%. The sensitivity of the assay was less than 0.5 pmol/injection.

Immunohistochemistry

For immunohistochemistry, animals were deeply anesthetized with ketamine/xylazine and perfused with PBS followed by 4 % PFA in PBS. Brains were then removed and post-fixed overnight (4 % PFA in PBS), and subsequently cryoprotected in 30 % sucrose and frozen. Coronal sections (50 µm thick) were cut with a cryostat and fluorescence immunohistochemistry was performed on free-floating sections. Immunohistochemistry was performed using a rabbit primary antibody against GFP (rabbit anti-GFP; 1:1000; Life technologies). As secondary antibody, we used cy3 donkey anti-rabbit (1:350; Jackson Immunoresearch). Antibodies were diluted in blocking solution containing 2 % bovine serum albumin and 0.2 % Triton X-100 dissolved in PBS. Sections were incubated in primary antibody for 24 h at 4° C, washed with PBS, and then incubated with secondary

antibody for 2 h at room temperature. After washing, sections were mounted with Vectashield mounting medium (Vector Labs).

Statistic analysis

Statistical analysis was performed using StatView 5.0 software (SAS Institute, Cary, NC). Data were analyzed using Student's t test, one-way, or two-way ANOVA with Student–Newman–Keuls posthoc testing; survival analysis, Kaplan–Meier, Logrank Mantel–Cox test; or non-parametric Mann–Whitney test as indicated. The criterion for significance for all analyses was $p < 0.05$. No statistically significant interactions of independent variables and gender were detected and therefore data were collapsed for sex. Results from data analyses are expressed as mean \pm SEM.

Results

5-HTT and MAOA blockade during early postnatal development increase adult anxiety/depression-like behavior

Early postnatal 5-HTT blockade increases anxiety/depression-like behavior in adult mice^{20, 21}. Because 5-HTT and MAOA blockade both increase 5-HT signaling, we hypothesized that early postnatal MAOA blockade would also increase adult anxiety/depression-like behavior. To test this hypothesis, we administered the 5-HTT blocker fluoxetine (FLX, 10 mg/kg/day, i.p.), the MAOA inhibitor clorgyline (CLO, 20 mg/kg/day, i.p.), or vehicle (VEH, 0.9% NaCl, i.p.) to mice in the early postnatal period (PN), from postnatal day 2 to 21 (P2–P21) and assessed emotional behavior in adulthood (P90). To investigate developmental specificity, we also tested mice that received FLX, CLO, and VEH in the peri-adolescent period (ADO), from postnatal day 22 to 41 (P22–P41). To control for the effects of the injection procedure, we included naïve littermates in our behavioral tests.

Using the novel open field, we assessed neophobic behaviors by measuring exploratory activity. We observed that FLX and CLO treatment from P2–P21 decreased total ambulatory time (treatment effect: $F_{(2,127)} = 6.225$, $p = 0.0026$) and total time spent rearing (treatment effect: $F_{(2,127)} = 5.42$, $p = 0.0055$; developmental timing effect: $F_{(1,127)} = 13.56$, $p = 0.0003$) when compared to VEH-treated mice (Fig. 1a and b). Neither FLX nor CLO treatment from P22–P41 altered exploratory behavior compared with VEH treated mice. Behaviors in VEH-treated mice and naïve littermates did not differ in either measure.

To further probe neophobia phenotypes, we used the novelty suppressed feeding paradigm, which assesses approach-avoidance behavior when a food-deprived animal is presented with a familiar food pellet placed in the center of a brightly-lit novel arena. Mice exposed to FLX or CLO from P2–P21 exhibited longer latencies to approach and feed when compared to PN–VEH controls (treatment effect: $p = 0.0001$, survival analysis, Kaplan–Meier, Logrank Mantel–Cox) (Fig. 1c). However, neither FLX treatment nor CLO treatment from P22–P41 produced such behavioral effect. No effect for treatment, developmental timing or treatment \times developmental timing was detected for weight loss during food deprivation (Fig. 1d). Behavior between VEH-treated mice and naïve littermates did not differ in either measure.

To assess behavioral response to stress, we examined escape latency in the shock escape paradigm. Consistent with our findings in tests of neophobia, mice exposed to FLX or CLO from P2-P21 but not P22-P41 exhibited significantly increased escape latencies when compared to PN-VEH controls (treatment effect: $F_{(2,133)} = 21.106, p < 0.0001$; developmental timing effect: $F_{(1,133)} = 46.749, p < 0.0001$; treatment x developmental timing interaction: $F_{(2,133)} = 24.613, p < 0.0001$) (Fig. 1e). No effect of treatment, developmental timing or treatment x developmental timing was detected for pre-shock activity in the dark chambers with the door open (Fig. 1f), indicating that the effect on escape latencies was not caused by reduced overall activity. Behavior between VEH-treated mice and naïve littermates did not differ in either measure.

Peri-adolescent MAOA blockade increases and 5-HTT blockade reduces adult aggressive behavior

Next we investigated aggressive behaviors in adult mice that had received FLX, CLO, or VEH during early postnatal or peri-adolescent development using the isolation-induced aggression paradigm. We measured the time mouse dyads spent engaged in aggressive behaviors consisting of mounting, tail rattling, or biting.

We found that CLO treatment from P22-P41 but not from P2-P21 increased aggressive behavior, when compared to VEH controls (effect of treatment: $F_{(2,69)} = 7.258, p = 0.0014$; effect of developmental timing: $F_{(1,69)} = 4.737, p = 0.033$; treatment x developmental timing interaction: $F_{(2,69)} = 3.828, p = 0.0265$) (Fig. 2a). FLX treatment reduced aggressive behavior when compared to VEH treatment, with no indication of developmental timing specificity. Aggressive behavior between VEH-treated mice and naïve littermates did not differ. Next we investigated period specificity for the effect of transient CLO-treatment on adult aggression, using mice that had received VEH or CLO treatment during adulthood (P182-P201) and were tested 4 months later (P300). We detected no effect of treatment on aggression (Fig. 2b).

Peri-adolescent MAOA blockade reduces the metabolism of 5-HT, NE and DA

The differential consequences of P22-P41 5-HTT and MAOA blockade on adult aggressive behavior suggest that non-5-HT effects of peri-adolescent MAOA blockade increase adult aggression. In peri-adolescence, *maoa* is expressed in 5-HTergic, DAergic and NEergic neurons²⁸. To determine the consequences of P22-P41 CLO treatment on brain monoamine signaling, we assessed tissue monoamine and metabolite levels at P23 (24h after treatment initiation) and at P42 (24 h after treatment cessation). Specifically, we quantified 5-HT and its main metabolite 5-hydroxyindoleacetic acid (5-HIAA), DA and its two main metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and NE. These measures serve as a proxy for enzyme activity.

CLO treatment significantly increased 5-HT and reduced 5-HIAA levels in the brainstem at P23 ($F_{(1,12)} = 51.972, p < 0.0001$ and $F_{(1,12)} = 31.92, p = 0.0001$, respectively) and P42 ($F_{(1,11)} = 62.475, p < 0.0001$ and $F_{(1,11)} = 18.8, p = 0.0012$, respectively), when compared to VEH treated controls (Fig. 3a–d). Norepinephrine (NE) levels in the brainstem were also increased by CLO treatment at P23 and P42 ($F_{(1,12)} = 68.717, p < 0.0001$ and $F_{(1,11)} =$

40.45, $p < 0.0001$, respectively) (Fig. 3e,f). Striatal DA levels were not affected at P23, but significantly increased at P42 after chronic CLO treatment when compared to VEH treated controls ($F_{(1,12)} = 0.033$, $p = 0.8587$ and $F_{(1,11)} = 61.745$, $p < 0.0001$, respectively) (Fig. 3g,h). Striatal DOPAC levels were lower at P23 and P42 ($F_{(1,12)} = 30.292$, $p = 0.0001$ and $F_{(1,11)} = 13.444$, $p = 0.0037$, respectively), while HVA levels were unchanged ($F_{(1,12)} = 0.003$, $p = 0.9586$ and $F_{(1,11)} = 2.41$, $p = 0.1489$, respectively) (Fig. 3i-l). These data demonstrate that MAOA blockade using CLO treatment during peri-adolescence inhibits 5-HT, NE, and DA metabolism and raises levels of 5-HT, DA and NE.

Peri-adolescent DAT but not NET blockade increases adult aggressive behavior

Next we sought to identify the monoamine component in P22-P41 MAOA blockade, which leads to increased adult aggressive behavior. Because peri-adolescent MAOA blockade robustly increases central 5-HT, NE and DA levels, we examined whether increased peri-adolescent NE or DA signaling could increase adult aggressive behavior. To selectively and transiently increase NE or DA signaling we administered drugs blocking the NE transporter (NET) or the DA transporter (DAT). We used desipramine (DMI, 20 mg/kg/day) to block the NET and GBR12909 (GBR, 20 mg/kg/day) to block the DAT. DMI treatment from P22-P41, either alone or in combination with FLX (10 mg/kg/day) treatment, did not result in increased aggressive behavior in adulthood (Fig. 4a). GBR treatment from P22-P41 however lead to increased time spent in active aggressive behaviors (Mann Whitney, $p = 0.0104$) (Fig. 4b).

Peri-adolescent MAOA but not 5-HTT blockade reduces adult brainstem 5-HIAA levels and increases striatal DA and DOPAC levels

To start investigating the underlying neural substrate which gives rise to altered adult aggressive behavior after peri-adolescent MAOA, 5-HTT, and DAT blockade, we next examined if these developmental interventions produce long-lasting changes in brain monoamine or metabolite levels. P22-P41 CLO-treatment significantly reduced 5-HIAA levels in the brain stem at P180 when compared to either VEH- or FLX-treatment ($F_{(2,15)} = 8.505$, $p = 0.0034$) (Table 1a). P22-P41 CLO treatment also significantly increased striatal DA ($F_{(2,15)} = 4.085$, $p = 0.0384$) and DOPAC levels ($F_{(2,15)} = 5.847$, $p < 0.0133$) at P180 when compared to VEH-treatment and VEH- or FLX-treatment, respectively (Table 1a). However, peri-adolescent FLX or GBR treatment did not produce significant changes in monoamines or their metabolites in the brainstem or striatum at P180, when compared to VEH treated controls (Table 1a,b). Taken together, this dissociation indicates that while altered tissue monoamine levels might be sufficient to alter adult aggressive behavior, they are not necessary.

Peri-adolescent MAOA or DAT blockade increases while 5-HTT blockade reduces the behavioral response to amphetamine in adulthood

Dopaminergic hyper-activity has been implicated in aggression²⁹. We find a positive association between aggression and striatal DA and DOPAC levels after peri-adolescent MAOA, but not 5-HTT or DAT blockade. Here we further investigate the hypothesis that dysfunction of the DAergic system might underlie altered aggression, using behavioral

response to amphetamine challenge (as opposed to total monoamine tissue levels) as a proxy for DAergic function.

First, we analyzed the behavioral response to amphetamine (3 mg/kg, i.p.) and VEH challenge in adult mice (P180), which had received VEH or CLO during P22-P41. Adult VEH injection did not alter behavior in P22-P41 VEH or CLO-treated mice (Fig. 5a); adult amphetamine injection increased ambulatory activity in P22-P41 VEH and CLO-treated mice (effect of time in the amphetamine treated group: $F_{(16,416)} = 18.812$, $p < 0.0001$, Fig. 5a); In comparison with P22-P41 VEH treated mice, P22-P41 CLO treated mice showed a significantly increased response to amphetamine (effect of peri-adolescent treatment: $F_{(1,26)} = 6.576$, $p = 0.0165$; effect of peri-adolescent x adult treatment interaction: $F_{(16,416)} = 3.810$, $p < 0.0001$; Fig. 5a). Analysis of only post-challenge activity confirmed these results: We detected an effect of peri-adolescent treatment, an effect of challenge, and an interaction between both ($F_{(1,26)} = 6.768$, $p = 0.0165$, $F_{(1,26)} = 45.932$, $p < 0.0001$, and $F_{(1,26)} = 5.549$, $p = 0.0263$, respectively; Fig. 5b). To test for period specificity, we next investigated the consequences of transient CLO-treatment during adulthood on behavioral response to amphetamine later in life. Specifically, mice, which had received VEH or CLO during P182-P201, were challenged with amphetamine (3 mg/kg, i.p.) or VEH 4 months later. We detected an effect of amphetamine but no effect of peri-adolescent treatment or peri-adolescent x adult treatment interaction ($F_{(1,44)} = 34.464$, $p < 0.0001$; Fig. 5c). Next, we investigated the behavioral response to amphetamine challenge in adult mice, which had received VEH or FLX during P22-P41. We detected an effect of peri-adolescent treatment, an effect of amphetamine and a peri-adolescent x adult treatment interaction ($F_{(1,18)} = 4.719$, $p = 0.0434$, $F_{(1,18)} = 4.480$, $p = 0.0485$, and $F_{(1,18)} = 4.484$, $p = 0.0484$, respectively; Fig. 5d). To our surprise, P22-P41 FLX treated mice displayed no behavioral response to amphetamine, while P22-P41 VEH treated littermates did respond as expected (Fig. 5d). We hypothesized that peri-adolescent FLX treatment shifts the amphetamine dose-response curve as opposed to making mice generally insensitive to amphetamine. Hence we tested the effect of amphetamine challenge at 5 mg/kg in adult mice, which had received VEH, CLO or FLX during P22-P41. Analyzing behavior as a function of amphetamine-dose, we detected an effect of peri-adolescent treatment and an effect of amphetamine-dose ($F_{(2,30)} = 6.364$, $p = 0.005$, $F_{(2,60)} = 58.639$, $p < 0.0001$, respectively; Fig. S1). Posthoc analysis revealed that FLX treated mice do behaviorally respond to amphetamine ($F_{(2,8)} = 6.259$, $p = 0.0231$), but that their response is diminished (Fig. S1). Finally, we tested if peri-adolescent GBR-treatment impacts adult amphetamine response. Strengthening the positive correlation between aggressive behavior and a sensitized/hyper-active DA system, we found that P22-P41 GBR treated mice display an exaggerated response to adult amphetamine challenge when compared to adult VEH injection or P22-P41 VEH treated mice (effect of peri-adolescent treatment: $F_{(1,21)} = 9.273$, $p = 0.0062$; effect of amphetamine challenge: $F_{(1,21)} = 40.730$, $p < 0.0001$; peri-adolescent treatment x adult amphetamine challenge interaction: $F_{(1,21)} = 6.847$, $p = 0.0161$, Fig. 5e). These data demonstrate that P22-P41 CLO, GBR, and FLX treatment differentially impact the sensitivity of the adult DA system. Furthermore, the behavioral sensitivity to amphetamine correlates positively with levels of adult aggression.

Optogenetic stimulation of VTA DAergic neurons increases aggressive behavior

Peri-adolescent monoamine levels impact adult amphetamine-response and aggressive behavior, suggesting that altered DAergic sensitivity is determining aggression levels. Alternatively, DAergic activity may be correlated with but not causally related to aggressive phenotypes. To investigate these alternative hypotheses we studied the direct causal relationship between DAergic neuronal activity and aggressive behavior using optogenetics.

To selectively target the dopaminergic system we crossed mice expressing Cre-recombinase under the control of the dopamine transporter promoter ($\text{DAT}^{\text{IRESCre}24}$) with a ROSA26-reporter line conditionally expressing ChR2-eYFP in a Cre-dependent fashion (ai32 line²⁵) (Fig. 6a). Optical fibers were implanted over the VTA region in double mutant mice ($\text{DAT}^{\text{IRESCre};\text{ai32}}$) and in single mutant controls (Fig. 6b, c). In order to assess aggressive behavior we used the isolation-induced aggression paradigm, pairing $\text{DAT}^{\text{IRESCre};\text{ai32}}$ mice with single mutant controls. Blue light pulses (473 nm, 20 Hz, 10 ms pulse duration) were delivered to the VTA for the duration of the test. Only one mouse of the pair was stimulated during each 10 min encounter. Under these conditions, we detected an effect of genotype on aggressive behavior ($F_{(1,27)} = 5.146, p = 0.0315$), with stimulation of $\text{Dat}^{\text{IRESCre};\text{ai32}}$ mice leading to significantly longer time engaging in aggressive contacts, when compared to stimulation of single mutant controls (Fig. 6d).

These data demonstrate that VTA DAergic neuronal activity is causally related to aggressive behaviors.

Discussion

Our experiments demonstrate the existence of two developmental periods during which early-life perturbation of monoamine signaling alters adult behavior: an early postnatal (P2-P21) 5-HT-sensitive period that affects anxiety and depression-related behaviors and a later peri-adolescent (P22-P41) 5-HT and DA-sensitive period altering aggression. Serotonergic and dopaminergic signaling perturbations during peri-adolescence also exert profound and opposite effects on the behavioral response to psychostimulant challenge in adulthood, suggesting that altered DA-function underlies the changes in aggressive behaviors. In support of this hypothesis we demonstrate that direct optogenetic stimulation of VTA DAergic neurons increases aggressive behavior.

Our findings can explain the paradoxical high neophobia/anxiety phenotypes of *5-htt*^{-/-} mice^{26, 30}, *maoa*^{-/-} mice¹¹⁻¹³, and pre-synaptic *htr1a* deficient mice³¹, because these mouse lines lack their respective gene products throughout life, including the sensitive P2-P21 period. Likewise, the model can explain the increased aggression seen in constitutive *maoa*, *dat*, and *comt* loss-of-function mouse lines^{11, 12, 32, 33}. The moderating effect of developmental 5-HTT blockade on adult aggressive behavior does not follow the strict timing specificity (5-HTT blockade during P2-P21 or P22-P41 both decreased adult aggression), but nonetheless supports a developmental mechanism for the low-aggression phenotype of *5-htt*^{-/-} mice³⁴. Because we performed developmental manipulations in wild-type mice but not in the respective mutant mouse lines, we can only compare phenotypes qualitatively but not quantitatively. Hence we cannot draw conclusions as to

whether the phenotypes we have observed are fully or only partially recapitulating the magnitude of behavioral consequences elicited by the life-long genetic mutations. Furthermore, we cannot exclude the involvement of off-target effects or downstream developmental effects in contributing to the phenotypes examined here. For example, even though clorgyline preferentially inhibits MAOA, it might also inhibit MAOB in our experiments. To overcome this drawback, we base our conclusions not on single drug effects but on the convergence of multiple pharmacological interventions that resonate with a large literature on genetic effects in both mice and humans. For example, our conclusion that increased DAergic activity during P22-41 increases adult aggression and the behavioral response to amphetamine, is supported by our finding that we can mimic the effect of CLO through DAT but not 5-HTT or NET blockade.

Our findings comport with human vulnerabilities to anxiety, depression and aggression conferred by functional genetic polymorphisms. For example, endophenotypes of affective disorders have been associated with low expressing alleles for the *5-htt*³⁵⁻³⁹, *maoa*^{9, 40} and presynaptic *htr1a*⁴¹. Likewise, aggressive behavior has been associated with loss-of-function and low-expressing *maoa* alleles^{7, 8, 42, 43}, the *10R* variant of *dat1*^{44, 45}, and the low activity *met* allele of the *comt*⁴⁶. Our model predicts that these risk alleles act primarily during sensitive developmental periods to alter brain maturation and circuit formation leading to altered behaviors. The temporal dynamics of transcription in the human brain and their genetic moderation support this hypothesis⁴⁷. An important aspect to consider when comparing our interventions or gene ablation studies in mice to genetic risk factors in humans is that human polymorphisms rarely lead to complete loss of function whereas the murine interventions often do. Thus, it is possible that in humans multiple risk factors that impact monoamine signaling during adolescence must converge to elicit a phenotype.

Based on our data, pharmacologic interventions during sensitive periods of human development would be predicted to impact affective and aggressive behavior. The murine P2-P21 period roughly corresponds to the third trimester of human gestation and early childhood, while P22-P41 corresponds to peri-adolescent development⁴⁸. Thus, SSRIs taken by pregnant mothers might impact fetal brain maturation and adult mood and anxiety, while stimulant exposure during peri-adolescence could alter adult aggressive behavior. Indeed, chronic stimulant exposure increases aggressive behavior in rodents, non-human primates and humans⁴⁹⁻⁵¹, even in abstinent stimulant users⁵². The long-term effects of gestational SSRI exposure on affect, aggression, and cognition are unknown^{15, 53}, but prenatal SSRI exposure has been associated with a 2-fold increased risk of autism spectrum disorder⁵⁴.

Our findings support the fundamental notion that neural circuitry and consequently behavior are most vulnerable to long lasting modulation during periods of maturation and high plasticity^{4, 55}. The 5-HT sensitive period described here coincides with the emergence of anxiety- and fear-related behaviors in rodents and the maturation of the underlying circuitry. For example, at around P10 the consequence of odor shock conditioning switches from preference to aversion, with amygdala engagement and hypothalamic-pituitary-adrenal axis integration^{56, 57}. Limbic structures including amygdala, prefrontal cortex and hippocampus are maturing during postnatal development and human *s* allele carriers as well as *5-htt-/-*

mice exhibit disrupted mPFC-amygdala functional connectivity that likely arises during early circuit maturation^{58, 59}. The DA-sensitive, peri-adolescent period coincides with the developmental onset of play fighting in rodents^{60, 61} and non-human primates⁶², and human adolescence is characterized by dynamic changes in aggressive behavior⁶³.

Insight into how developmental monoamine signaling establishes long-lasting alterations in brain function and behavior remains scarce. The best-studied example is the establishment of the cyto- and circuit-architecture in the somatosensory cortex of mice. MAOA and 5-HTT deficient mice lack topographically defined barrel fields^{17-19, 64}. This deficit is caused by increased 5-HT signaling through 5-HT1B receptors located on thalamocortical axon terminals, which may inhibit the glutamate release¹⁷ necessary for the formation of barrel columns⁶⁵. The sensitive period for this developmental process starts at P0 and ends at P4¹⁹, and therefore partially dissociates from the 5-HT and fully dissociates from the DA-sensitive period under investigation here. Thus, thalamocortical wiring abnormalities are unlikely to contribute to the emotional and aggressive phenotypes. Focusing on the peri-adolescent period we identified changes in monoamine levels and more importantly, changes in behavioral reactivity to amphetamine challenge that correlate with aggressive behavior. Specifically, we report that increased adult aggressive behavior elicited by peri-adolescent MAOA or DAT blockade correlates with increased locomotor-stimulating effects of amphetamine challenge in adulthood. Conversely, reduced adult aggressive behavior elicited by peri-adolescent 5-HTT blockade correlates with decreased locomotor-stimulating effects of amphetamine challenge in adulthood. Supporting phylogenetic conservation, human individuals with antisocial traits also show mesolimbic dopamine hypersensitivity to amphetamine, as impulsivity is positively correlated with the magnitude of amphetamine-induced DA release in the striatum⁶⁶. Highlighting the notion that altered DAergic signaling impacts adult behavior as a function of developmental timing, transient *pre*-adolescent methylphenidate exposure *decreases* responsiveness to cocaine's locomotor-activating effects in adult rats⁶⁷. In contrast, transient adult methylphenidate exposure does not alter cocaine's locomotor-activating effects in later adulthood⁶⁷.

The positive correlation between levels of aggression and behavioral sensitivity to amphetamine across our peri-adolescent treatment regimes comports with the DA-hypothesis of aggression, which states that DAergic hyperfunction increases aggression^{29, 68}. However, the DA hypothesis of aggression is largely based on correlative data and has thus far not been tested using direct and specific activation of DAergic neuronal activity. We here therefore performed such an experiment using optogenetics and demonstrate the existence of a direct causal relationship between DAergic neuronal activity and aggressive behavior. Hence, our data support a model in which monoamine signaling during peri-adolescence permanently alters the sensitivity/activity of DAergic neurons/pathways to impact adult aggression. However, many questions remain unanswered: are DAergic changes located pre-synaptically or post-synaptically? Do they occur at the cell-intrinsic level or do they involve altered micro- or macro-circuitry? How do DA and 5-HT exert their opposing effects on behavior and how is DA-dominance established (aggressive MAOA deficient mice have both, elevated 5-HT and DA levels)? Future studies examining these questions will be critical to understand both the mechanism for the establishment of

DAergic sensitivity levels during development as well as the nature of alterations in adult DA-related circuit function.

Taken together, our findings support the existence of sensitive periods that influence lifelong vulnerability to anxiety, depression, aggression, and substance abuse. Such sensitive periods have been most extensively characterized for sensory systems (e.g. visual cortex), but conceptually similar principles may apply to the development and organization of brain circuitry that mediate the more complex behaviors described here. With increased knowledge about sensitive periods during which genetic and environmental factors act to confer risk for neuropsychiatric disorders, we will not only be able to improve classification and diagnosis, but also treatment and prevention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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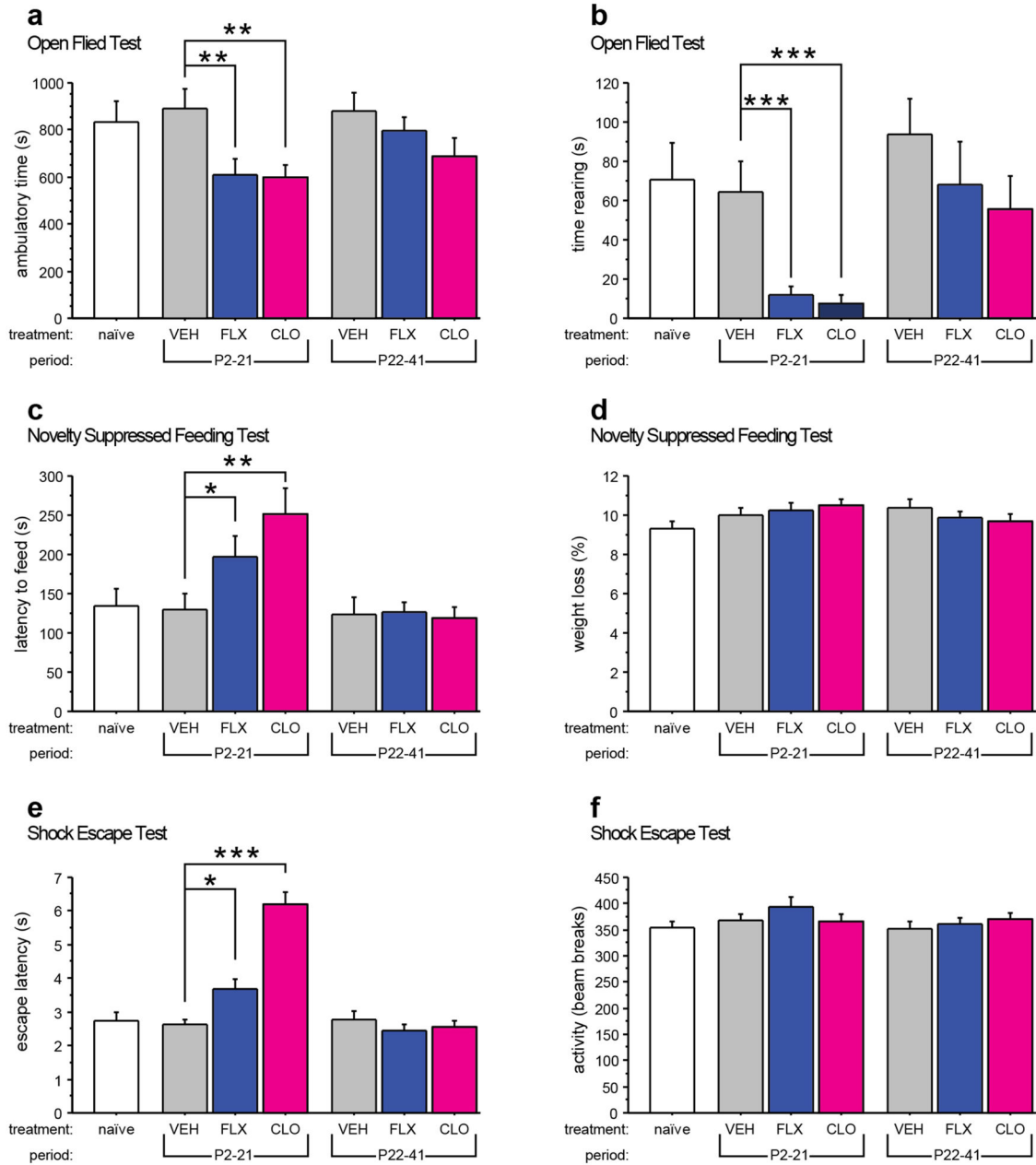


Fig. 1. Increased adult anxiety/depression-like behavior after early postnatal 5-HTT or MAOA blockade

Anxiety/depression-like behavior was assessed in mice using the open field test (**a**, **b**), the novelty suppressed feeding test (**c**, **d**), and the shock escape test (**e**, **f**). FLX or CLO treatment from P2-P21 reduced the time ambulating (**a**) and the time rearing (**b**) when compared to control mice treated with VEH from P2-P21. FLX or CLO treatment from P2-P21 increased the latency to feed when compared to control mice treated with VEH from P2-P21 (**c**). No effect of treatment, period, or treatment x period interaction was detected for the weight loss after 24 hours of food deprivation (**d**). FLX or CLO treatment from P2-P21 increased the latency to escape when compared to control mice treated with VEH from P2-

P21 (e). No effect of treatment, period, or treatment x period interaction was detected for activity in the shuttle box before the onset of shock (f). (n = 17–32 mice per group). (*p < 0.05; **p < 0.01, ***p < 0.001).

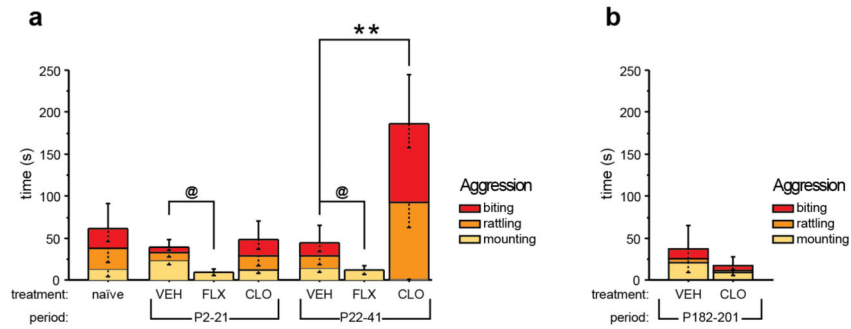


Fig. 2. Altered aggression after developmental 5-HTT or MAOA blockade
 Isolation induced aggressive behavior was assessed in mice by scoring the time spent mounting, tail rattling, or biting during a 10-minute encounter. **(a)** Aggressive behavior was increased in mice treated with CLO from P22-P41 when compared to control mice treated with VEH from P22-P41. Mice treated with FLX displayed reduced aggression when compared to VEH-treated control mice. Of note, FLX treated mice did not display any tail rattling or biting behavior. (n = 7–16 pairs per group). **(b)** Aggressive behavior was unaffected by transient CLO-treatment from P182-P201 (n = 7 pairs per group). (@ indicates main effect of treatment: p < 0.05; **p < 0.01).

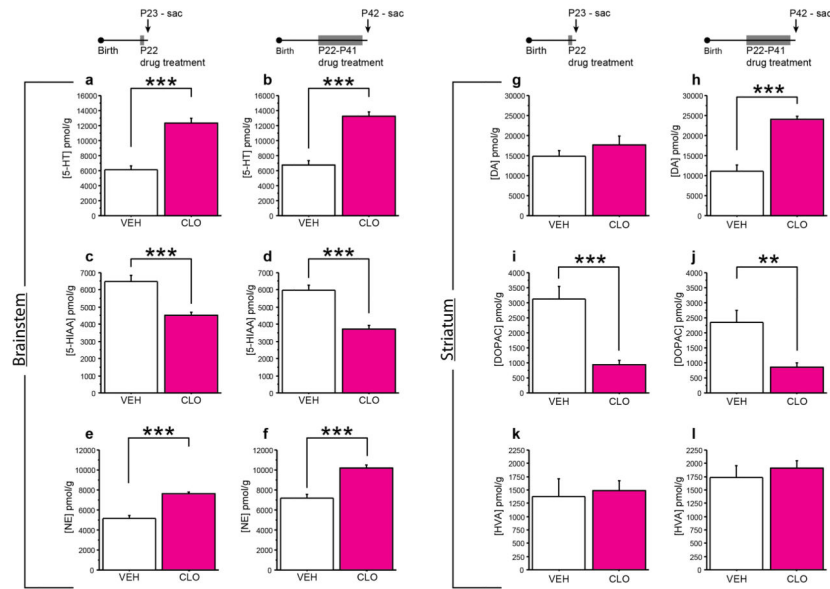


Fig. 3. Altered monoamine and –metabolite levels during and after peri-adolescent MAOA blockade

Mice were injected daily with CLO from P22-P41 and tissue monoamine and monoamine-metabolite levels were assessed on P23 (a, c, e, g, i, k) and P42 (b, d, f, h, j, l) by high performance liquid chromatography. 5-HT (a, b), 5-HIAA (c, d), and NE (e, f) were measured in the brainstem (a–f). DA (g, h), DOPAC (i, j), and HVA (k, l) levels were measured in the striatum (g–l). Monoamine levels were increased and monoamine-metabolite levels were decreased during CLO treatment when compared to VEH treatment as indicated. (n = 6–8 mice per group); (**p < 0.01; ***p < 0.001).

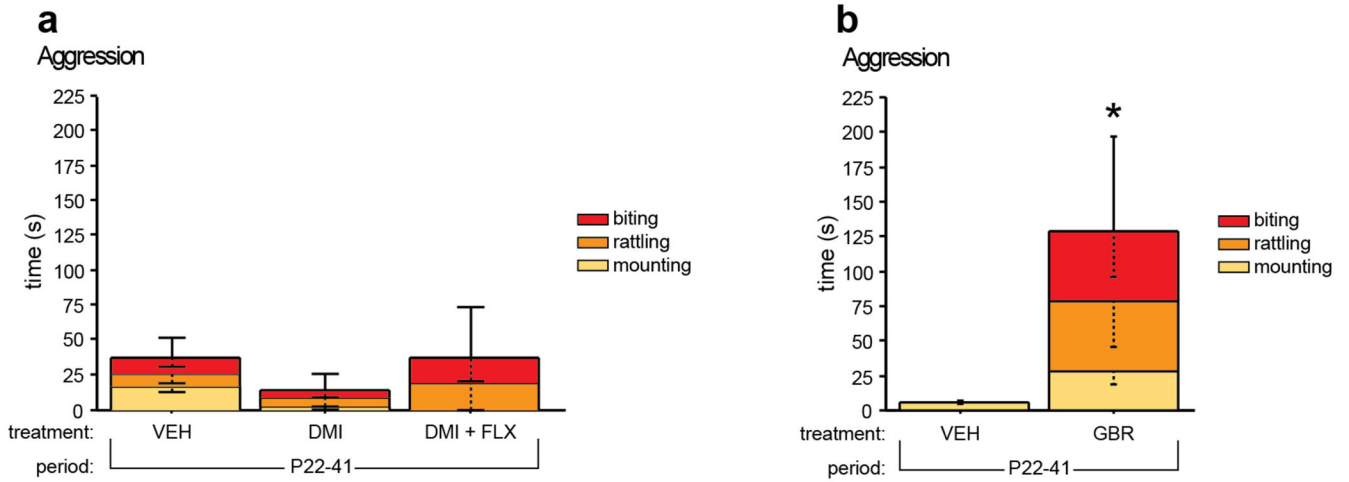


Fig. 4. Increased adult aggressive behavior after peri-adolescent DAT blockade

Isolation induced aggressive behavior was assessed in mice by scoring the time spent mounting, tail rattling, or biting during a 10 minute encounter (**a**, **b**). Aggressive behavior was not altered in mice treated with DMI or DMI+FLX from P22-P41 when compared to VEH treated control mice (**a**). Aggressive behavior was increased in mice treated with GBR from P22-P41 when compared to VEH treated control mice (**b**). (n = 5-15 per group) (*p < 0.05).

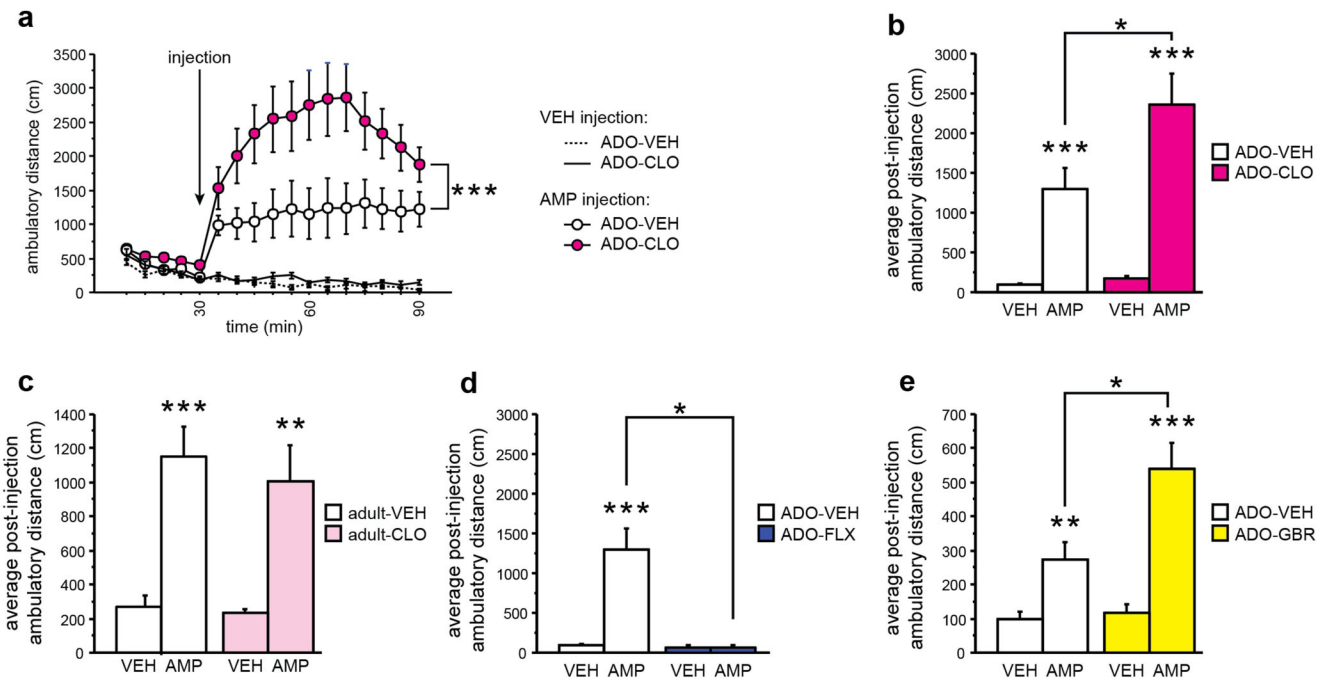


Fig. 5. Altered behavioral response to amphetamine in adulthood after peri-adolescent MAOA, 5-HTT, or DAT blockade

Behavioral amphetamine response was assessed using the open field. **(a)** Amphetamine injection (AMP, 3 mg/kg, i.p.) induced locomotor hyperactivity in ADO-VEH and ADO-CLO treated mice. Peri-adolescent CLO-treatment altered the response to AMP (indicated significance refers to peri-adolescent treatment x time interaction). **(b)** Post-injection activity demonstrates increased behavioral response to AMP in ADO-CLO when compared to ADO-VEH. **(c)** Transient adult CLO treatment did not impact behavioral response to AMP challenge. **(d)** ADO-FLX treatment abolished the behavioral response to AMP challenge. **(e)** ADO-GBR treatment increased the behavioral response to AMP challenge. (n = 5-15 per group) (*p < 0.05, ***p < 0.001).

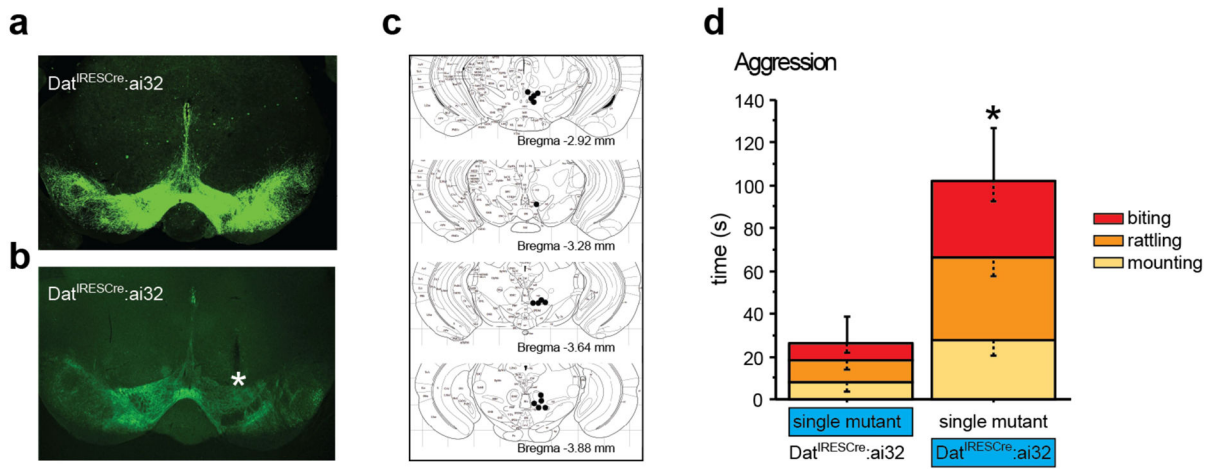


Fig. 6. Optogenetic stimulation of DAergic VTA neurons increases aggression

DAT^{IRESCre:ai32} double mutant mice expressing ChR2-eYFP in VTA neurons as visualized by immunohistochemistry against eYFP (a) and autofluorescence (b). A fiberoptic cable track demarks the position of an implant with its tip just dorsal of the VTA (indicated by a star, b). Tip locations were assessed histologically after behavioral experiments were concluded (c). *DAT^{IRESCre:ai32}* double transgenic mice and single mutant controls were co-housed in mixed pairs and isolation induced aggressive behavior was assessed by scoring the time spent mounting, tail rattling, or biting during a 10 minute encounter (d). Only one mouse in a pair was stimulated (blue). Aggressive behavior was increased in pairs when *DAT^{IRESCre:ai32}* double mutant mice were stimulated when compared to pairs where the single mutant controls were stimulated (d). (n = 11-18 per group) (*p < 0.05).

Table 1
Adult Monoamine and –metabolite levels after peri-adolescent MAOA, 5-HTT and DAT blockade

Tissue 5-HT, 5-HIAA, NE, DA, DOPAC, and HVA levels were measured by high performance liquid chromatography. (a) Consequences of ADO-CLO and ADO-FLX on adult monoamine and –metabolite levels. (b) Consequences of ADO-GBR on adult monoamine and –metabolite levels. (n = 5-7 mice per group; colored fields indicate differences compared to VEH with *p* at least < 0.05; red indicates a decrease and green indicates an increase when compared to VEH treatment).

		p180											
		VEH (N=5)		CLO (N=7)		FLX (N=6)		treatment		VEH vs. CLO		VEH vs. FLX	
		mean	SE	mean	SE	mean	SE	p %	change	p %	change	p	
Brainstem	5-HT (pmol/g)	8699	283	8250	355	9547	323	0.037	-5.2	0.362	9.7	0.107	
	5-HIAA (pmol/g)	9471	191	7553	414	8555	238	0.003	-20.3	0.001	-9.7	0.078	
	NE (pmol/g)	6754	1122	5355	868	6451	1122	0.492					
Striatum	DA (pmol/g)	16931	605	19217	460	17903	651	0.038	13.5	0.013	5.7	0.266	
	DOPAC (pmol/g)	2493	190	3206	229	2299	172	0.013	28.6	0.029	-7.8	0.534	
	HVA (pmol/g)	2957	174	3605	321	3026	199	0.174					

		P180				
		VEH (N=7)		GBR (N=6)		treatment
		mean	SE	mean	SE	p
Brainstem	5-HT (pmol/g)	9476	994	9150	1327	0.845
	5-HIAA (pmol/g)	7223	286	9124	1075	0.094
	NE (pmol/g)	3753	511	3671	508	0.913
Striatum	DA (pmol/g)	15626	373	15576	373	0.946
	DOPAC (pmol/g)	4051	327	4513	391	0.380
	HVA (pmol/g)	1555	31	1614	103	0.569