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Prenatal broad-spectrum cannabidiol administration prevents an autism-like phenotype in male offspring from a maternal stress/terbutaline rat model

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ABSTRACT

Recently, the diagnosis of autism spectrum disorder (ASD) has increased from 1 in 150 to every 1 in 36 children in the United States, warranting a need for novel prevention and therapeutic strategies. Broad-spectrum cannabidiol oil, free from delta-9-tetrahydrocannabinol, the psychoactive component of cannabis, may be one such therapeutic. It has a high safety profile and is frequently used as a complementary and integrative intervention by persons experiencing symptoms of anxiety, stress, and inflammation. Using a neurodevelopmental rat model of ASD (based on neuroinflammation induced by stress and terbutaline exposure during pre- and postnatal development), we sought to prevent the development of ASD-like behaviors in male offspring by administering broad-spectrum cannabidiol oil to dams throughout pregnancy (10 mg/kg, i.p., daily, embryonic days 3–16). To assess an ASD-like phenotype in the offspring, we used three behavioral measures relevant to three core ASD symptoms: 1) social communication (time spent vocalizing when alone); 2) repetitive behavior (marbles buried during a marble burying test); and 3) social interaction (time spent interacting with a novel conspecific during the three-chamber social interaction test). Broad-spectrum cannabidiol oil given during pregnancy decreased scores for all three ASD-related behavioral responses, resulting in an overall significant prevention of the ASD-like phenotype. These findings highlight the potential of broad-spectrum cannabidiol oil as a complementary and integrative approach for prevention of stressor-induced sequelae relevant to development of an ASD-like phenotype.

1. Introduction

Autism spectrum disorder (ASD) has risen in prevalence from 2000 to 2020 in the U.S. (Zablotsky et al., 2019; Maenner et al., 2023). This increase in prevalence highlights a need for prevention strategies (Insel and Scolnick, 2006). A suspected causal component of ASD is the pattern of cytokines expressed by maternal immune cells during pregnancy (Meyer et al., 2006, 2008, 2011). Recently, a paradigm shift in thought concerning stress and inflammation has spurred the development of environmental rodent models of ASD (Bronson et al., 2014; Bercum et al., 2015; Fonken et al., 2018; Frank et al., 2020; Smith et al., 2020). Indeed, within our rat developmental model of ASD, which utilizes maternal stress and terbutaline administration in early postnatal development of the offspring (terbutaline is a tocolytic β2 adrenergic receptor agonist known to be an environmental risk factor for ASD in humans and ASD-like behaviors in rats), an increase in neuroinflammatory markers have been observed (Bercum et al., 2015; Smith et al., 2020). Additionally, rat offspring in this model display behavioral deficits in social interaction, repetitive behaviors, and social communication, all of which are hallmark characteristics of ASD (American Psychiatric Association, 2013; Bercum et al., 2015; Smith et al., 2020).

Broad-spectrum cannabidiol (CBD) hemp extract oil may be an

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effective intervention for preventing neuroinflammation and/or cytokine imbalances in maternal, placental, and fetal tissue. Cannibidiol acts as an agonist at cannabinoid receptor 2 on microglia, which results in microglial cells expressing anti-inflammatory molecules (Komorowska-Muller and Schmole, 2020). Additionally, CBD is lipophilic and capable of crossing the placenta (Chayasirisobhon, 2020), which is important because maternal cytokines are capable of crossing the placental barrier as well. Here, we conducted a brief investigation of CBD's therapeutic potential as a preventative intervention in our rat developmental model of ASD by assessing behavioral outcomes in a small cohort of male rats. We hypothesized that daily administration of CBD given to dams during pregnancy would prevent the development of ASD-like behaviors in male offspring.

2. Materials and methods

On E1 (embryonic day 1), timed mated Sprague Dawley dams were shipped and arrived on E3 (Fig. 1, $N = 12$; ENVIGO (now Inotiv), Indianapolis, IN, USA). Dams were randomly assigned to one of four groups: No-Stress/No-Terbutaline + Vehicle (No ST/Veh), No-Stress/No-Terbutaline + CBD (No ST/CBD), Stress/Terbutaline + Vehicle (ST/Veh), Stress/Terbutaline + CBD (ST/CBD) ($n = 3$ per group). Dams were given diamond twists (Teklad 7979C.CS Certified/Irradiated, Cat. No. NC1738670, ENVIGO) for environmental enrichment and were single housed. Food and water were provided *ad libitum* (20 ± 1 °C; 12 h light/dark cycle, 0700–1900). All procedures were approved by the University of Colorado Boulder Institutional Animal Care and Use Committee and were conducted in an AAALAC-accredited facility.

2.1. Cannabidiol administration

Dams received CBD daily from E3 – E16. CBD was administered (1000 h) 1 h prior to the shock stress regimen. CBD solutions were made fresh immediately before injection (dissolved in 2% Tween 80 in sterile saline, 10 mg/kg, i.p.; 100 mg/mL CBD, organic MCT oil, CO_2 extracted, batch #CB20219, Colorado Botanicals, Denver, CO, USA). Supplemental Fig. 1 contains the certificate of analysis performed by Botanacor Laboratories™ (Denver, CO, USA).

2.2. Maternal stress shock regimen

Dams began the maternal stress shock regimen on E3 (see Smith et al., 2020 for details). Briefly, the regimen consisted of an initial habituation day (E3) followed by a shock day (E4; two 1 mA foot shocks, 1 s duration, 60 s interval; Modular Shock Floor Model H10-11R/M-TC-SF and H13-15 Precision-Regulated Shocker, Coulbourn Instruments, Allentown, PA, USA) and two context days (E5 and E6). This schedule (shock-context-context) was repeated from E7 - E20 (E10 and E17 were rest days). This procedure was conducted between the hours 1100–1300.

2.3. Cross-fostering

Dams and their litters were left undisturbed from E21 through P1 (postnatal day 1). On P2, pups were counted, females were culled ($n = 59$), and males were cross-fostered to dams within their treatment groups ($N = 36$). Only male pups were examined in this study due to the prevalence of ASD among boys being 3.8 times higher than for girls (Maenner et al., 2023). No dam fostered more than six pups.

2.4. Limited bedding

On P2 – P7, dams and litters assigned to the stress condition were housed in custom cages (25.4 cm diameter x 30.4 cm high cylindrical plastic cage) containing ~21 g of bedding, one paper towel (22.8 cm × 10.1 cm), six pieces of food (~30 g, daily), and *ad libitum* water. Cage bottoms were changed daily with fresh bedding, food, and paper towel (Ivy et al., 2008; Molet et al., 2014; Smith et al., 2020).

2.5. Maternal separation

On P2 – P7, dams were separated from their litters and each pup was placed into an individual cage with one paper towel to lay on for 3 h (room temperature 21 °C).

2.6. Terbutaline administration

As rats are an altricial species, P2–P5 corresponds to the second or third trimester in humans. Terbutaline has been used to delay preterm labor occurring during these two periods, but can have detrimental effects on development as it can cross the placenta (Rhodes et al., 2003; Perna et al., 2014). Terbutaline was administered on P2 – P5 (10 mg/kg, 5 mg/mL, max volume injected ~ 0.028 mL at P5, USP, i.p., dissolved in sterile saline; CAS 23031-32-5, Spectrum Chemical Mfg. Corp., New Brunswick, NJ, USA).

2.7. Behavioral tests

Behavioral tests were conducted on days P10 (ultrasonic vocalizations (USV)), P72–P83 (marble burying test), and P86–P89 (three-chamber social interaction test) (Fig. 1).

2.7.1. Ultrasonic vocalizations

Pups were placed in a plastic mouse cage with two paper towels on the bottom for 3 min alone inside a sound-attenuating chamber. A Ciel Electronique (Cat. No. CDP102, St Andre De La Roche, France) bat detector was set to transduce frequencies between 40 and 50 kHz and Audacity® open source sound software (version 3.3.3, London, UK) was used to record. Recordings were analyzed using custom-written Matlab software (R2021b, version 9.11.0.1769968, Natick, MA, USA).

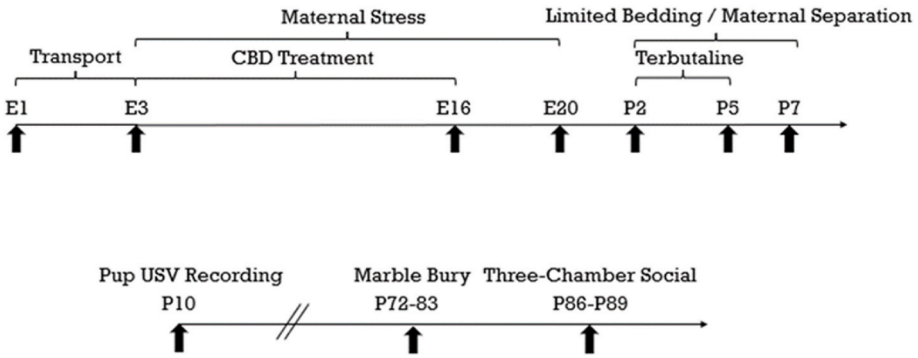

Fig. 1. Experiment timeline. *Abbreviations*: CBD, broad-spectrum cannabidiol oil; E, embryonic day; P, postnatal day; USV, ultrasonic vocalization.

2.7.2. Marble burying test

Rats were randomized into four equally sized cohorts and assigned a three-day testing schedule (see Smith et al., 2020 for details). Briefly, rats underwent two days of 30-min habituation sessions to the test environment followed by a test day. On test day, animals were given 10 min alone in a plastic rat cage containing 18 marbles in a 3 x 6 grid on ~5 cm of bedding. The number of marbles buried were counted at the end of the test. A marble was considered buried if: 1) at least 2/3 of the marble was buried and 2) it appeared to have not been walked on.

2.7.3. Three-chamber social interaction test

Animals were randomized into four equally sized cohorts and

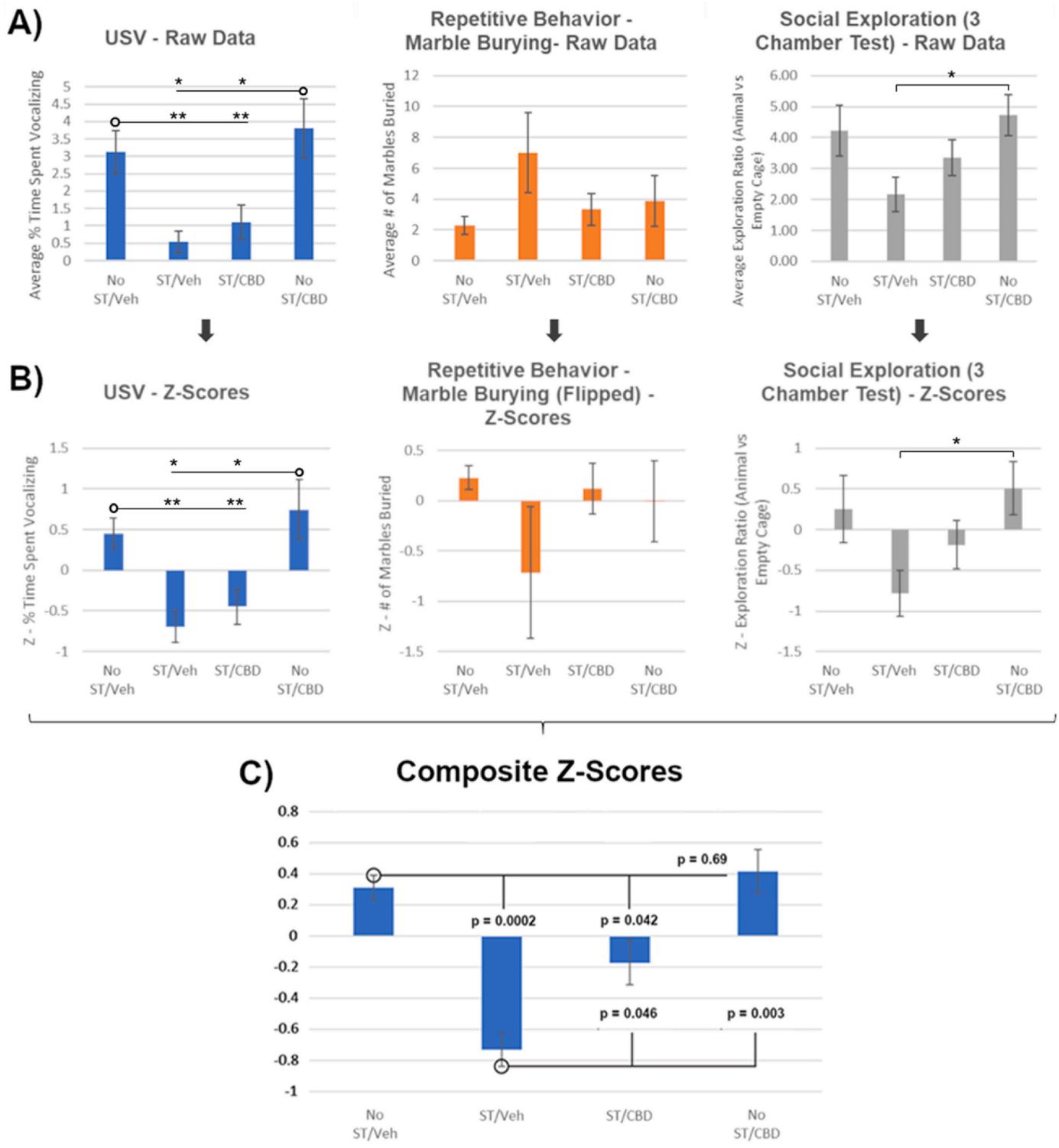

Fig. 2. Effects of prenatal administration of CBD on stress/terbutaline-induced behaviors relevant to an ASD-like phenotype in male offspring assessed during adolescence and adulthood. A) Raw averaged group data for three behavioral tests, which were converted into Z-scores (B), and then averaged together to create an ASD-like composite *Z*-score (C). Sample sizes: No ST/Veh, $n = 7$; ST/Veh, $n = 5$; ST/CBD, $n = 9$; No ST/CBD, $n = 9$. *Abbreviations*: CBD, broad-spectrum cannabidiol oil; ST = Stress + Terbutaline; USV, ultrasonic vocalization; Veh = Vehicle; $p < 0.05$ (*), $p < 0.01$ (**).

assigned a testing day. The amount of time spent socially exploring a conspecific juvenile was measured (see Smith et al., 2020 for details). Briefly, the test consisted of three stages run in succession. These stages were: 1) a 5-min habituation stage, 2) an animal vs empty cage stage, and 3) a familiar animal vs unfamiliar animal stage. Social exploration was considered to be any behavior displaying direct active engagement, interest, or investigation of the conspecific animal(s).

2.8. Data and statistical analysis

An ASD-like score was created by converting the three behavioral test scores into *Z*-scores ($Z = \frac{x-\mu}{\sigma}$), which were then averaged to form a composite score (El-Kordi et al., 2013; Bercum et al., 2015; Smith et al., 2020). The marble burying test *Z*-scores were inverted so that when combined with the USV and three-chamber social interaction test scores, ASD-like behaviors would all be heading in the same direction and accurately depict a negative score.

3. Results

One dam in the No ST/CBD group was excluded from the study due to dehydration on E8. All other dams gave birth (No ST/Veh, mean litter size = 11.5 ± 0.14, male $n = 7$, female $n = 16$; ST/Veh, mean litter size = 7.6 ± 1.22, male $n = 9$, female $n = 14$; ST/CBD, mean litter size = 7.3 ± 0.96, male $n = 10$, female $n = 12$; No ST/CBD, mean litter size = 9 ± 0.9, male $n = 10$, female $n = 17$). One dam from each group (not including the No ST/CBD group) was excluded from fostering due to cross-fostering constraints. A dam in the ST/Veh group developed a mammary infection, which led to significant litter weight loss and euthanasia of her litter on P12 ($n = 4$). One pup in the No ST/CBD group and one in the ST/CBD group were found dead on P3 and P4, respectively.

Fig. 2A and B depict the three behavioral tests (raw data and *Z*-scores, respectively). There was a trend toward increased ASD-like behavior in the ST/Veh rats, reflected in all 3 behavioral tests, with a main effect of ST on USVs and Social Exploration reaching significance via a two-way ANOVA ($F_{(1,26)} = 17.102$, $p = 0.0003$ and $F_{(1,26)} = 5.34$, $p = 0.029$, respectively). Post hoc analysis (Fisher's LSD) for USVs found ST/Veh rats were significantly different compared to No ST/Veh and No ST/CBD rats ($p = 0.0017$ and $p = 0.0169$, respectively), but for Social Exploration ST/Veh rats were only significantly different from No ST/CBD rats ($p = 0.0228$). Behavioral severity seen in ST/Veh rats decreased (non-significantly) with CBD treatment (ST/CBD) and approached that of No ST/Veh and No ST/CBD rats.

As ASD is composed of deficits in three core behavioral domains, we combined these tests into one ASD-like score. To justify this combination, a multivariate ANOVA was first conducted to compare the effects of treatment on the separate tests. There was a main effect of CBD treatment ($F_{(2,81)} = 7.65$; $p = 0.0009$) but no main effect of the separate behavioral tests ($F_{(2,81)} = 0.24$; $p = 0.7887$) or interaction ($F_{(4,81)} = 0.88$; $p = 0.4793$). As there was no effect of separate tests and to decrease their variability, we proceeded with combining them into a composite *Z*-score reflecting an ASD-like behavioral measure (Fig. 2C). Two-way ANOVA of the ASD-like composite *Z*-score revealed a significant effect of stress/terbutaline ($F_{(1,26)} = 16.80$, $p = 0.0003$) but no effect of CBD ($F_{(1,26)} = 2.80$, $p = 0.1060$) or interaction ($F_{(1,26)} = 1.49$, $p = 0.2329$). Post-hoc analysis (Fisher's LSD) indicated a significant behavioral abnormality for the ST/Veh rats ($p = 0.0002$) compared to No ST/Veh rats. CBD reduced ASD-like behavioral composite *Z*-scores significantly ($p = 0.046$). There was no effect of CBD alone relative to No ST/Veh rats.

4. Discussion

Exposure to stress and terbutaline resulted in an overall ASD-like behavioral phenotype similar to that previously observed in male rats (Bercum et al., 2015; Smith et al., 2020). These offspring had impairments to social communication by P10 and social interaction by P86. When combined with our repetitive behavioral measurement, a clear deviation from normal behavior was observed.

Administering CBD during pregnancy provided protection to the stress/terbutaline offspring. However, from these data it is unclear if CBD was protecting against prenatal stress (E1 - E20) or all adverse manipulations. CBD is lipophilic and found in mouse pups for up to four days after last prenatal exposure; it's therefore possible that CBD was still present in the rat pups postnatally (Swenson et al., 2023). Additionally, CBD may have been present in the dams' breast milk. Cannabinoids can be detected in breast milk of nursing human mothers up to six days from last use (smoked cannabis) and, in studies utilizing rats and mice, were discovered to accumulate in adipose, liver, and muscle tissue (Bertrand et al., 2018; Moss et al., 2021; Child et al., 2022; Johnson et al., 2022). As such, CBD may have been present in small quantities that were slowly being released by adipose tissues used for metabolism and breast milk production.

If CBD was not present in the rat pups or dams during postnatal manipulations, the protection observed here may have stemmed from lasting prenatal neuronal and microglial changes. Previous research using this model has explored an alternative prevention strategy utilizing heat-killed *Mycobacterium vaccae* NCTC 11659 (*M. vaccae*) (Reber et al., 2016; Smith et al., 2020; Amoroso et al., 2021). *M. vaccae* has been found to immunize against psychosocial stressors that elicit sterile inflammation and has demonstrated highly significant behavioral protection against the development of ASD-like behaviors (Reber et al., 2016; Smith et al., 2020; Amoroso et al., 2021). The two behaviors assessed by Smith et al. (2020) were social interaction and repetitive behaviors. We observed similar results, indicating that CBD may be acting through comparable immunoregulatory mechanisms. *M. vaccae* induces regulatory T cell expansion in peripheral tissues and is capable of inducing expression of the anti-inflammatory cytokine interleukin (IL) 4 in the central nervous system (Fonken et al., 2018; Frank et al., 2020; Smith et al., 2020). Interestingly, CBD also induces expansion of regulatory T cells (Dhital et al., 2017; Nichols and Kaplan, 2020; Angelina et al., 2022). As CBD can bind to endocannabinoid receptors and these receptors are present in immune cells, one speculative explanation of how CBD is protecting against the observed behavioral deficits may be that CBD is acting through anti-inflammatory and immunoregulatory mechanisms.

Understanding the potential negative effects of CBD administration during pregnancy is important. Negative effects of prenatal CBD administration (50 mg/kg, oral gavage) were noticed only in demanding behavioral test paradigms in mice (Swenson et al., 2023). Here, it is a promising finding that a lower prenatal dosage of CBD (10 mg/kg, i.p.) did not produce readily observable negative behavioral outcomes.

5. Conclusions

This study examined the potential for prenatal administration of CBD to prevent negative neurodevelopmental behavioral effects of stress/terbutaline exposures. The behavioral improvements in the male offspring of CBD-treated animals are promising. Future research will be needed to uncover the mechanisms underlying the protective effects of CBD.

CRediT authorship contribution statement

Jeremy A. Taylor: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Zachariah Z. Smith:** Methodology. **Michael E. Anderson:** Methodology. **Evan M. Holbrook:** Formal analysis, Data curation. **Isabella S. Elkinbard:** Methodology. **Jon D. Reuter:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Christopher A.**

Lowry: Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Daniel S. Barth:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2024.100828.

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