



## ARTICLE

# Medial orbitofrontal cortex and nucleus accumbens mediation in risk assessment behaviors in adolescents and adults

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Risk assessment behaviors are necessary for gathering risk information and guiding decision-making. Risky decision-making heightens during adolescence, possibly as a result of low risk awareness and an increase in sensitivity to reward-associated cues and experiences. Higher adolescent engagement in high-risk behaviors may be, in part, due to developing circuits that contribute to risk assessment behaviors. Nucleus accumbens (NAc) activity is linked to risky decision-making and receives inputs carrying sensory and emotional information. Namely, the medial orbitofrontal cortex (MO) contributes to behavior guided by reward probability and sends direct projections to the NAc (MO→NAc), which may permit risk assessment in a mature circuit. Here, we evaluated risk assessment behaviors in adult and adolescent rats during elevated plus maze (EPM) exploration, including stretch and attend postures, head dips, and rears. We found that adolescents exhibited fewer EPM risk assessment behaviors than adults. We also quantified MO→NAc projections using a fluorescent anterograde tracer, Fluoro-Ruby, in both age groups. Labeled MO→NAc pathways exhibited greater total fluorescence in adults than in adolescents, indicating MO→NAc fibers increase over development. Using a disconnection approach to measure the contribution of the MO-NAc pathway in adults, we found that ipsilateral inactivation of the MO-NAc did not alter risk assessment behavior; however, MO-NAc disconnection reduced the number of stretch-and-attend postures. Together, this work suggests that the development of MO-NAc pathways can contribute to age-dependent differences in risk assessment.

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## INTRODUCTION

Adolescence is a dynamic, transitional period defined by many behavioral changes, including riskier decision-making and risky behaviors relative to adulthood [1–6]. Taking risks can have positive or negative outcomes, but risky adolescent behaviors are associated with increased motor-vehicle accidents/fatality, substance abuse, and sexually-transmitted infections [7–10]. Neurodevelopmental theories suggest the adolescent prefrontal cortex (PFC) has less top-down control over reward-circuits than in adults, leading to risky decision-making and increased impulsivity [11–13]. However, low risk assessment and risk awareness may precede these risky adolescent behaviors.

Developing inputs from the medial orbitofrontal cortex (MO), a PFC division, into the nucleus accumbens (NAc) likely incorporate risk information. MO lesion and inactivation biases choices during decision-making tasks when risk-benefit probability or reward retrieval latency are manipulated [14–16]. Additionally, MO activity is responsive to cues that signal reward or reward value changes during decision-making tasks [17, 18]. These findings suggest the MO encodes and updates the value of options, factoring risk-benefits, to guide behavioral choices. The MO sends dense projections to the NAc [19], a structure linked to risky behavior [20–25]. The activity of MO neurons projecting to the NAc (MO→NAc) differs between in adults and adolescents [26],

suggesting functional shifts in MO→NAc interactions over development. In a series of financial risk or risk-taking tasks, NAc activation precedes risky choices [27–29]. Conversely, NAc inactivation decreases preference for large, risky rewards during probabilistic discounting tasks [21]. Given the links of MO and NAc to risk-related behaviors, developing MO-NAc interactions may contribute to risk assessment behaviors.

Risk assessment is the process of gathering information and is an initial step in decision-making. Risk assessment during novel environment has been characterized in rodents [30, 31], including orientating towards new environments, approaching and scanning, and retracting the body away from a targeted location [30]. However, little is known about rodent risk assessment behaviors during adolescence. Adolescents exhibit elevated risk-related behaviors in comparison to adults [2], which may be due to reduced risk evaluation. Differences in risk evaluation can be measured through changes to risk assessment behaviors on the elevated plus maze (EPM) [32–40]. Risk assessment behaviors include rearing, head-dipping, and stretch and attend postures (SAPs) [30]. Rearing is the vertical posture in which the majority of the rat's body weight is on its hind legs, often seen as movement against closed arms walls [30, 32, 33]. Head-dipping is the exploratory movement of the head and shoulders extended over the EPM open arms' edge [30, 32, 33]. SAPs are positions in which

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the torso stretches forward and retracts without forward locomotion [31, 33], and its frequency is sensitive to threatening stimuli [30] and anxiolytic or anxiogenic drugs [35, 41].

Here, we test the hypothesis that adults exhibit more EPM risk assessment behaviors than adolescent subjects. Additionally, we assess MO→NAC fiber density differences between adolescent and adult subjects. Last, we test if MO-NAC interactions contribute to adult risk assessment behaviors using a disconnection approach.

## MATERIALS AND METHODS

### Subjects

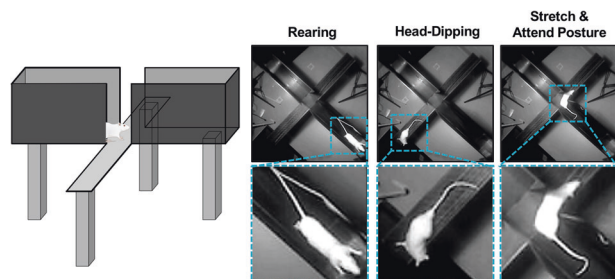
A total of 131 adult (post-natal day (PND) 70–90) and 35 adolescent (PND 30–40) male Sprague Dawley rats (Envigo, Indianapolis, IN) were group-housed and provided ad libitum access to food and water. PND 28–52 defines rat adolescence, which factors growth spurt timing, nest habitat emergence, sexual organ development, and peak social behavior/exploration that is homologous to human adolescent behaviors [42–46]. Subjects were habituated to the animal facility for one week before experimentation. Colonies were maintained on a twelve-hour reverse light cycle, with lights off at 8:00 A.M. Behavioral testing occurred only during the lights-off phase after 9:00 A.M. Experiments were conducted under the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by Rosalind Franklin University of Medicine and Science's Institutional Animal Care and Use Committee.

### Elevated plus maze apparatus

The EPM has been used as a context where rodents exhibit discrete risk assessment behaviors [32–40], which are independent of learned operant behaviors and sensitive to NAc manipulation [47]. The EPM (Scientific Design, Pikesville, MD) consisted of four arms: two open arms (width × length: 5" × 20") and two closed, walled arms (width × length × wall height: 5" × 20" × 18") (Fig. 1). All arms connect to a square center (width × length: 5" × 5") and are raised 32" above the ground (Fig. 1). At the beginning of each session, the subject was placed at the EPM's junction facing an open arm. Animal behavior was recorded in dim lighting (25–30 lux) for five-minutes with an IR-sensitive camera and analyzed on a personal computer.

### Behavioral scoring

EPM exploration videos were analyzed by video tracking software (AnyMaze, Stoelting, Wood Dale, IL). The software traced each subject's movement on the EPM and provided time and distance measurements. The time spent in the open arms was used as an index of anxiety-like behavior. The number of closed arm entries and distance run were used as indicators of locomotion. Latency to enter open arm was recorded as a supportive measure as it can reflect hesitancy to explore. Many subjects moved into the open arm upon placement and exhibited open arm entry latencies of 0 s. Videos were also scored by an experimenter, blinded to treatment, to count risk-assessment behaviors: rearing, head-dipping, and SAPs (Fig. 1) [30].



**Fig. 1 Model of the elevated plus maze and quantified risk assessment behaviors.** Left: The elevated plus maze (EPM) comprises two open, unprotected arms and two closed, walled arms raised 32" off the ground. The EPM was used to study age- and MO-NAC-dependent influences in risk assessment behaviors. Right: Images of scored risk-assessment behaviors during subjects' exploration of the EPM. Quantified risk-assessment behaviors include rearing, head-dipping, and stretch and attend postures (SAP).

### Surgeries

Adult subjects were anesthetized (2–4% isoflurane), body temperature was monitored via a rectal thermometer and held between 36–37 °C via heating pad (Kent Scientific Corporation, Torrington, CT). For cannulations, four burr holes were drilled in the skull to hold two stainless steel skull screws and two 26-gauge guide cannula (Plastics One Inc., Roanoke, VA). Coordinates were drawn from a flat skull from the intersection of Bregma and the sagittal suture (MO: Anterior/Posterior (A/P) + 4.2 mm, Medial/Lateral (M/L) + 0.4 mm, Dorsal/Ventral (D/V) – 4.2 mm from Bregma; NAc cannula angled at 10°: A/P + 1.4 mm, M/L + 1.5 mm, D/V – 7.0 mm from Bregma). MO and NAc guide cannula were implanted ipsilateral or contralateral to each other and counterbalanced between subjects. Cannulas were secured with cyanoacrylate glue (Gorilla Glue Co., Cincinnati, OH) and dental acrylic. Cannula dummies were inserted into guide cannulas and left until infusions were performed.

For anterograde tracer injections, adolescent (PND 29–31 on surgery date) and adult (PND 66–80 on surgery date) subjects received a unilateral MO infusion (Adult: A/P + 4.2 mm, M/L + 0.4 mm, D/V – 4.1 mm from Bregma; Adolescent: A/P + 4.1 mm, M/L + 0.2 mm, D/V – 4.0 mm from Bregma). Injections were counterbalanced between subjects though the majority of accurately placed injections were in the left MO ( $n = 7$ ). Anterograde fluorescent tracer, Fluoro-Ruby (Fluorochrome, LLC, Denver, CO), was infused using a 10  $\mu$ L Hamilton syringe with a 22-gauge needle (World Precision Instruments, Sarasota, FL) mounted onto a stereotaxic infusion pump (World Precision Instruments). 20 nL of 10% Fluoro-Ruby in ddH<sub>2</sub>O [48] was infused at a rate of 7.5 nL/minute and the needle was left in place for an additional 5-min for diffusion.

Meloxicam (1 mg/kg, subcutaneous injection) was administered as an analgesic during surgery and daily for two-days following surgery. Cannulated subjects were given a minimum of one-week of recovery time before habituation and EPM testing.

### Cannula infusion

Before testing, animals were habituated to the infusion procedure and environment three times over two-three days. Thirty-minutes before EPM exploration, 0.5  $\mu$ L of vehicle (artificial cerebral spinal fluid, aCSF (in mM): 148 NaCl, 3 KCl, 1 NaH<sub>2</sub>PO<sub>4</sub>, 22.2 D-glucose (dextrose), 0.8 MgCl<sub>2</sub>, 1.4 CaCl<sub>2</sub>, and 2.5 NaOH (Fisher Scientific, Pittsburgh, PA; Sigma-Aldrich, St. Louis, MO)) or a 1:1 mixture of GABA agonists (125 ng/site baclofen and muscimol, Sigma Aldrich; R&D Systems, Minneapolis, MN) in aCSF were infused through MO and NAc cannulas. Infusions were administered by 33-gauge injector cannula that protruded 0.5 mm past the guide cannula at 0.4  $\mu$ L/min via infusion pump (Harvard Apparatus, Holliston, Massachusetts). Injectors remained in the guide cannula an additional minute post-injection for diffusion.

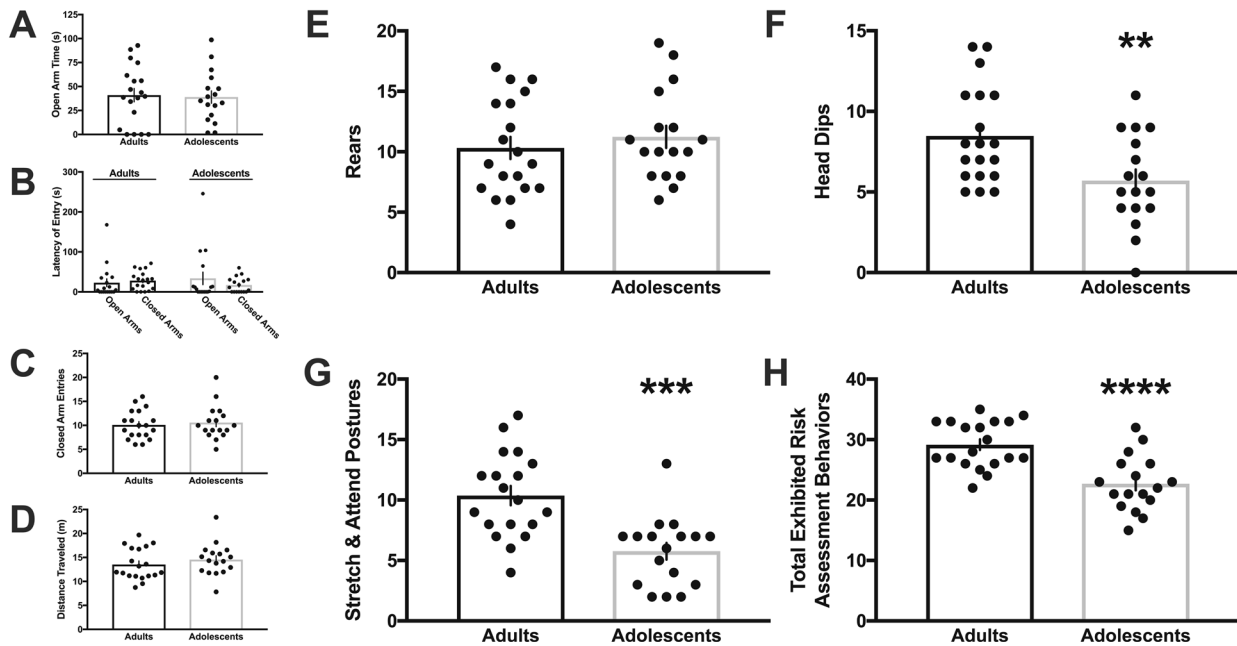
### Histology

Following EPM testing, cannulated subjects were euthanized in a carbon dioxide chamber. Alexa Fluor 594 (Fisher Scientific) was infused through the cannula using the same infusion methods. Brains were fixed in 4% paraformaldehyde (PFA) overnight. Fluoro-Ruby-injected subjects were anesthetized with isoflurane and perfused with 0.1 M phosphate buffer saline (PBS) followed by 4% PFA six-days post-operation, an optimal time period for transport to terminals [49].

Brains were switched into 0.1 M PBS and sliced into 40  $\mu$ m sections on a Leica VT1000 S vibratome (Leica Biosystems, Buffalo Grove, IL). Sections containing the MO, NAc, and/or cannula track marks were mounted onto charged slides and cover-slipped with Fluoroshield mounting medium with DAPI (Sigma-Aldrich). MO and NAc core anatomical boundaries were defined by a brain atlas [50]. Infusion sites were visualized by detecting Alexa Fluor 594/Fluoro-Ruby labeling and residual cannulas/injectors track marks. Cannulated subjects without both MO and NAc core placements ( $n = 60$ ) and Fluoro-Ruby-injected subjects sans MO injection (adults,  $n = 9$ ; adolescents,  $n = 14$ ) were excluded from analyses.

### Fluoro-ruby microscopy

Regions of interest (ROI) were captured on a Nikon Eclipse E600 microscope (Melville, NY, USA) using a 4x objective lens. For each subject, the MO Fluoro-Ruby injection and the ipsilateral NAc core at three coronal planes (Rostral: A/P + 2.08 mm – + 2.16 mm; Mid-NAc: A/P + 1.56 mm – + 1.68 mm; Caudal: A/P + 0.84 mm – + 1.08 mm) were captured. A z series stack of images was acquired for each section using 4  $\mu$ m step intervals for five consecutive focal planes using a linear LUT. Using Fiji software (Madison, WI), z series images



**Fig. 2 Adolescents exhibited fewer elevated plus maze risk assessment behaviors than adults.** **A–D** Adults (PND 70–90) and adolescents (PND 30–40) do not exhibit significant differences in the amount of time spent in the open arms, the amount of time to explore closed or open arms, closed arm entries, and distance traveled. **E–G** Adults and adolescents do not exhibit significant differences in rearing frequency during EPM exploration. **F–G** Adolescents exhibit significantly less head-dips and SAPs during EPM exploration. **H** Adolescents exhibit significantly less combined risk-assessment behaviors during EPM exploration. \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ , unpaired  $t$  test (adults,  $n = 19$ ; adolescents,  $n = 17$ ).

were stacked to represent fluorescent intensity. The NAc core and background were outlined with the freehand ROI tool and processed through Fiji preset measurements to obtain integrated density, area, and mean gray value. Total fluorescence value of the NAc core was calculated using the formula: Corrected Total Fluorescence<sub>NAc</sub> = Integrated Density<sub>NAc</sub> – (Area<sub>NAc</sub> × Mean Gray Value<sub>Background</sub>). The sum of the three corrected total fluorescence values was quantified to represent the presence MO→NAc fibers in each subject. Corrected total fluorescence of each coronal section was compared between age groups to assess rostral/caudal differences in MO→NAc fiber presence.

### Data analysis & statistics

We analyzed our results with PRISM statistical software (GraphPad, La Jolla, CA) or SPSS (v28, IBM, Armonk, NY) and expressed data as the group mean  $\pm$  SEM. A value of  $p < 0.05$  was used to determine statistical significance. To compare age groups, we used an unpaired  $t$  test. In disconnection experiments, we used a one-way analysis of variance (ANOVA) for between-group comparisons. The post hoc Tukey's multiple comparisons test was used when appropriate. In the Fluoro-Ruby injection study, we used a two-way RM ANOVA and the post hoc Holm's-Sidak multiple comparisons test to evaluate age-dependent differences across NAc coronal sections.

## RESULTS

### Adolescent rats exhibit less EPM risk assessment behaviors than adults

We hypothesized that adults would exhibit more risk assessment behaviors than adolescents. Adolescent and adult rats were allowed to explore the EPM, and the number of risk assessment behaviors during the five-minute exploration was measured (Fig. 1).

The total time spent in the open arms or latency to enter open or closed arms between age groups were comparable, indicating similar baseline exploration (Fig. 2A; open arm time: adults  $41.0 \pm 30.6$  s,  $n = 19$ ; adolescents  $39.0 \pm 26.6$  s,  $n = 17$ ,  $t(34) = 0.21$ ,  $p > 0.05$ , unpaired  $t$  test; Fig. 2B; latency to enter open arms: adults  $23.0 \pm 41.8$  s,  $n = 19$ ; adolescents  $33.9 \pm 64.9$  s,  $n = 17$ ,  $t(33) = 0.60$ ,

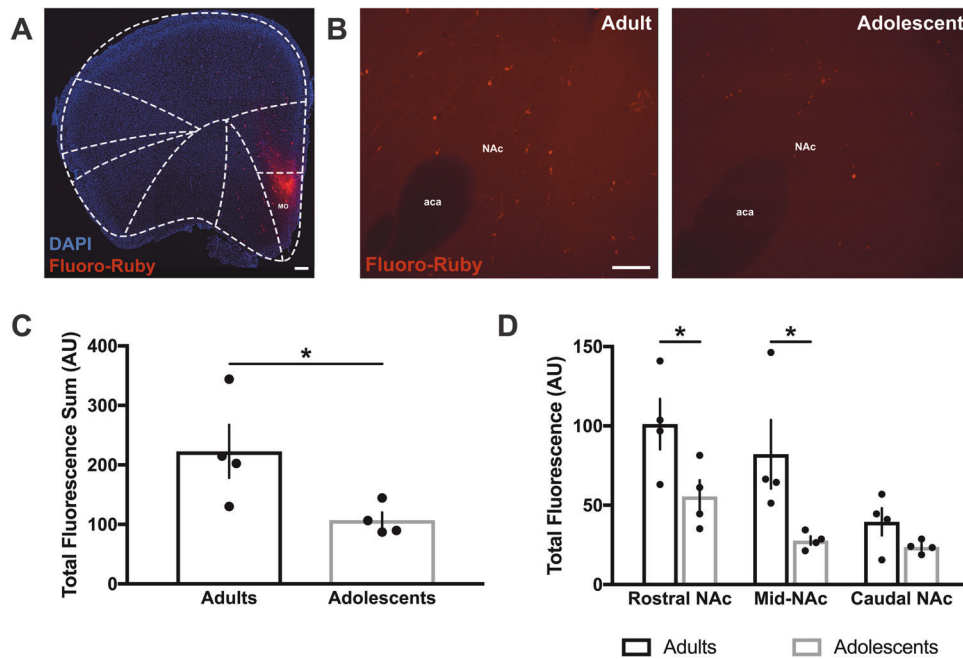
$p > 0.05$ , unpaired  $t$  test; latency to enter closed arms: adults  $28.1 \pm 23.2$  s,  $n = 19$ ; adolescents  $17.2 \pm 19.3$  s,  $n = 17$ ,  $t(34) = 1.53$ ,  $p > 0.05$ , unpaired  $t$  test). We also did not find any age-dependent differences in closed arm entries or distance traveled between groups, suggesting there were no locomotor differences between groups (Fig. 2C; closed arm entries: adults  $10.1 \pm 3.0$ ,  $n = 19$ ; adolescents  $10.6 \pm 3.5$ ,  $n = 17$ ,  $t(34) = 0.44$ ,  $p > 0.05$ ; Fig. 2D; distance traveled: adults  $13.5 \pm 0.7$  m,  $n = 19$ ; adolescents  $14.6 \pm 0.8$  m,  $n = 17$ ,  $t(34) = 0.93$ ,  $p > 0.05$ , unpaired  $t$  test).

Adolescents and adults did not exhibit differences in rear frequency (Fig. 2E; rears: adults  $10.3 \pm 4.0$ ,  $n = 19$ ; adolescents  $11.2 \pm 3.8$ ,  $n = 17$ ,  $t(34) = 0.71$ ,  $p > 0.05$ , unpaired  $t$  test). Adolescents showed less head dipping and SAPs than adults (Fig. 2F; head dips: adults  $8.5 \pm 3.0$ ,  $n = 19$ ; adolescents  $5.7 \pm 2.9$ ,  $n = 17$ ,  $t(34) = 2.82$ ,  $p < 0.01$ , unpaired  $t$  test; Fig. 2G; SAPs: adults  $10.4 \pm 3.5$ ,  $n = 19$ ; adolescents  $5.8 \pm 2.9$ ,  $n = 17$ ,  $t(34) = 4.28$ ,  $p < 0.005$ , unpaired  $t$  test). Overall, adolescent rats exhibited fewer EPM-related risk assessment behaviors than adults (Fig. 2H; total exhibited risk assessment behaviors: adults  $29.2 \pm 3.9$ ,  $n = 19$ ; adolescents  $22.7 \pm 4.6$ ,  $n = 17$ ,  $t(34) = 4.59$ ,  $p < 0.0001$ , unpaired  $t$  test).

### MO-NAc projections increase from adolescence to adulthood

Fluoro-Ruby was injected into the MO (Fig. 3A) of adolescent and adult subjects to visualize descending fibers and axon terminals. Fluorescent indicators were found in the NAc core (Fig. 3B). All analyzed rats had similar sized injection sites with the majority of the injection restricted within MO anatomical boundaries.

Fibers from MO Fluoro-Ruby injections were quantified in the NAc core at three rostral/caudal planes. The sum of total fluorescence from three coronal planes in the NAc was greater in adults than in adolescents (Fig. 3C; Total Fluorescence Sum: adults  $251.4 \pm 46.8$ ,  $n = 4$ ; adolescents  $126.6 \pm 10.7$ ,  $n = 4$ ,  $t(6) = 2.60$ ,  $p < 0.05$ , unpaired  $t$  test; Fig. 3D; main effect of age:  $p < 0.05$ ,  $F(1, 6) = 6.76$ , Two-Way RM ANOVA). Total fluorescence of MO Fluoro-Ruby indicators in the NAc was also dependent on the rostral/caudal plane of the NAc (Fig. 3D; main effect of rostral/



**Fig. 3** Density of MO projections terminating in the NAc is greater in adults than adolescents. **A** The anterograde tracer, Fluoro-Ruby (red), was infused into the MO of subjects and counterstained with DAPI (blue) (MO, medial orbitofrontal cortex). **B** Anterograde labels from MO Fluoro-Ruby injections in the ipsilateral NAc core in adult (PND 70–90) and adolescent (PND 30–40) subjects (aca anterior commissure, NAc nucleus accumbens). **C** The summation of NAc total fluorescence from MO Fluoro-Ruby injections of all three NAc rostral/caudal regions is greater in adults than in adolescents. **D** NAc total fluorescence from MO Fluoro-Ruby injections is greater in adults than in adolescents in rostral NAc and mid-NAc sections. Scale bars, 10  $\mu$ m. \* $p < 0.05$ , unpaired *t* test; two-way RM ANOVA, Holm-Sidak's multiple comparisons test (adults,  $n = 4$ ; adolescents,  $n = 4$ ).

caudal plane:  $p < 0.0001$ ,  $F(2, 12) = 22.61$ ; age  $\times$  rostral/caudal plane:  $p < 0.05$ ,  $F(2, 21) = 4.23$ , Two-Way RM ANOVA). Specifically, total NAc fluorescence in rostral NAc and mid-NAc sections was greater in adults than in adolescents (Fig. 3D; Rostral NAc,  $p < 0.05$ ; Mid-NAc,  $p < 0.05$ ; Caudal NAc,  $p > 0.05$ , post hoc Holm-Sidak's multiple comparisons test).

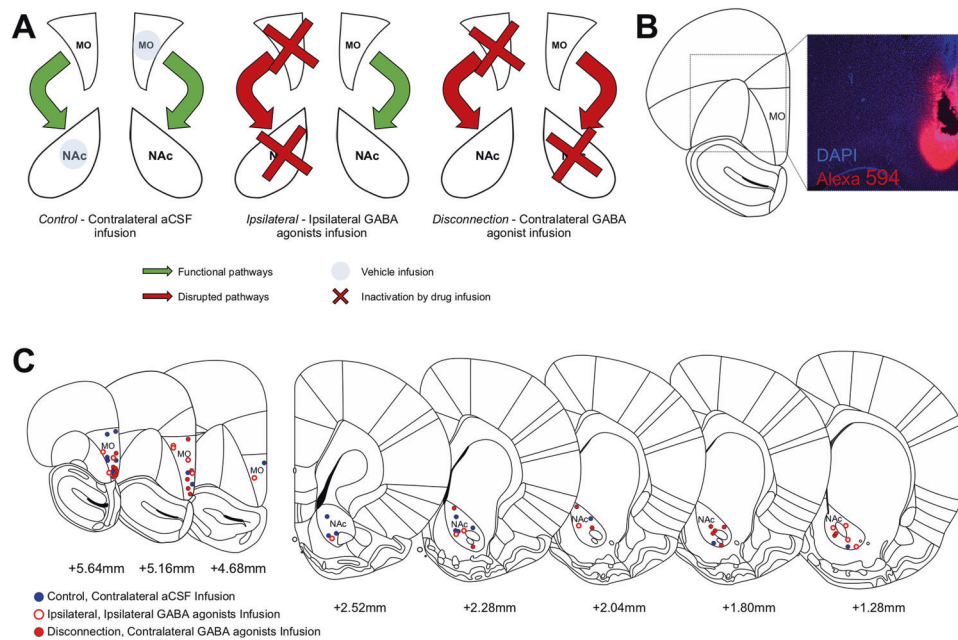
#### MO-NAc disconnection decreases stretch and attend postures

We used a disconnection approach to understand the contributions of MO-NAc interactions in risk assessment behaviors during EPM exploration. Three groups were evaluated: Control (contralateral vehicle infusion), Ipsilateral (ipsilateral drug infusion), and Disconnection (contralateral drug infusion) (Fig. 4). Since the presence of MO→NAc fibers was significantly greater in adults relative to adolescents (Fig. 3), we analyzed the effect of MO-NAc disconnection in adult subjects.

We predicted that specific risk assessment behaviors would be limited to distinct zones (e.g., head-dipping is primarily performed in the open arms). Consistent with this, and regardless of treatment, subjects that spent more time in the open arms exhibited less rears, more head dips and less SAPs (Supplementary Fig. 1A–C; Rears to Open Arm Time:  $p < 0.0001$ ; Head Dips to Open Arm Time:  $p < 0.05$ ; SAPs to Open Arm Time:  $p < 0.005$ , two-tailed Pearson's *r*). Open arm time was also negatively correlated to the total number of risk assessment behaviors exhibited by subjects (Supplementary Fig. 1D; Total Exhibited Risk Assessment Behaviors to Open Arm Time:  $p < 0.005$ , two-tailed Pearson's *r*). Although no significant differences were found in open arm time, open or closed arm entry latency, closed arm entries, or distance traveled between treatment cohorts (Supplementary Fig. 1E–L; control,  $n = 12$ ; ipsilateral,  $n = 9$ ; disconnection,  $n = 18$ ), there were subjects that displayed very low open arm time, which can be problematic as low open arm time reduces head-dipping and increases rearing opportunity. This could lead to misinterpretation.

To ensure comparisons were not biased due to differences in arm exploration (or differences in the opportunity to demonstrate specific risk assessment behaviors), we established inclusion criteria to compare risk assessment behaviors made across groups with similar explorative drive. We selected one standard deviation from the control group open arm time average as a threshold (open time average (s) – standard deviation (s)). To be included in the analysis, subjects must have spent over 45-seconds in the open arms. Subjects that did not meet this threshold were omitted (control,  $n = 2$ ; ipsilateral,  $n = 1$ ; disconnection,  $n = 7$ ; see Supplementary Fig. 1 for full data set). Excluded animals across control and disconnection groups had similar risk assessment behaviors, indicating that bias was not introduced by exclusion criteria (rears: control + ipsilateral  $15.0 \pm 2.0$ ,  $n = 3$ , disconnection  $13.1 \pm 2.1$ ,  $n = 7$ ; head dips: control + ipsilateral  $1.7 \pm 0.3$ , disconnection  $3.9 \pm 1.2$ ; SAPs: control + ipsilateral  $12.3 \pm 2.7$ , disconnection  $7.1 \pm 1.2$ ; total exhibited risk assessment: control + ipsilateral  $29.0 \pm 4.5$ , disconnection  $24.1 \pm 2.3$ ).

Upon criteria application, we observed similar exploration levels between treatment groups. There was no significant difference in the total time spent in the open arms or latency to enter open or closed arms between treatment groups (Fig. 5A; open arm time: control  $107.3 \pm 9.6$  s,  $n = 10$ ; ipsilateral  $109.6 \pm 9.3$  s,  $n = 8$ ; disconnection  $112.0 \pm 19.7$  s,  $n = 11$ ;  $F(2, 26) = 0.03$ ,  $p > 0.05$ ; Fig. 5B; latency to enter open arms: control  $0.8 \pm 0.4$  s,  $n = 10$ ; ipsilateral  $4.0 \pm 4.0$  s,  $n = 8$ ; disconnection  $1.8 \pm 0.9$  s,  $n = 11$ ;  $F(2, 26) = 0.77$ ,  $p > 0.05$ ; latency to enter closed arms: control  $40.5 \pm 8.4$  s,  $n = 10$ ; ipsilateral  $44.5 \pm 12.9$  s,  $n = 8$ ; disconnection  $59.3 \pm 18.0$  s,  $n = 11$ ;  $F(2, 26) = 0.52$ ,  $p > 0.05$ , one-way ANOVA). Additionally, there was no difference in closed arm entries or distance traveled between groups, suggesting similar locomotion (Fig. 5C; closed arm entries: control  $14.4 \pm 1.8$ ,  $n = 10$ ; ipsilateral  $14.5 \pm 2.0$ ,  $n = 8$ ; disconnection  $10.9 \pm 2.0$ ,  $n = 11$ ;  $F(2, 26) = 1.18$ ,  $p > 0.05$ ; Fig. 5D; distance traveled: control  $13.3 \pm 1.9$  m,  $n = 10$ ; ipsilateral  $15.9 \pm 1.8$  m,  $n = 8$ ;



**Fig. 4** Disconnection approach for the inactivation of MO-NAc interactions. **A** The MO and NAc were infused contralaterally with artificial cerebral spinal fluid (aCSF, *Control*), ipsilaterally with baclofen and muscimol (125 ng/site) (*Ipsilateral*), or contralaterally with baclofen and muscimol (*Disconnection*). **B** Alexa Fluor 594 (red) was infused through cannulae post mortem, and tissue sections were counterstained with Fluoroshield mounting media with DAPI (blue). **C** The implanted cannula locations in the MO and NAc were verified based on the post-experimental histological reconstruction. Each infusion type is indicated by color: control, contralateral aCSF infusion (blue), ipsilateral, ipsilateral GABA agonists infusion (white, red border), and disconnection, contralateral GABA agonists infusion (red). Reconstructed images do not represent the specific hemisphere where cannulae were implanted.

disconnection  $10.2 \pm 1.6$  m,  $n = 11$ ;  $F(2, 26) = 0.01$ ,  $p > 0.05$ , one-way ANOVA).

Disconnection of MO-NAc interactions did not have a significant effect on the total number of risk assessment behaviors (Fig. 5E; total number of risk assessment behaviors: control  $22.0 \pm 2.7$ ,  $n = 10$ ; ipsilateral  $21.9 \pm 3.0$ ,  $n = 8$ ; disconnection  $16.6 \pm 2.2$ ,  $n = 11$ ;  $F(2, 26) = 1.50$ ,  $p > 0.05$ , one-way ANOVA). When considered by risk assessment behavior type, ipsilateral or contralateral MO-NAc inactivation did not affect rearing or head dipping frequency (Fig. 5F; rears: control  $8.3 \pm 1.7$ ,  $n = 10$ ; ipsilateral  $9.8 \pm 2.1$ ,  $n = 8$ ; disconnection  $6.6 \pm 1.2$ ,  $n = 11$ ;  $F(2, 26) = 0.94$ ,  $p > 0.05$ ; Fig. 5G; head dips: control  $6.6 \pm 1.3$ ,  $n = 10$ ; ipsilateral  $8.1 \pm 1.0$ ,  $n = 8$ ; disconnection  $6.9 \pm 1.6$ ,  $n = 11$ ;  $F(2, 26) = 3.13$ ,  $p > 0.05$ , one-way ANOVA). However, MO-NAc disconnection significantly altered the number of exhibited SAPs (Fig. 5H; SAPs: control  $7.1 \pm 1.4$ ,  $n = 10$ ; ipsilateral  $4.0 \pm 0.9$ ,  $n = 8$ ; disconnection  $3.1 \pm 0.7$ ,  $n = 11$ ;  $F(2, 26) = 4.18$ ,  $p < 0.05$ , one-way ANOVA). Specifically, MO-NAc disconnection reduced the number of SAPs relative to the aCSF control group (Control vs. Disconnection:  $p < 0.05$ ,  $q = 3.96$ , post hoc Tukey's multiple comparisons test). While ipsilateral inactivation groups showed fewer SAPs than controls, there was not a statistical difference between these groups (Control vs. Ipsilateral:  $p = 0.13$ ,  $q = 2.81$ , post hoc Tukey's multiple comparisons test) or between ipsilateral inactivation and disconnection groups (Ipsilateral vs. Disconnection:  $p = 0.82$ ,  $q = 0.84$ , post hoc Tukey's multiple comparisons test).

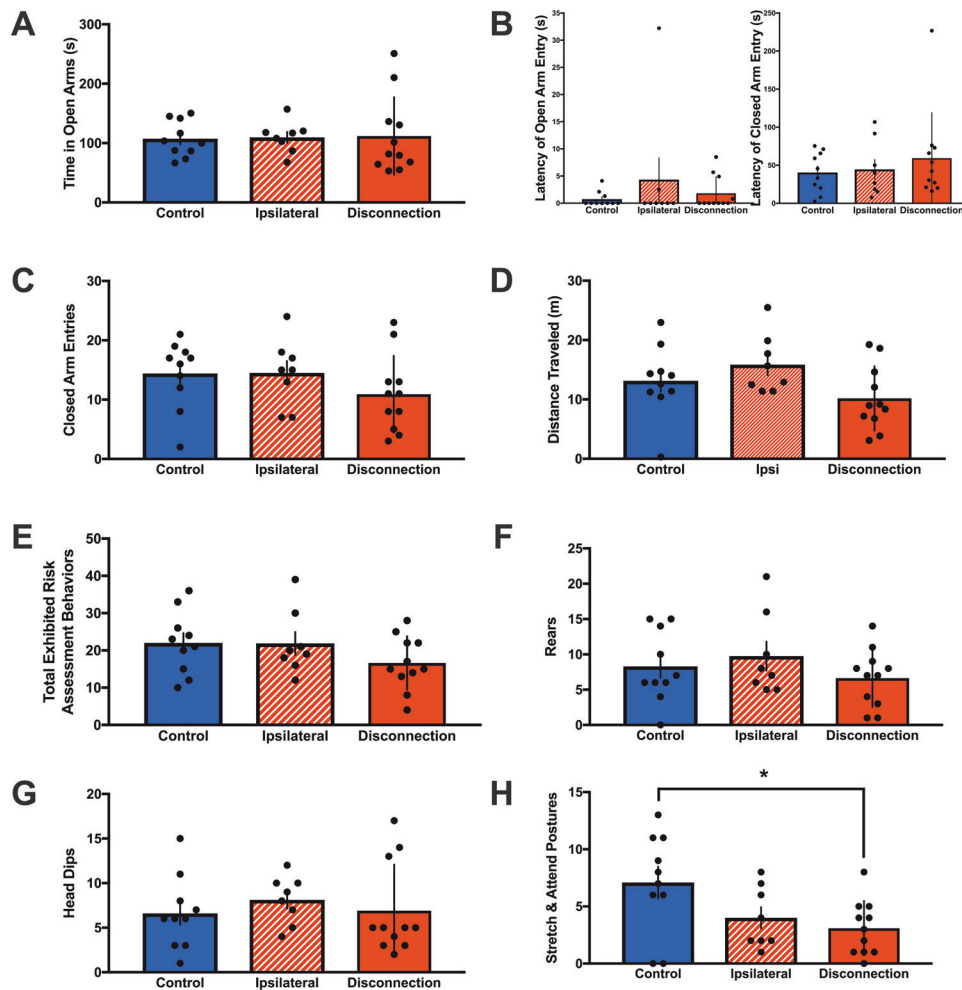
Exclusion of data points based on lack of exploration in open arms was the most direct way to control for potential artifactual effects caused by reduced opportunity to display risk assessment behaviors. We also tested whether group differences would still emerge if open arm exploration is controlled. With open arm time used as a co-variate, there was still a significant effect of disconnection on SAPs ( $F(2, 39) = 4.03$ ,  $p = 0.027$ , ANCOVA, Control vs. Disconnection:  $p < 0.05$ ,  $q = 3.97$ ), but not head-dips ( $F(2, 39) = 0.37$ ,  $p > 0.05$ , ANCOVA) or rearing ( $F(2, 39) = 1.24$ ,  $p > 0.05$ , ANCOVA).

## DISCUSSION

Prior work has focused on adolescent risk-taking behaviors that taper into adulthood. However, studies have not addressed if risky behaviors during adolescence is a product of low risk assessment. Our findings support the hypothesis that adolescents exhibit fewer risk assessment behaviors than adults during exploration of the same novel environment. Although EPM exploration was similar between age groups, adolescents exhibited less head dipping and SAPs than adults. Interestingly, rearing was comparable between ages. Rearing is nearly exclusively exhibited in the closed arms, which restricts exploratory information to inside the walls of the EPM. The similar frequency of rearing between age groups may reflect the less risky nature of closed arm exploration relative to open arm exploration.

The EPM has been validated to quantify risk assessment and anxiety-like behaviors [32–40, 51, 52]. A decrease in open arm activity (duration or entry) is interpreted as a heightened anxiety-like state [53–55], and open arm activity is sensitive to rodent stressors [56–58]. Additionally, risk assessment behaviors are influenced by anxiety states, where acute stressors before exploration can increase the occurrence of risk assessment behaviors [30, 36], which are also sensitive to anxiolytics or substances of abuse [35, 36, 59, 60]. We did not find a difference in the time occupied in the open arms between age groups (Fig. 2A) but still found differences in risk assessment; thus, the results of age-dependent differences in head-dipping and SAPs between age groups were likely not confounded by anxiety-like behaviors.

While SAPs can reflect risk assessment, an argument can be made that open arm exploration reflects risky behavior. Open arm time and latency of open/close arm entry were comparable between age groups. However, it's unclear if this is due to similar risk behaviors, that risk is assessed and determined to be low, or because exploration in the open arm isn't a pure measure of risk behavior, and reflects other factors, including a balance between caution and novelty-seeking. Future studies could analyze the serial order of behaviors to test if adolescents/adults enter the



**Fig. 5 Disconnection of MO-NAc interactions reduces stretch and attend posture exhibition.** **A–D** No significant differences were found in the amount of time spent in the open arms, latency to enter either open or closed arms, the number of closed arm entries, and distance traveled between cohorts. **E–G** No significant differences were found in combined risk assessment behaviors exhibited during EPM exploration between cohorts. **H** Disconnected subjects exhibited less stretch & attend postures during EPM exploration than control subjects. \* $p < 0.05$ , one-way ANOVA (Control,  $n = 10$ ; Ipsilateral,  $n = 8$ ; Disconnection,  $n = 11$ ).

open or closed arms after assessing risk, and if risk information causes further exploration in a riskier zone or retreat from the open arms.

Outputs from the MO and other PFC regions are not fully developed before adolescence [61, 62], and may contribute to age-related behavioral differences. Inputs from other PFC regions to NAc strengthen from adolescence to adulthood [63]. As NAc activity is linked to risky decision-making [21, 27], one potential mechanism underlying increased risk assessment from adolescence to adulthood is the development of MO-NAc pathways. Our findings led to a refinement of the initial hypothesis, and suggest that MO-NAc interactions permit specific risk assessment behaviors, SAPs. MO-NAc fibers increased from adolescence to adulthood (Fig. 3C), which parallels the increase of MO-NAc driven SAPs. However, there may be other contributing factors. Other brain regions also develop during this time period, some of which are involved with responses to threat (e.g., cortico-amygdala circuits) [64–66]. It's also possible that adolescent rats assess risk as much as adults, but use a behavior that has thus far been unidentified.

MO-NAc disconnection decreased SAP frequency but did not affect head-dip or rear frequency (Fig. 5). It's unclear why SAPs were more sensitive to MO-NAc disconnection. SAPs engage

forward locomotive posture and are exhibited on all EPM arms. Conversely, rearing is unlikely to provide significant information about open areas due to height of closed arms, and head-dipping is only performed when already in open arms. The decrease in SAP is unlikely driven by an impairment to stretch because rearing often requires stretching of the body up the wall. Although head-dipping and rearing behaviors have been characterized as risk assessment poses, they were unaffected by MO-NAc disconnection (Fig. 5) and are instead sensitive to manipulations of other neural regions. Superior colliculus (SC) lesion decreased rearing in open fields [67–69], and decreased head-dips through a hole-board [70], as did lesion of hippocampus regions [71, 72]. Phasic activity of the ventral hippocampus CA1 region is observed during EPM head-dipping in mice [73] and open field rearing increases high-frequency theta power in multiple hippocampal subregions [74], which are an important element for organization of spatial maps. Thus, head-dipping and rearing also serve as exploratory behaviors to gain spatial information. Overall, this suggests a specific functional purpose of SAPs and reliance on MO-NAc, while other brain regions (e.g., hippocampus and SC) may be critical for other risk-assessment behaviors.

The specific effect of MO-NAc disconnection on SAPs complement NAc's role in forward approach movement [75]. NAc core

lesions do not abolish locomotion but do reduce approach to reward-predictive stimuli [76], suggesting MO-NAc inactivation diminishes forward-directed risk assessment without impairing general locomotion. Additionally, treatment groups had comparable closed arm entries and distance traveled, countering the possibility that a reduction in SAPs is due to reduced locomotive ability.

Interestingly, ipsilateral inactivation of MO-NAc pathways trended towards SAP reduction during EPM exploration but was not significant (Control vs. Ipsilateral:  $p = 0.13$ ,  $q = 2.18$ , post hoc Tukey's multiple comparisons test). Disconnection approaches have historically included ipsilateral inactivation treatment groups to demonstrate that unilateral inactivation of bilateral structures would not disrupt behavior, as the contralateral non-manipulated circuit should partially compensate for the unilateral disruption. However, the trend toward decrease of SAPs upon ipsilateral MO-NAc inactivation was not surprising as the MO sends both ipsilateral and contralateral descending projections into the NAc [19]. Ipsilateral inactivation may be sufficient to partially reduce SAP frequency. Additionally, NAc cannula severs some corpus callosum fibers, which permit communication between hemispheres. However, ipsilateral infusions only weakly effects, while disconnection of MO-NAc pathways by contralateral inactivation significantly reduced SAPs during EPM exploration.

## CONCLUSIONS

Our findings demonstrate the contribution of the MO-NAc pathway to select risk assessment behaviors. Adults exhibit more risk assessment behaviors than adolescents and have more MO→NAc fibers. Additionally, MO-NAc disconnection reduces SAPs in adults. These findings represent the next step in understanding the contribution of MO-NAc pathways to the larger, developing circuits underlying the acquisition of risk information. Immaturity of the MO-NAc pathway may contribute to low risk assessment and risk awareness in adolescents. The absence of risk information can skew decision-making choices, where reward value and immediacy are prioritized, and produce impulsive, risky behaviors. Future work is still needed to determine if differences in risk assessment contribute to age differences in risky behaviors.

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## AUTHOR CONTRIBUTIONS

Conceptualization: MKL and JAR; Methodology: MKL, NCF, and JAR; Data Curation: MKL and JMT; Validation: MKL; Analysis: MKL; Resources: JAR; Original Draft preparation: MKL; Draft Revisions & Edits: MKL, NCF, and JAR; Funding Acquisition: JAR.

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## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

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