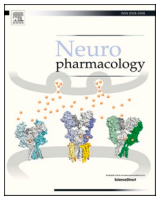
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N-acetylcysteine decreases toluene conditioned place preference and modulates corticostriatal activity in adolescent rats

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ABSTRACT

Inhalant (toluene) use disorder (IUD) continues to be a public health problem especially among Filipino adolescents. Animal models of IUD have not yet explored neurobiological changes from chronic toluene exposure, or possible treatments for long-lasting toluene-induced deficits. In rats repeatedly exposed to 3000 ppm of toluene vapor over 12 days, we report that daily administration of N-acetylcysteine (NACys; 100 mg/kg i.p. or 0.9 g/l oral), but not melatonin (10 mg/kg i.p.), during 8 days of abstinence reduced toluene conditioned place preference in both male and female adolescent Sprague Dawley rats. Using immunofluorescence, we found that toluene-exposed rats showed decreased c-Fos densities in the dorsomedial striatum (DMS), dorsolateral striatum, and ventrolateral orbitofrontal cortex (vlOFC) after 8 days of abstinence. NACys partially rescued c-Fos expression in vlOFC. We found that c-Fos densities in both vlOFC and prelimbic cortex were significantly correlated with CPP values and with c-Fos densities in the DMS across treatment groups, suggesting that a history of toluene exposure may act by reducing cortical modulation of the dorsal striatum to promote long-lasting drug context seeking. Our findings suggest that NACys may be a promising treatment for IUD in adolescent males and females.

1. Introduction

Inhalant use disorder (IUD) is a persistent health problem that affects male and female adolescents worldwide (Cruz and Bowen, 2021; Nguyen et al., 2016). Inhalants are the fifth most commonly used drug among patients in treatment facilities in the Philippines (Dangerous Drugs Board, 2025) and are highly used by Filipino adolescents, particularly street children (Miguel-Baquilod, 2004; Njord et al., 2010). However, there is still no established pharmacological treatment for IUD (Lail et al., 2025). Substance use disorders, including IUD, are chronic disorders (Bachi et al., 2025) characterized by periods of abstinence and relapse, which have been hypothesized to reflect long-lasting drug-induced changes in brain function (Volkow and Blanco, 2023). Therefore, understanding the long-term behavioral and neurobiological effects of inhalants is key in developing treatments for IUD.

We have previously reported that repeated exposure to toluene, the most-studied component of inhaled solvents (Cruz and Bowen, 2021), produces long-lasting conditioned place preference (CPP) and impairs social behavior during prolonged abstinence in both male and female adolescent rats (Asis et al., 2025). With respect to the brain, *acute* toluene exposure has been shown to increase neuronal activity in addiction-related corticostriatal regions such as the prefrontal cortex, orbitofrontal cortex, and dorsal striatum in male rats using c-Fos immunohistochemistry (Lo and Chen, 2005; Perit et al., 2012). However, the effects of repeated exposure to toluene on the brain have not been examined to our knowledge.

N-acetylcysteine (NACys) is an amino acid supplement that is available over the counter and has been shown to reduce craving and alleviate withdrawal symptoms in clinical trials for nicotine, cannabis, cocaine, amphetamine, and alcohol use disorders (Chang et al., 2021;

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Duailibi et al., 2017; but see Winterlind et al., 2024) The effect of NACys in IUD, however, has not been tested clinically. In male mice, NACys has been shown to decrease the rewarding effects of an acute injection of toluene (Wu et al., 2018). Melatonin is an indoleamine hormone supplement that is available over the counter with potent antioxidant properties (Leung et al., 2020), and has been shown to rescue dendritic spines in rats acutely exposed to toluene (Montes et al., 2019; Pascual et al., 2011). Melatonin decreases CPP for cocaine (Barbosa-Méndez et al., 2021) and morphine (Alghamdi and Alshehri, 2021) in rats. Neither NACys nor melatonin have been explored for reversing the effects of chronic toluene exposure.

In this study, we exposed male and female adolescent rats to toluene vapors over 12 days using the CPP procedure. We then assessed whether NACys or melatonin, when administered for 8 days during the abstinence period, would decrease toluene-induced CPP, restore social novelty preference, or reverse toluene-induced changes in neuronal activity using c-Fos immunofluorescence. Our goals were to characterize the long-lasting effects of toluene on the brain and to evaluate NACys and melatonin as potential low-cost supplemental treatments to behavioral therapy for IUD in adolescents.

2. Materials and methods

2.1. Animals

Male and female Sprague Dawley (SD) rats at 21 days of age (P21) were purchased from BioLASCO (Taiwan) and were given treatments via intraperitoneal (i.p.) injection. A separate cohort of female rats were purchased from M-CLEA Bioresource (Thailand) as a follow-up experiment and were given treatments via drinking water to address the possible confounding effects of female-specific injection-induced stress. Rats of the same sex were pair-housed in polypropylene cages (425 × 266 × 185 mm) with corn cob beddings and given ad libitum access to water and feeds (Altromin 1324, Germany). The animal facility was a temperature- and humidity-controlled environment (20–22 °C, 40–70% humidity) with a 12-h reversed light-dark cycle (lights on: 1900–0700). Experimental procedures were performed in adolescent (P29-P37) rats after at least 7 days of acclimatization, during the dark phase. All experiments were approved by the University of the Philippines Manila Institutional Animal Care and Use Committee (protocol no. 2021-008).

2.2. Drugs and chemicals

Liquid toluene (99.5% analytical grade, RCI Labscan, Thailand) was vaporized using custom-made bubblers attached to computer-controlled airflow regulators (La Jolla Alcohol Research, Inc. (LJARI), USA) at 2-3 lpm. We have previously validated that an airflow rate of 2-3 lpm vaporizes liquid toluene and results in stable toluene vapor levels of 3000 ppm for 30 min in our CPP conditioning chamber when measured with the Cosmos XP-3360II (DOD Technologies, USA) portable toluene gas detector (Asis et al., 2025). N-acetylcysteine (NACys) (Sigma-Aldrich, USA) was dissolved in 0.9% saline for injections. Melatonin (Sigma-Aldrich, USA) was dissolved in ethanol (99.9%; Scharlab Inc., Spain) and diluted in 0.9% saline for injections. The final ethanol concentration in the melatonin solutions was 1.5%. All injections were freshly prepared daily. For oral administration, NACys powder was dissolved in drinking water and drinking bottles were replaced daily.

2.3. Toluene conditioned place preference

The toluene conditioned place preference (CPP) protocol (Fig. 1) was performed as in our previous study (Asis et al., 2025). The CPP apparatus (LJARI, USA) consisted of three distinct chambers: a smaller middle chamber with gray walls and a smooth acrylic floor (21 × 13 × 21 cm), a conditioning chamber with white walls and a smooth rubber floor (21 × 27 × 21 cm), and a second conditioning chamber with black walls and a textured rubber floor (21 × 27 × 21 cm). Manually operated guillotine doors separated the chambers. Toluene was only given in the black conditioning chamber due limitations in the chamber design, as the white conditioning chamber did not have an inlet for toluene vapor and was not manufactured to be airtight.

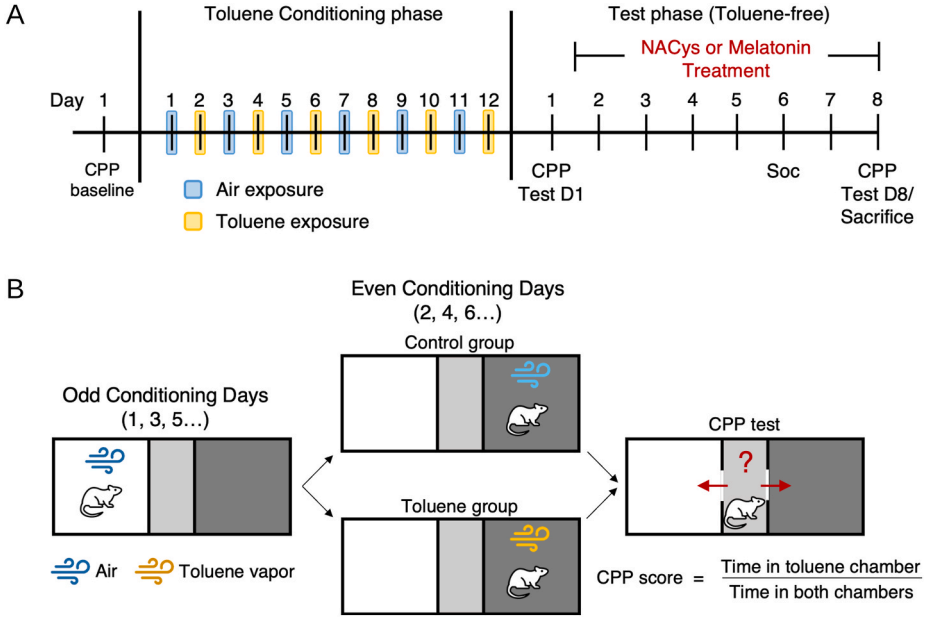


Fig. 1. Experimental design. A) Rats were exposed to toluene or air for 6 days (over a 12-day period) in a CPP apparatus during the conditioning phase. One day after the last toluene exposure (test D1), rats underwent behavioral tests during the test phase, during which they also received daily injections of saline, NACys, or melatonin. Rats were sacrificed and brains were collected for c-Fos immunofluorescence after the CPP test on D8. CPP = conditioned place preference; Soc = social novelty test; NACys = N-acetylcysteine. B) CPP conditioning was performed using a three-chambered apparatus in which rats were exposed to air in a white chamber and to toluene vapor in a black chamber. Control rats were exposed to air in both chambers. During test, rats were tested for chamber preference and a CPP score was calculated.

The CPP protocol consisted of three phases (Fig. 1A): preconditioning (baseline), conditioning, and test. *Preconditioning phase:* Adolescent rats (P29 to P37) were placed in the gray middle chamber of the CPP apparatus with the guillotine doors closed. After 2 min, the doors were opened, and the rats were allowed to explore all three chambers for 15 min. Each rat's baseline preference score was calculated by the time spent in the black conditioning chamber (where toluene will be given) over the sum of the time spent in both black and white conditioning chambers. Rats were group-assigned with the intention to match the average baseline preference scores across the air group (control) and the three toluene groups. *Conditioning phase (*Fig. 1B): Toluene groups were exposed to 3000 ppm toluene vapor in the black chamber and clean air in the white chamber with the guillotine doors closed. The control group was exposed to clean air in both conditioning chambers. For 12 days, rats underwent one 30-min conditioning session per day. Rats received air on the first day, then air (control group) or toluene vapor (toluene groups) the next day, with alternating air and toluene conditioning sessions on subsequent days. The toluene groups received a total of six air and six toluene conditioning sessions, while the control group received 12 air conditioning sessions. *Test phase* (Fig. 1B*):* One day after the last toluene conditioning day (day 13, termed test day 1 (D1)), rats were placed in the gray middle chamber with the guillotine doors closed. After 2 min, the doors were opened, and the rats were allowed to explore all three chambers for 15 min. The CPP test was repeated 8 days after the last toluene conditioning day (test day 8 (D8; Fig. 1A)). A CPP ratio score was calculated as the time spent in the black conditioning chamber (where toluene was given) divided by the sum of the time spent in both black and white conditioning chambers (Asis et al., 2025; Jia et al., 2023).

2.4. Social novelty test

The social novelty test (Fig. 3A) was performed as previously described (Asis et al., 2025; Moy et al., 2004), with some modifications. The social test apparatus (Ugo Basile, Italy) consisted of three chambers with transparent acrylic walls, and each chamber measured 40 × 40 × 40 cm. Manually operated guillotine doors separated the chambers. Two identical cylindrical enclosures (25 cm high × 15 cm in diameter) with metal railings and polyvinyl chloride roofs and floors were placed in the center of the left and right chambers.

The social novelty test consisted of two phases: habituation and social novelty test. *Habituation:* On test day 4 (D4), test rats were initially placed in the middle chamber of the social test apparatus with the guillotine doors closed. After 2 min, the doors were opened, and the rats were allowed to explore all three chambers and empty cylindrical enclosures for 10 min. *Social novelty test:* On test day 6 (D6), sex- and age-matched demonstrator rats were placed in both cylindrical enclosures, one of which is a familiar rat (rat that was introduced to the test rat a day prior), and the other is a novel rat (previously unencountered by the test rat). The position of the demonstrator rats was counterbalanced for all the groups. Exploration time was defined as the time spent sniffing or moving within 3 cm of the cylindrical enclosures that contained the demonstrator rats, with the head oriented towards the enclosures. The social novelty score was calculated as the exploration time for the cylindrical enclosure with the novel rat divided by the sum of the exploration times for both cylindrical enclosures.

2.5. c-Fos immunofluorescence

Immunofluorescence was performed on selected brain regions as previously described (Quiñones-Laracuente et al., 2021), with some modifications. Ninety mins after the CPP test on D8, rats were deeply anesthetized with sodium pentobarbital (450 mg/kg, i.p.) and then perfused transcardially with 100 ml of 0.9% saline followed by 100 ml of cold fresh 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Brains were extracted and fixed overnight in 4% paraformaldehyde, then transferred to 30% sucrose in 0.1 M PBS for cryoprotection at 4 °C until sectioning. Brains were frozen with optimal cutting temperature (OCT) compound and were cut coronally (40 μm) with a cryostat (Leica CM1850). Brain sections containing the prefrontal cortex, ventrolateral orbitofrontal cortex, insular cortex, nucleus accumbens, dorsal striatum, retrosplenial cortex, dorsal hippocampus, amygdala, ventral hippocampus, midline thalamus, and ventral tegmental area were obtained.

Free-floating sections were rinsed with PBS and blocked for 2 h in a normal donkey serum + Triton X-100 (NDST) solution containing 5% normal donkey serum (NDS; SouthernBiotech, USA) and 0.2% Triton X-100 (Sigma-Aldrich, USA) in 0.1 M PBS. These were then incubated overnight at room temperature (RT, 25 °C) in a primary antibody solution containing 1:2500 rabbit anti-c-Fos (Synaptic Systems, Germany) in NDST. Sections were rinsed then incubated for 2 h in a secondary antibody solution containing 1:1000 donkey anti-rabbit Alexa Fluor 488 (Invitrogen, USA) in NDST. Sections were rinsed with PBS, mounted onto charged slides, cover slipped with Fluoromount-G with DAPI (SouthernBiotech, USA), and examined under a fluorescent microscope (Olympus BX53).

2.6. Experiment 1: Effect of NACys or melatonin in treating toluene-induced changes in CPP and social behavior

Male (n = 24) and female (n = 24) adolescent SD rats were exposed to air (n = 6 per sex) or toluene (n = 18 per sex) under the CPP procedure and their CPP scores were measured on test D1 (Figs. 1A and 2A). One hour after the D1 CPP test, toluene-exposed rats were divided into three treatment groups (Fig. 2B) and were injected i.p. with 5 ml/kg of saline (toluene-saline group), NACys (toluene-NACys group), or melatonin (toluene-melatonin group). NACys was given at a dose of 100 mg/kg (Moussawi et al., 2011; Wu et al., 2018) while melatonin was given at a dose of 10 mg/kg (Montes et al., 2019; Pascual et al., 2011). Rats received daily treatment injections during the test phase, with their last injection on test D8 given 1 h before the CPP test. They also underwent the social novelty test on test D6 (Fig. 1A). In the social novelty test, one male rat (toluene-NACys group) with a habituation score of > 0.8 was excluded from the analysis.

In a separate CPP experiment, toluene-exposed female rats (n = 12) received daily treatments during the test phase via oral administration (Fig. 2C) of NACys-infused water (0.9 g/l; toluene-NACys group, n = 6) or regular drinking water (toluene-water group, n = 6). NACys powder was dissolved in drinking water at a concentration of 0.9 g/l, as previously described (Bühner et al., 2022; De Felice et al., 2024).

2.7. Experiment 2: Determination of changes in neuronal activity associated with toluene CPP and its treatment with NACys

After the D8 CPP test (Fig. 1A), a subset of toluene-exposed rats from experiment 1 (Fig. 2B) treated with i.p. injections of saline or NACys were sacrificed for c-Fos immunofluorescence (n = 6 per group per sex). Air-exposed rats from experiment 1 (Fig. 2A) also received daily saline i.p. injections (air-saline group; n = 6 per sex) during the test phase and were also sacrificed for c-Fos immunofluorescence. Thus, we compared c-Fos expression in three groups of rats: air-saline, toluene-saline, and toluene-NACys.

2.8. Data analyses

Rat behaviors were recorded with digital video cameras, and behavioral measures for CPP and social novelty test were obtained using ANY-Maze version 7.33 (Stoelting Co., USA). c-Fos immuno-labeled cells were counted automatically at 10X magnification using QuPath version 0.6.0 (Bankhead et al., 2017) with a fixed intensity threshold for all sections per structure within a defined counting box. Counting artifacts were manually removed. Counting was performed by an experimenter

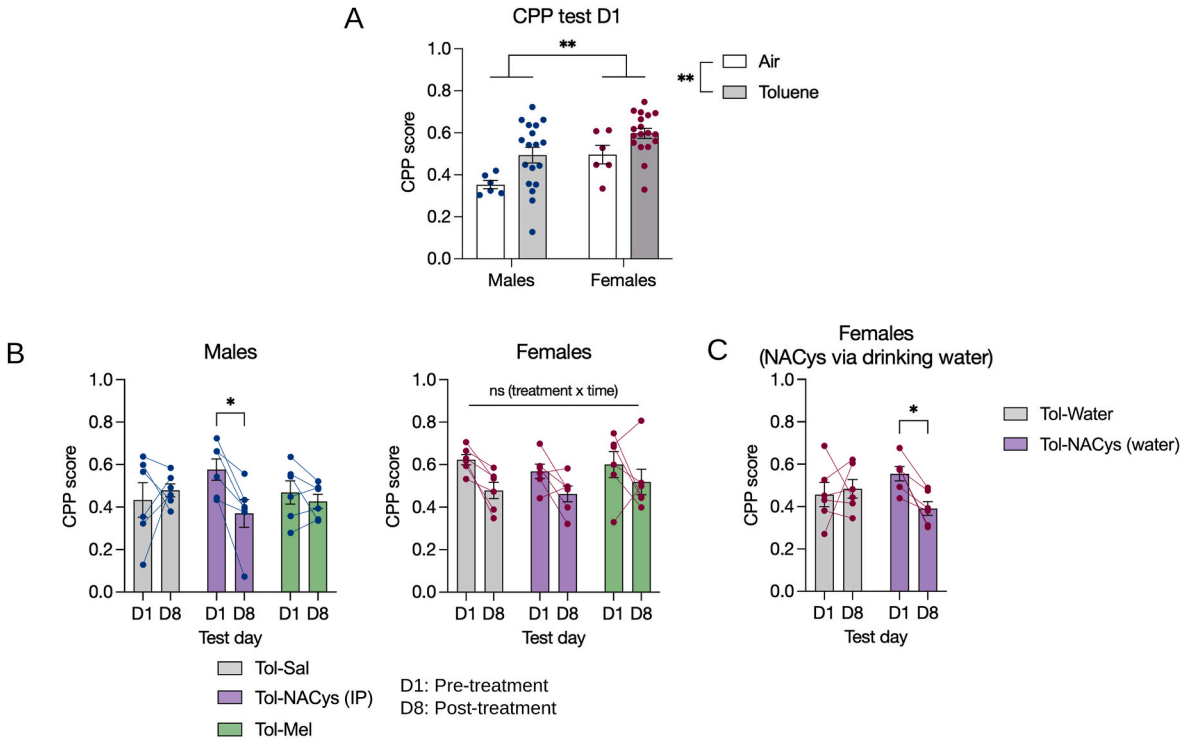


Fig. 2. NACys treatment decreased toluene conditioned place preference. A) On test D1, toluene-exposed male and female rats (n = 18 per sex, including saline, NACys, and melatonin treatment groups) showed significantly increased CPP scores compared to air-exposed controls (n = 6 per sex). B) In toluene-exposed males, NACys injections significantly decreased the CPP score from D1 (pre-treatment) to D8 (post-treatment), while no significant change was observed in rats injected with either saline or melatonin (n = 6 per group). In toluene-exposed females, there was a main effect of time (decrease from D1 to D8), but no significant group differences (n = 6 per group). C) In toluene-exposed females that received NACys via drinking water, CPP score was significantly decreased only in the NACys group (n = 6 per group). Tol-Sal = toluene-saline, Tol-NACys = toluene-N-acetylcysteine, Tol-Mel = toluene-melatonin, Tol-Water = toluene-water, IP = intraperitoneal. ns = not significant, *p < 0.05, **p < 0.01.

who was blind to the identity and experimental group of the rats. Counts from both hemispheres were averaged using three different sections for each structure. c-Fos density was calculated by dividing the number of c-Fos-positive cells by the total area of the counting box for each region (counts/mm^2).

All graphs and statistical analyses were generated using Prism version 10.6.1 (GraphPad Software, USA) at alpha = 0.05. We used the two-tailed unpaired *t*-test, two-way ANOVA, two-way repeated measures ANOVA with Šidák's multiple comparisons test, and three-way repeated measures ANOVA to analyze behavioral data. Kruskall-Wallis test with Dunn's multiple comparisons test was used to analyze the c-Fos data. Correlation coefficients were obtained using Spearman correlation.

Outliers in the c-Fos data were identified independently for each brain region using the Robust Regression and Outlier Removal (ROUT) method (Q = 1%) (Motulsky and Brown, 2006) and were excluded from the c-Fos analysis. In the male data, a total of eight observations were partially excluded from the c-Fos analysis: except for DLS (two outliers), only one outlier each was removed from six brain regions (out of 23 total). In the female data, a total of four observations were partially excluded from the c-Fos analysis: one outlier each was removed from four out of 23 brain regions.

3. Results

3.1. NACys decreases toluene CPP in both males and females

We exposed male and female rats to 3000 ppm toluene vapor for 6 days (over a period of 12 days), with control groups that were only exposed to air. Toluene-exposed rats had higher CPP scores on test D1 compared to air-exposed rats in both males and females (Fig. 2A; two-way ANOVA: significant main effect of drug ($F_{1,44} = 8.597$, $p = 0.005$) and sex ($F_{1,44} = 8.964$, $p = 0.005$), but not a drug × sex interaction ($F_{1,44} = 0.220$, $p = 0.642$)).

After the D1 CPP test, we administered daily injections of saline, NACys (100 mg/kg i.p.), or melatonin (10 mg/kg i.p.) to toluene-conditioned rats across 8 days of abstinence and performed another CPP test on D8 post-treatment (Fig. 2B). CPP scores differed across treatment, sex, and time (three-way ANOVA: significant treatment × sex × time interaction $F_{2,48} = 4.939$, $p = 0.011$). Data were then separated by sex and analyzed with two-way ANOVAs. In male rats, only the toluene-NACys group showed decreased CPP scores from test D1 to test D8 (two-way ANOVA: significant treatment × time interaction $F_{2,15} = 3.941$, $p = 0.042$; Šidák's $p = 0.018$). In female rats, the average CPP scores decreased from D1 to D8 (two-way ANOVA: significant main effect of time $F_{1,15} = 11.73$, $p = 0.004$), but we did not observe any significant group differences.

We have previously shown that toluene CPP persists until D22 of abstinence in female rats (Asis et al., 2025). Hence, the unexpected CPP reduction from D1 to D8 in the female toluene-saline group may be due to sex-specific stressful effects of daily i.p. injections. Repeated stress exposure during the abstinence period has been shown to decrease methamphetamine CPP in mice (Seo et al., 2018), and it has been documented that female mice show more anxiety-like and depressive-like behaviors after an intraperitoneal saline injection compared to males (Moraes et al., 2025). To address the potential confounding effects of daily i.p. injections, and to determine whether NACys is effective in reducing CPP in females (as in males), we repeated the experiment in females but administered NACys via drinking water over 8 days (0.9 g/L; Fig. 2C). We found that only the toluene-NACys group showed decreased CPP scores from test D1 to test D8 (two-way ANOVA: significant treatment × time interaction $F_{1,10} = 5.024$, $p = 0.049$; Šidák's $p = 0.043$). There was no difference in the average daily water consumption between the group with plain drinking water and the group with NACys-infused water (unpaired *t*-test: Tol-Water 52 ml/day vs Tol-NACys 51 ml/day, $t_{22} = 0.468$, $p = 0.645$). With ~40% oral bioavailability of NACys in female rats (Zhang, 2002), the estimated amount of NACys in the bloodstream of the Tol-NACys group was 90.65 mg/kg/day, which was comparable to the injected dose of 100 mg/kg/day. We also measured social behavior on test D6 and found a preference for social novelty (Fig. 3), but we did not see any sex or group differences (three-way ANOVA: significant main effect of time $F_{1,29} = 16.82$, $p < 0.001$).

3.2. Toluene decreases neuronal activity in cortical-striatal regions

We next performed c-Fos immunofluorescence as a marker of neuronal activity (Cruz et al., 2013) to determine changes in brain regions that are associated with toluene CPP and NACys treatment effects (melatonin was not examined with c-Fos) (Fig. 4A). A subset of the toluene-exposed rats that were treated with saline or NACys injections (Fig. 2B), and air-exposed rats (Fig. 2A) that were injected with saline, were sacrificed 90 min after the D8 CPP test, and their brains were extracted for c-Fos immunofluorescence analysis.

In the male c-Fos dataset, we found significant group differences in the ventrolateral orbitofrontal cortex (Fig. 4B vlOFC; Kruskal-Wallis test $H_{3,17} = 6.413$, $p = 0.032$) but not in the prelimbic cortex (Fig. 4B PL; Kruskal-Wallis test $H_{3,18} = 3.415$, $p = 0.187$). We also found significant group differences in subregions of the dorsal striatum, namely the dorsomedial striatum (Fig. 4C DMS; Kruskal-Wallis test $H_{3,17} = 6.110$, $p = 0.039$) and dorsolateral striatum (Fig. 4C DLS; Kruskal-Wallis test $H_{3,16} = 6.255$, $p = 0.041$). Compared to air-exposed rats, toluene-exposed rats treated only with saline showed significantly decreased c-Fos densities in the DMS (Dunn's $p = 0.041$) and a trend for decreased c-Fos densities in the vlOFC (Dunn's $p = 0.068$) and DLS (Dunn's $p = 0.055$). Compared to the toluene-saline group, toluene-exposed rats treated with NACys showed a trend for increased c-Fos densities in the vlOFC (Dunn's $p = 0.090$).

We found that the c-Fos densities in the cortical regions vlOFC and PL were significantly inversely correlated with the D8 CPP score (Fig. 4D; vlOFC: $r_s = -0.564$, $p = 0.020$; PL: $r_s = -0.642$, $p = 0.007$). However, we did not find correlations between the D8 CPP score and c-Fos densities in DMS ($r_s = -0.430$, $p = 0.087$) and DLS ($r_s = -0.147$, $p = 0.586$). Both vlOFC (also termed VOLO) and PL are cortical areas known to project to DMS (Haber, 2022; Heilbronner et al., 2016), while vlOFC is known to project to DLS (Heilbronner et al., 2016). Accordingly, c-Fos densities in DMS were significantly correlated with c-Fos densities in both vlOFC (Fig. 4E; $r_s = 0.764$, $p < 0.001$) and PL (Fig. 4E; $r_s = 0.806$, $p < 0.001$). There was no significant correlation between c-Fos densities in vlOFC and DLS ($r_s = 0.154$, $p = 0.588$).

We did not find group differences in the 19 other brain regions we examined in males (Table 1) or in any of the 23 brain regions that we analyzed in females (Table 2). These results suggest that a history of toluene exposure in males leads to a long-lasting decrease in c-Fos expression in the DMS, which may be regulated upstream by signals from PL and vlOFC (Fig. 4F). NACys may act to restore cortical control of striatal activity, especially from vlOFC to DMS. However, since this was an exploratory neuroanatomical screen, regional analyses were interpreted cautiously and require replication.

4. Discussion

We found that N-acetylcysteine, when given daily during the 8-day abstinence period, decreases toluene CPP in both sexes (through different routes of administration), whereas melatonin had no effect on toluene-induced behaviors. We demonstrate decreased c-Fos densities in DMS, and a trend in vlOFC and DLS, in male toluene-exposed rats compared to air-exposed controls. NACys treatment tended to reverse these changes in c-Fos expression. Furthermore, c-Fos densities in PL and vlOFC were negatively correlated with CPP values and positively correlated with DMS c-Fos densities. Corticostriatal projections may be key targets for determining the mechanisms of persistent toluene addiction.

4.1. Toluene causes long-lasting decreases in corticostriatal activity

Prior work on toluene's effect on c-Fos expression has only examined acute exposure (Lo and Chen, 2005; Perit et al., 2012; Tomaszycki et al., 2013). Here, we show that rats repeatedly exposed to toluene had decreased c-Fos densities in the dorsal striatum (significant in DMS and a trend in DLS) following 8 days of abstinence. Similar reductions in c-Fos expression were reported in the dorsal striatum during 74h to 7d of abstinence from chronic ethanol exposure (Smith et al., 2020), and during 1 month of abstinence from methamphetamine self-administration (Krasnova et al., 2013).

We also show a tendency for decreased vlOFC c-Fos expression in toluene-exposed rats during the D8 CPP test. Prior work has shown that 6 weeks of abstinence from morphine impaired reversal learning and decreased c-Fos expression in vlOFC (Piao et al., 2017). Similarly, we have previously observed a toluene-induced impairment in reversal learning during abstinence (Asis et al., 2025). vlOFC is important in

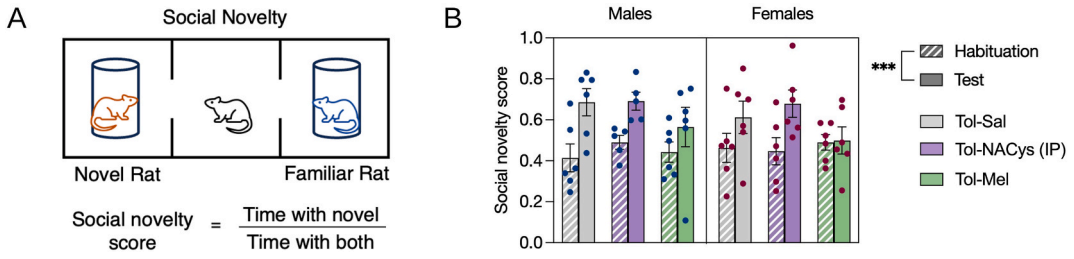


Fig. 3. Toluene did not impair social novelty preference. A) Three-chamber design for social novelty preference test. B) Social novelty scores increased from habituation to test phase across all treatment groups in both males (Tol-Sal n = 6, Tol- NACys n = 5, Tol-Mel n = 6) and females (n = 6 per group), but there were no significant group differences. Tol-Sal = toluene-saline, Tol-NACys = toluene-N-acetylcysteine, Tol-Mel = toluene-melatonin. ***p < 0.001

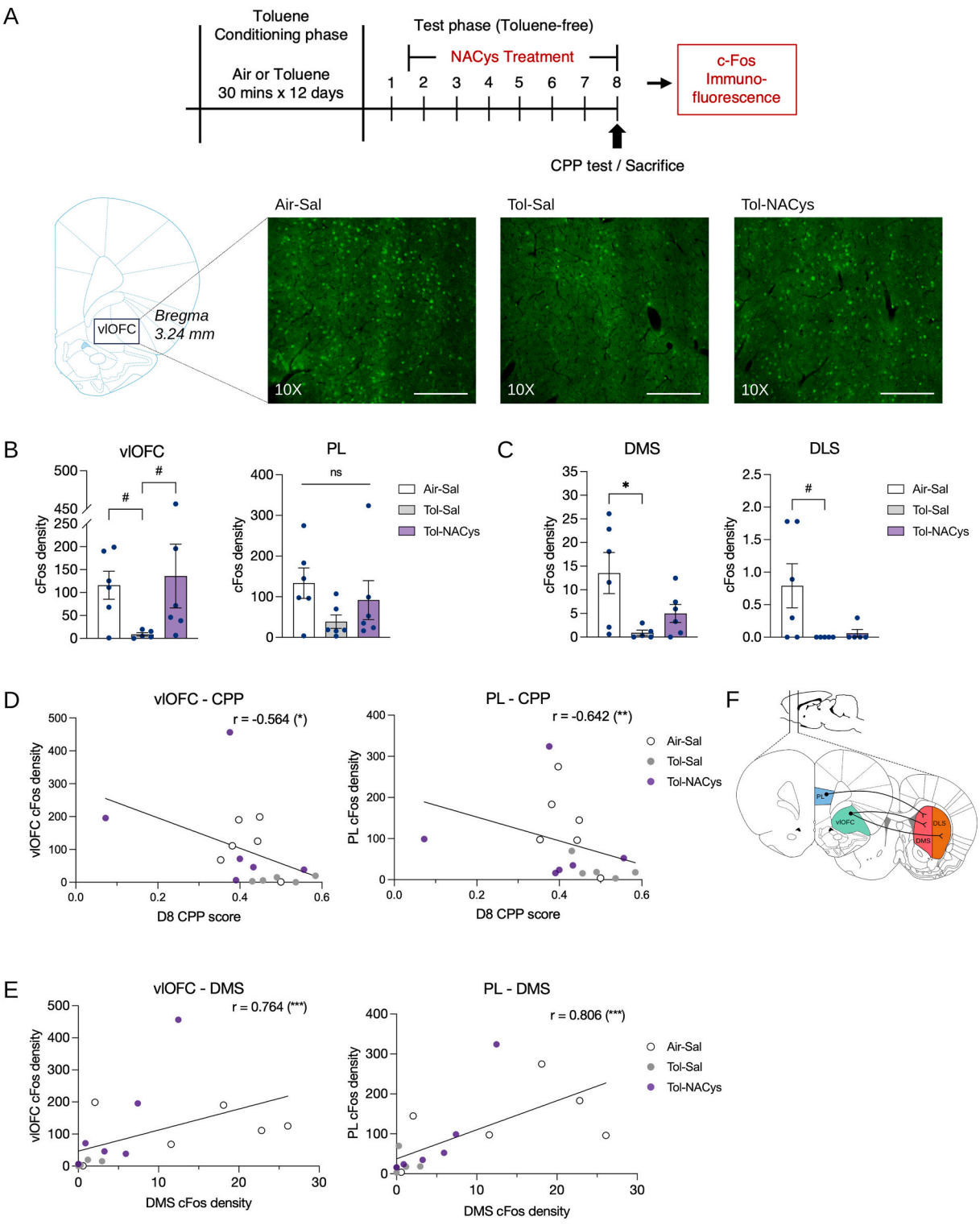


Fig. 4. The effects of exposure to toluene and NACys on c-Fos expression in males. A) Experimental timeline (top) and representative images of vlOFC showing c-Fos-positive cells across treatment groups (bottom). B) Toluene-saline rats (n = 5) showed a trend for decreased c-Fos densities in vlOFC compared to air-saline rats (n = 6), while toluene-NACys rats (n = 6) showed a trend for increased c-Fos densities compared to toluene-saline rats. There were no group differences in PL c-Fos densities (n = 6 per group). C) For striatal areas, toluene-saline rats (n = 5) showed significantly decreased c-Fos densities in the DMS and a trend for decreased c-Fos densities in the DLS compared to air-saline rats (n = 6). D) c-Fos densities in vlOFC and PL significantly inversely correlated with the CPP scores on D8. E) c-Fos densities in vlOFC and PL significantly positively correlated with c-Fos densities in DMS. F) Diagram showing PL and vlOFC projections to DMS and DLS in the rat brain. vlOFC = ventrolateral orbitofrontal cortex, PL = prelimbic cortex, DMS = dorsomedial striatum, DLS = dorsolateral striatum. Rat brain atlas image was adapted from (Paxinos and Watson, 2018) and c-Fos positive cells were counted at 10x magnification. Scale bars 200 μm. Air-Sal = air-saline, Tol-Sal = toluene-saline, Tol-NACys = toluene-N-acetylcysteine. ns = not significant, #p < 0.10, *p < 0.05, **p < 0.01, ***p < 0.001.

Table 1
c-Fos immunoreactivity values in male rats.

Males	Mean c-Fos density (counts/mm^2) ± SEM			Kruskal-Wallis test
	Air-Saline	Toluene-Saline	Toluene-NACys	p-value (main effect)
Prelimbic cortex	133.5 ± 37.44	38.80 ± 16.62	91.82 ± 47.98	0.187
Infralimbic cortex	145.8 ± 38.38	51.99 ± 22.53	88.14 ± 39.66	0.248
Nucleus accumbens core	58.81 ± 18.37	17.26 ± 7.520	27.33 ± 14.16	0.160
Nucleus accumbens shell	56.29 ± 13.09	16.37 ± 8.595	24.28 ± 13.85	0.071
Dorsomedial striatum	13.54 ± 4.350	0.890 ± 0.563	4.992 ± 1.890	0.039 (*)
Dorsolateral striatum	0.791 ± 0.340	0.000 ± 0.000	0.059 ± 0.059	0.041 (*)
Orbitofrontal cortex	115.9 ± 30.57	8.801 ± 3.763	135.9 ± 69.74	0.032 (*)
Insular cortex	3.950 ± 0.558	4.203 ± 1.519	4.763 ± 1.718	0.936
Retrosplenial agranular cortex	68.85 ± 14.64	105.2 ± 31.98	58.02 ± 14.86	0.505
Retrosplenial granular cortex	74.27 ± 9.163	106.3 ± 27.21	55.05 ± 11.56	0.124
Basal amygdala	48.38 ± 7.934	30.40 ± 3.509	37.68 ± 3.854	0.260
Lateral amygdala	33.24 ± 4.735	24.43 ± 7.387	17.05 ± 2.064	0.052
Central nucleus of the amygdala	9.765 ± 2.482	7.543 ± 2.197	8.531 ± 3.388	0.726
Dorsal hippocampus - CA1	94.33 ± 14.08	72.94 ± 14.16	56.04 ± 7.045	0.114
Dorsal hippocampus - CA3	67.67 ± 6.628	60.37 ± 8.957	61.26 ± 5.658	0.794
Dorsal hippocampus - dentate gyrus	74.95 ± 13.82	53.17 ± 21.21	33.94 ± 7.358	0.186
Dorsal hippocampus - hilus	27.69 ± 5.362	31.34 ± 11.39	20.41 ± 5.228	0.678
Ventral hippocampus - CA1	34.97 ± 6.941	22.72 ± 10.83	31.95 ± 13.95	0.493
Ventral hippocampus - CA3	16.53 ± 4.766	12.14 ± 3.090	13.35 ± 3.324	0.784
Ventral hippocampus - dentate gyrus	371.2 ± 49.88	346.4 ± 65.05	252.3 ± 46.83	0.336
Paraventricular thalamus	177.3 ± 38.97	196.6 ± 80.45	160.9 ± 73.76	0.798
Intermediodorsal thalamic nucleus	60.72 ± 15.85	41.88 ± 19.15	49.56 ± 19.02	0.848
Ventral tegmental area	57.64 ± 19.62	70.93 ± 23.38	55.44 ± 21.60	0.910

Table 2
c-Fos immunoreactivity values in female rats.

Females	Mean c-Fos density (counts/mm^2) ± SEM			Kruskal-Wallis test
	Air-Saline	Toluene-Saline	Toluene-NACys	p-value (main effect)
Prelimbic cortex	154.8 ± 19.61	106.6 ± 23.32	82.40 ± 39.88	0.080
Infralimbic cortex	216.4 ± 48.21	151.7 ± 36.16	107.4 ± 35.35	0.276
Nucleus accumbens core	67.8 ± 27.05	20.68 ± 5.313	17.44 ± 6.889	0.238
Nucleus accumbens shell	36.69 ± 9.458	28.59 ± 6.022	21.22 ± 7.469	0.399
Dorsomedial striatum	17.30 ± 7.675	14.20 ± 6.327	10.65 ± 3.374	0.770
Dorsolateral striatum	6.766 ± 0.963	10.43 ± 3.245	7.986 ± 1.297	0.409
Orbitofrontal cortex	85.99 ± 30.32	80.31 ± 27.18	26.18 ± 9.337	0.102
Insular cortex	2.185 ± 1.235	6.584 ± 3.542	2.522 ± 0.574	0.505
Retrosplenial agranular cortex	26.4 ± 9.658	25.33 ± 8.831	15.09 ± 4.679	0.742
Retrosplenial granular cortex	34.62 ± 10.14	36.47 ± 10.39	22.00 ± 10.96	0.597
Basal amygdala	14.71 ± 1.843	21.38 ± 2.266	21.41 ± 5.362	0.056
Lateral amygdala	21.31 ± 4.239	28.70 ± 7.854	12.79 ± 2.519	0.138
Central nucleus of the amygdala	7.184 ± 1.532	11.22 ± 4.596	5.388 ± 1.790	0.601
Dorsal hippocampus - CA1	12.41 ± 3.614	23.45 ± 10.44	15.35 ± 7.609	0.829
Dorsal hippocampus - CA3	16.65 ± 2.130	23.60 ± 7.337	13.80 ± 1.697	0.749
Dorsal hippocampus - dentate gyrus	52.32 ± 17.74	85.70 ± 18.70	42.99 ± 8.760	0.246
Dorsal hippocampus - hilus	23.56 ± 5.955	34.98 ± 7.975	18.95 ± 3.510	0.245
Ventral hippocampus - CA1	18.95 ± 6.638	24.79 ± 5.292	16.21 ± 5.315	0.396
Ventral hippocampus - CA3	6.669 ± 1.148	9.953 ± 3.327	10.75 ± 3.190	0.589
Ventral hippocampus - dentate gyrus	264.8 ± 52.54	207.6 ± 51.56	220.9 ± 68.65	0.844
Paraventricular thalamus	197 ± 43.78	239.4 ± 44.02	137.4 ± 46.89	0.248
Intermediodorsal thalamic nucleus	56.29 ± 13.69	39.48 ± 11.02	38.64 ± 13.94	0.551
Ventral tegmental area	82.19 ± 17.99	108.0 ± 27.30	68.39 ± 38.04	0.453

reward devaluation and stimulus-outcome reversal learning (Haber, 2022), hence decreased vlOFC activity during expression of toluene CPP may reflect the inability of toluene-exposed rats to update their previously learned drug-CPP chamber association.

We found that the c-Fos expression in DMS was correlated with the c-Fos expression in both vlOFC and PL, suggesting co-activation of these subregions. c-Fos expression in vlOFC and PL, but not DMS, were significantly inversely correlated with the D8 CPP scores. Hence, we hypothesize that decreased input from both vlOFC and PL to DMS may be important in persistent toluene CPP. Reduction of inputs (Kreitzer, 2009) from the cortex to the striatum disrupts inhibitory control over drug-associated behaviors (Everitt and Robbins, 2005; Volkow et al., 2019). Specifically, loss of cortical control from PL and vlOFC may lead to inability to change drug-seeking behavior (Kalivas, 2008). In ethanol-dependent mice, decreased output from vlOFC to DMS has been associated with a loss of goal-directed behavior towards more habitual behavior (Renteria et al., 2018).

4.2. NACys reverses toluene-induced behaviors and partially restores vlOFC activity during abstinence

We found that daily administration of NACys during the abstinence period was effective in decreasing toluene CPP scores of both male and female adolescent rats (albeit through different routes of administration). This is consistent with previous animal studies using NACys as a treatment for cocaine-primed drug seeking (Baker et al., 2003), morphine CPP (Katebi et al., 2021, 2024), ethanol drinking (Quintanilla et al., 2018), and nicotine CPP reinstatement (Quintanilla et al., 2018).

We observed intact social novelty preference in all groups, including the Tol-Sal group, in both sexes. Due to the absence of a deficit in social behavior in the Tol-Sal group, we could not evaluate NACys or melatonin's treatment effects. This is in contrast to our previous finding that repeated exposure to toluene impairs preference for social novelty (Asis et al., 2025). However, in contrast to our previous report, the rats in this study received daily intraperitoneal injections which may have interfered with toluene-induced effects on social behavior. NACys effects on social novelty preference may be conducted with oral treatment administration in future studies.

We found that NACys-treated rats showed a trend for increased c-Fos densities in vlOFC, and we hypothesize that this may reflect restored signaling to DMS to exert control over drug context seeking (Gremel et al., 2016). In ethanol-dependent mice, restoration of glutamatergic transmission from vlOFC to DMS was sufficient to restore goal-directed control of behavior (Renteria et al., 2018). NACys may decrease toluene CPP by increasing extracellular glutamate levels in the striatum. NACys was previously shown to restore decreased striatal glutamate levels during withdrawal from cocaine (Baker et al., 2003). NACys is oxidized to cysteine in the brain and activates the cystine-glutamate antiporter (xCT) on astrocytes, which shuttles glutamate out of the cell in exchange for cystine, thereby increasing extracellular glutamate levels (Berk et al., 2013). Prior work has shown that NACys's ability to prevent the behavioral effects of acute toluene exposure and increase extracellular glutamate levels required the activity of xCT (Wu et al., 2018). NACys's treatment effects in other drugs have also been associated with increased striatal activity of xCT (Ezquer et al., 2022; Moro et al., 2020; Quintanilla et al., 2020).

NACys's beneficial effects for SUD may also be attributed to its antioxidative and/or anti-inflammatory effects (Ezquer et al., 2022; Quintanilla et al., 2018, 2020) via upregulating the synthesis of glutathione, a key endogenous antioxidant molecule (Bavarsad Shahripour et al., 2014). Toluene inhalation has been documented to induce oxidative damage in the cerebral cortex, hippocampus, and cerebellum, and specifically in pyramidal neurons (Reiter et al., 2010). Toluene-induced oxidative damage in the striatum has not yet been documented; thus, it would be interesting to see whether NACys also acts to restore oxidative balance in the striatum.

In humans, a meta-analysis of 16 randomized controlled trials showed that NACys significantly reduced craving for various addictive drugs (Chang et al., 2021), but a more recent meta-analysis of 9 trials found that NACys did not outperform placebo in reducing craving and relapse (Winterlind et al., 2024). However, NACys's potential in the treatment of inhalant use has not been clinically explored.

4.3. Conclusions and future directions

We propose that NACys warrants further exploration as a therapeutic candidate for adolescent inhalant use due to its effect in decreasing toluene context seeking and its potential in rescuing toluene-induced deficits in striatal and cortical activity. We found that NACys reduced CPP in male rats when administered via intraperitoneal injections and in female rats when administered orally via drinking water. Future studies should explore whether NACys will also be effective when administered orally in male rats because oral administration is more translatable to humans. Measurement of glutamate release and/or markers of oxidative damage in the striatum should also be explored to determine NACys's mechanism of action in the treatment of toluene-induced deficits. We found trends for the effect toluene and NACys on c-Fos expression in the vlOFC and DLS, and future studies should consider confirming the effects on these structures with a larger sample size. Detection of c-Fos expression can be combined with other techniques such as retrograde tracing to establish whether projections from PL and vlOFC to DMS are important in the persistence of toluene CPP.

CRediT authorship contribution statement

Ajina C. Carampel: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Joannes Luke B. Asis:** Writing – review & editing, Investigation, Formal analysis. **Johanna C. Munar:** Writing – review & editing, Investigation, Formal analysis. **Cynthia Grace C. Gregorio:** Writing – review & editing, Formal analysis. **Paul Mark B. Medina:** Writing – review & editing, Formal analysis. **Gregory J. Quirk:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Rohani Cena-Navarro:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

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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.

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