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Authors

Shab, Gabriella
Fultz, Elissa K
Page, Ariana
et al.

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The motivational valence of methamphetamine relates inversely to subsequent methamphetamine self-administration in female C57BL/6J mice.

Gabriella Shab[a], **Elissa K. Fultz**[a], **Ariana Page**[a], **Michal A. Coelho**[a], **Lindsey W. Brewin**[a], **Nicholas Stailey**[a], **Chelsea N. Brown**[a], **Camron D. Bryant**[b], **Tod E. Kippin**[a,c,d], **Karen K. Szumlinski**[a,c]

[a]Department of Psychological and Brain Sciences, University of California at Santa Barbara, Santa Barbara, CA

[b]Department of Pharmacology and Experimental Therapeutics and Psychiatry, Boston University School of Medicine, Boston, MA

[c]Department of Molecular, Cellular and Developmental Biology and the Neuroscience Research Institute, University of California at Santa Barbara, Santa Barbara, CA

[d]Institute for Collaborative Biology, University of California at Santa Barbara, Santa Barbara, CA

Abstract

Understanding the mechanisms underpinning individual variance in addiction vulnerability requires the development of validated, high-throughput screens. In a prior study of a large sample of male isogenic C57BL/6J mice, the direction and magnitude of methamphetamine (MA)-induced place-conditioning predicts the propensity to acquire oral MA self-administration, as well as the efficacy of MA to serve as a reinforcer.. The present study examined whether or not such a predictive relationship also exists in females. Adult C57BL/6J females underwent a 4-day MA place-conditioning paradigm (once daily injections of 2 mg/kg) and were then trained to nose-poke for delivery of a 20 mg/L MA solution under increasing schedules of reinforcement, followed by dose-response testing (5–400 mg/L MA). Akin to males, 53% of the females exhibited a conditioned place-preference, while 32% of the mice were MA-neutral and 15% exhibited a conditioned place-aversion. However, unlike males, the place-conditioning phenotype did not transfer to MA-reinforced nose-poking behavior under operant-conditioning procedures, with 400 mg/L MA intake being inversely correlated place-conditioning. While only one MA-

To whom correspondence should be addressed: Karen K. Szumlinski, Ph.D., Department of Psychological and Brain Sciences, University of California at Santa Barbara, Santa Barbara, CA, USA 93106-9660, Phone: 1-805-893-2984, Fax: 1-805-893-4303, szumlinski@ucsb.edu.

conditioning dose has been assayed to date, these data indicate that sex does not significantly shift the proportion of C57BL/6J mice that perceive MA's interoceptive effects as positive, neutral or aversive. However, a sex difference appears to exist regarding the predictive relationship between the motivational valence of MA and subsequent drug-taking behavior; females exhibit MA-taking behavior and reinforcement, despite their initial perception of the stimulant interoceptive effects as positive, neutral or negative.

Keywords

1. Introduction

Methamphetamine (MA) is an amphetamine derivative with high abuse potential that leads to devastating psychophysiological and socioeconomic consequences (e.g., Gailbraith, 2015). Excluding nicotine and alcohol, amphetamine-type stimulants constitute the third most abused drug globally (United Nations, 2018) and within the United States, overdose deaths related to amphetamine-type stimulants (including MA) have risen 7.5 times between 2007 and 2017, with 15 percent of all drug overdose deaths involving methamphetamine (Center for Disease Control). MA-taking elicits a variety of drug effects in the individual user that range from those typically perceived to have positive motivational/affective valence (e.g., euphoria and high energy) to those often perceived to have negative motivational/affective valence (anxiety, dysphoria, headaches, cardiovascular hyperactivity) (Cruickshank and Dyer 2009; Sheridan et al. 2009). Clinical and epidemiological data point to individual differences in the perception of a drug's effects as appetitive or aversive as having a major influence upon the risk of continued drug abuse and subsequent addiction (e.g., Chait 1993; Davidson et al. 1993; de Wit et al. 1986; DiFranza et al. 2004; Fergusson et al. 2003; Schuckit et al. 1997). However, a large number of confounding variables (incl. other drug abuse or therapeutic drug treatment, trauma &/or frequent aversive life events, duration of drug abstinence etc.) render it difficult to disentangle cause-effect relations between subject factors, biomarkers and addiction vulnerability/resiliency in human subjects, as it is impossible to study these relations in any systematic, experimentally-controlled, fashion.

Clinical and epidemiological evidence indicate that women begin using MA use at an earlier age and develop a more severe addiction than men (Brecht et al., 2004; Dluzin and Liu, 2008; Hser et al., 2005; Rawson et al. 2005; United Nations 2019). Indeed, female humans and rodents are more sensitive than males to the psychomotor-activating properties of MA (Johnson et al., 2000; Mayo et al., 2019; Milesi-Hallé et al. 2007; Ohia-Nwoko et al. 2017; Schindler et al., 2002) and female rodents exhibit greater motivation to self-administer MA and to reinstate MA-seeking behavior (Cox et al. 2013; Reichel et al., 2012; Ruda-Kucerova et al. 2015). Less is known regarding sex differences in the initial perception of MA's effects as appetitive versus aversive and how this initial perception might contribute to subsequent addiction vulnerability.

Developing and validating animal models of addiction vulnerability/resiliency are critical to elucidating the interactions between genetic and environmental subject factors in the addiction etiology that cannot be readily disentangled in studies of human drug users. The large-scale, population-based, nature of studying individual differences in addiction requires an animal model that is procedurally facile, high throughput, relatively non-invasive and inexpensive. Arguably, such a model should be able to index both the positive and negative motivational/affective valence of a drug to identify both addiction-vulnerable and -resilient individuals. To gauge individual differences in drug preference/aversion, choice procedures have been implemented within the contexts of both intravenous and oral self-administration models, involving both operant and non-operant procedures, (e.g., Banks et al. 2015; Caprioli et al. 2015; Czoty and Nader 2015; Lenoir et al. 2013; c.f. Bell et al. 2014; Crabbe et al. 2013; Rodd et al. 2004; Vandaele et al. 2016). The application of choice self-administration procedures have resulted in the generation of a large number of different selected rodent lines for the study of genetic variance in addiction-related traits, in particular drug intake (e.g., P vs. NP rat, HAD vs. LAD rat, HAP vs. LAP mouse, SHAC vs. SLAC mouse, MAHDR1/2 vs. MALDR1/2 mouse etc; c.f., Bell et al. 2006, 2014; Crabbe 2014; McBride et al. 2014; Reed et al., 2020; Wheeler et al. 2009). While possessing high face validity for human addiction, voluntary drug self-administration methods do not tend to control for the amount or the timing of drug exposure across individuals. This fact limits their utility for studying the biobehavioral correlates of *idiopathic* addiction vulnerability/resiliency, as results are confounded by individual variance in drug-intake/dosing (Sanchis-Segura and Spanagel 2006).

In contrast, place-conditioning methods have significant advantages over the more conventional self-administration models of addiction in that place-conditioning provides the simultaneous assessment of both the positive & negative motivational/affective valence of a drug in an undrugged state, which is essential for understanding the underpinnings of preference/aversion without confounding drug effects upon psychomotor activity or motivational measures. More importantly, the non-contingent drug regimens employed to elicit a drug-conditioned response provide control over drug exposure (see Bardo and Bevins 2000; Sanchis-Segura and Spanagel 2006; Tzschentke 2007). This latter fact is critical for disentangling the relative contribution of drug vs. other subject factors in the manifestation of an addiction vulnerable/resilient phenotype and the biobehavioral correlates of that phenotype. It has been argued that place-conditioning methods have low face validity for addiction in humans as the motivational valence of a drug is assessed in subjects treated non-contingently with drug. However, this approach completely avoids the "chicken-egg" issue resulting from individual variability in drug intake/dosing that confounds data interpretation & reduces the significance of findings obtained under self-administration methods (c.f., Bardo and Bevins 2000; Sanchis-Segura and Spanagel 2006; Tzschentke 2007). Genetic variance in oral MA intake generalizes to behavior under MA-induced place-conditioning procedures in mice (Eastwood et al. 2014; Shabani et al. 2011, 2012a, 2012b; Wheeler et al., 2009) and we have identified marked behavioral heterogeneity in place-conditioning induced by a moderate MA dose (2 mg/kg) within commercially available male, inbred, C57BL/6J (B6) mice obtained from the Jackson Laboratory (Sacramento, CA). Of relevance to this report, the magnitude and direction of the place-conditioned response was positively

correlated with subsequent MA self-administration behavior under operant-conditioning procedures in males, indicating that the motivational valence of MA predicts subsequent drug self-administration, at least in male mice (Szumlinski et al. 2017).

Given the purported sex differences in the psychomotor-activating and reinforcing properties of MA, in this study, we replicated our prior study of male mice (Szumlinski et al. 2017) in females to test the hypotheses that (1) a greater proportion of a population of female mice will perceive the subjective effects of a moderate MA dose as positive, compared to that observed in males and (2) the motivational valence of MA expressed under place-conditioning procedures will correlate with subsequent MA-taking behavior, as observed in males.

2. Materials and Methods

2.1 Mice:

C57BL/6J (**B6J**) inbred females (Jackson Laboratories, Sacramento, CA; 10–12 weeks of age) were housed in groups of four within polycarbonate cages under standard conditions. Mice were housed under a regular 12-hour light/dark cycle (lights on: 07:00) during the place-conditioning phase of the study. In line with prior MA-induced place-conditioning studies by our group (Fultz and Szumlinski, 2018; Ruan et al., 2020; Sern et al., 2020; Szumlinski et al., 2017), place-conditioning was conducted during the light phase to minimize spontaneous activity and facilitate detection of group differences in MA-induced hyper-activity, as well as maximize the extent of the conditioned response (see Brown et al., 2020 for discussion). As conducted in our prior study of male B6J mice (Szumlinski et al., 2017), upon completion of place-conditioning procedures, mice were relocated to an adjacent colony room and housed under a reversed 12-hour light/dark cycle (lights off: 11:00) for a minimum of 7 days prior to operant-conditioning. As such, operant-conditioning procedures occurred during the dark/active phase of the cycle when animals exhibit higher levels of arousal and attention to facilitate learning. Food and water were available *ad libitum* with the exception of the time animals were engaged in behavioral testing. As the goal of this study was to extend our prior work in males to females, the estrous cycle was not monitored to avoid introducing a procedural confound. The experiments followed a protocol consistent with NIH guidelines presented in the recently revised *Guide for Care and Use of Laboratory Animals* (2014) and approved by the IACUC of the University of California, Santa Barbara.

2.2 Place-conditioning:

To identify variance in the ability of MA to induce place-conditioning, we employed a conditioning dose (2 mg/kg, IP), coupled with a relatively short conditioning period (15 min). Using this approach, we identified subpopulations of male B6J mice that exhibited high CPP (conditioned place-preference), high CPA (conditioned place-aversion), as well as relative place-ambivalence (a.k.a., Neutral mice). Importantly, when the entire cohort of female B6J mice was considered, the 2 mg/kg dose produced an overall CPP, which is consistent with other reports in B6J male mice (Bryant et al. 2012; Lominac et al. 2014, 2016; Takamatsu et al. 2006, 2011; Watanabe 2015). Overall, the selection of dose, as well

as the duration and number of conditioning sessions proved to be effective in inducing place conditioning in the majority of mice (see Results). Further, critical for the goals of the present study, induced differential valence place-conditioning in subpopulations of female B6J mice.

The procedures to induce place-conditioning were identical to those employed in our previous study of male mice (Szumlinski et al 2017). The paradigm involved 4 pairings each of saline (SAL) and 2 mg/kg MA (vol=10 ml/kg) with distinct compartments of a 2-chamber apparatus (46 cm long × 24 cm high × 22 cm wide) that differed in wall pattern and floor texture. One side was wall-papered with a wood-paneling design and had a smooth floor, while the other side was wall-papered with a marble design and had a rough floor. Digital video-tracking (ANYMaze, Stoelting Co., Wood Dale, IL USA) automatically recorded the time spent in each of the two compartments, as well as the total distance traveled by the mice to index locomotor hyperactivity and the development of locomotor sensitization during conditioning. Place-conditioning procedures commenced with a 15-min habituation session during which animals had free-access to both compartments. Each mouse was then assigned to receive MA in either the marble or wood sides of the chamber in an unbiased fashion. Each conditioning session was 15 min in duration and was initiated immediately after injection. The conditioning sessions occurred twice daily, with SAL pairings occurring in the mornings and MA pairings taking place at least 5 h later in the afternoon and mice underwent a total of 4 days of conditioning. The day following the last conditioning session, , a 15-min post-conditioning test was performed in which animals had free-access to both compartments in a MA-free state (Post-test) and the CPP Score was calculated as the time spent on the MA-paired versus SAL-paired compartment (in sec). Positive CPP Scores indicate a preference for the MA-paired compartment, while negative CPP Scores indicate a preference for the SAL-paired compartment (i.e. MA-aversion). As conducted in our prior study (Szumlinski et al. 2017), we phenotyped mice as "CPP" (conditioned place-preference) if their CPP Score was >+100 sec, "CPA" (conditioned place-aversion) if their CPP Score was <−100 sec & "Neutral" if their CPP Score fell between −99 and 99 sec. The data were analyzed by analyses of variance (ANOVA), followed by t-tests or LSD post-hoc tests as appropriate and α=0.05. Pearson correlational analyses (corrected for multiple comparisons) were also conducted to relate CPP Scores to locomotor activity during different phases of the study. The available number of operant-chambers limited the total number of mice that could be tested under operant-conditioning procedures for any given place-conditioning cohort. Given the distribution of subjects, priority was given to obtaining operant data from CPA mice, followed by Neutral and then CPP mice. To maximize control over environmental factors and avoid single-housing of mice, we opted to conduct operant-conditioning procedures only in CPP mice co-housed with CPA and/or Neutral animals. In other words, the selection of CPP mice for operant testing was independent of their actual CPP Score. With this approach, 37 of the 54 mice phenotyped as CPP underwent our operant-conditioning procedures, while brain tissue was collected from the remaining CPP mice to create a tissue bank for future study.

2.3   Oral MA Reinforcement:

The procedures used to induce operant-conditioning for an oral MA reinforcer were also identical to those described in our previous report of male mice (Szumlinski et al., 2017). Following initial phenotyping under our MA place-conditioning procedures, groups of CPP, Neutral & CPA mice were trained in daily 1-h sessions to nose-poke for delivery of unadulterated MA solutions (prepared in tap water; reinforcer volume=20 μl). The apparatus employed to assess operant-conditioning for MA reinforcement consisted of standard mouse operant-conditioning chambers (Med Associates, St Albans, VT), fitted with 2 nose-poke holes and a liquid receptacle located between the nose-poke holes, all housed within ventilated sound-attenuated chambers. Responses in the active (MA-associated) hole resulted in the activation of the infusion pump, delivery of 20 μl of the MA reinforcer into the receptacle, and the presentation of a 20-sec light/tone compound stimulus. During the 20-sec MA-delivery period, further responding in the active hole was recorded but had no programmed consequences. Throughout the session, responding in the inactive hole had no programmed consequences but was recorded to index the selectively of responding and general motor activity.

CPP, CPA and Neutral mice were first trained to nose-poke for delivery of a 20 mg/L MA solution. We selected this MA concentration for response acquisition in our original study (Szumlinski et al. 2017) as mice on a mixed B6-D2 background will voluntarily consume this concentration in the home cage (e.g., Kamens et al. 2005; Wheeler et al. 2009). Indeed, male B6 mice exhibiting a CPP readily meet acquisition criterion for operant-conditioning within the first 5 days of training under this procedure, while Neutral and CPA males meet acquisition criteria within 2 weeks of training (Szumlinski et al. 2017). Thus, this low-dose training procedure can distinguish between CPP, Neutral and CPA male mice. Self-administration training commenced under a fixed ratio 1 (FR1) schedule of reinforcement (+ 20-sec time-out) and mice were trained daily until they earned a minimum of 10 reinforcers/session, with greater than 70% of their responding directed towards the active lever. We next progressively increased the number of nose-pokes required for delivery of 20 mg/L MA (maintaining the 20-sec time-out) over subsequent days (4–5 days/schedule) in order to increase behavioral output and engender more selective responding in the active hole. As MA intake dropped as a function of reinforcement schedule, we then returned the mice to an FR1 (+ 20-sec time-out) schedule of reinforcement and a dose-response study of MA reinforcement and intake (5–400 mg/L MA) was conducted (3–5 days/dose).

At the end of each 1-h operant session, the volume of solution remaining in the receptacle was determined by pipetting (Szumlinski et al. 2017) and mice were returned to the colony room and left undisturbed until the next day. Total MA intake was determined each day by subtracting the volume of MA remaining in the receptacle from the total volume delivered and was expressed as a function of body weight (in mg/kg), which was determined weekly. The data were analyzed by a mixed ANOVA, with repeated measures on the factors of Training Day, Reinforcement Schedule, and MA Dose. Two-tailed Pearson correlational analyses were also conducted to relate dependent measures with CPP Score. α=0.05 for these analyses.

3. Results

3.1 MA-induced place-conditioning.

Of the 102 female mice tested, 54 exhibited a CPP (52.9%), 33 exhibited no conditioning (32.4%), while only 15 mice exhibited a CPA (14.7%). There was little variability in the place-conditioning data for any of the groups and, as expected given their categorization, the group differences in CPP Score were statistically significant from each other (Fig. 1A; one-way ANOVA, F(2,101)=144.22, p<0.0001; LSD post-hoc tests]. The place-conditioning phenotype was not related to, or predicted by, their initial locomotor reactivity to the place-conditioning apparatus (Fig.1B, Pre-Test; one-way ANOVA, p=0.64; r=0.05, p=0.62), the locomotor response to an acute injection of either saline (Fig.1B, SAL1; one-way ANOVA, p=0.45; r=0.08, p=0.44) or 2 mg/kg MA (Fig.1B, MA1; one-way ANOVA, p=0.60; r=0.06, p=0.56). Nor were group differences noted for the locomotor activity expressed during the Post-Test (Fig.1B, Post-Test; one-way ANOVA, p=0.43; r=0.05, p=0.62). Interestingly, group differences were observed with respect to the extent to which saline-induced locomotion habituated over the 4 saline-conditioning sessions [F(1,101)=3.13, p=0.048]. While both CPP and Neutral mice exhibited the expected reduction in saline-induced locomotion, CPA mice exhibited an increase in locomotor hyperactivity that was significantly different from that of the other two groups (Fig.1C, Habituation; LSD post-hoc tests: CPA vs. CPP, p=0.03, CPA vs. Neutral p=0.02) and the extent of locomotor habituation inversely correlated with the direction and magnitude of conditioned response in female mice (r=−0.25, p=0.01). Likewise, a modest group difference was apparent in the reduction in locomotor activity expressed between the Pre- and Post-Tests, when mice had access to both compartments [Test effect: F(1,99)=11.19, p=0.001; Test by Phenotype interaction: F(2,99)=2.67, p=0.07], that reflected significantly less between-test habituation in CPA mice versus both CPP and Neutral animals (LSD post-hoc tests: vs. CPP, p=0.03; vs. Neutral, p=0.04). Further, correlational analyses supported a significant inverse relationship between CPP Score and the extent to which mice habituated from the Pre - to Post-Tests (r= −0.22, p=0.03). However, no group differences were observed with respect to the extent of MA-induced locomotor sensitization (Fig.1C; one-way ANOVA, p=0.76), nor was there a significant correlation between the magnitude of locomotor sensitization and CPP Score (r= −0.10, p=0.30).

3.2 Acquisition of Operant Responding for Oral MA.

Mice were then trained over the course of 2 weeks to nose-poke for 20 mg/L MA under an FR1 (+ 20-sec time-out) schedule of reinforcement and the data for this initial acquisition phase is presented in Fig. 2. We first examined for group differences in initial responding for oral MA by comparing operant behavior over the first 5 days of self-administration training and also examined the average behavioral responses during the last 3 days of the training phase of this experiment. No group differences were observed for the decrease in MA-reinforced nose-poking behavior exhibited by female mice during the initial 5-day training period (Fig.2A) [Session effect: F(4,352)=9.62, p<0.0001; Phenotype effect and interaction, p's>0.60]. Further, no group differences were apparent for the average active nose-pokes over the last 3 days of training (Fig.2A'; one-way ANOVA, p=0.29), and CPP Score was not predictive of active nose-poking behavior (r=−0.14, p=0.10, n=91). No group differences

were noted for the decrease in inactive hole-pokes during initial training (Fig.2B) [Session effect: $F(4,352)=35.52$, $p<0.0001$; Phenotype effect and interaction, p's>0.60] or for the average inactive hole-pokes at the end of training (Fig.2B'; one-way ANOVA, $p=0.67$), and CPP Score was not correlated with inactive hole-poking behavior ($r=-0.06$, $p=0.57$, $n=91$). Not surprisingly, there was no group differences in the allocation of responding on the active lever during early training (Fig.2C) [Session effect: $F(4,352)=9.65$, $p<0.0001$; Phenotype effect: $p=0.59$; interaction: $p=0.92$] or late training (Fig.2C'; one-way ANOVA, $p=0.86$; correlation: $r=-0.03$, $p=0.78$). Finally, there were also no group differences in MA intake during the first 5 days of training (Fig.2D) [Session effect: $F(3,352)=7.48$, $p<0.0001$; Phenotype effect and interaction, p's>0.40: $p=0.43$] or late training (Fig.2D'; one-way ANOVA: $p=0.34$; correlation: $r=-0.13$, $p=0.22$, $n=91$).

3.3 Demand-Response Testing.

Having established no phenotypic differences in the acquisition of operant-responding for 20 mg/L MA, we next established demand-response curves for reinforcement by this MA concentration and the data are presented in Fig.3. Unlike males (Szumlinski et al., 2017), female mice increased their active nose-poking for 20 mg/L MA in response to increasing response demand [Schedule effect: $F(2,164)=54.55$, $p<0.0001$], but there were no group differences in this regard (Fig.3A; Phenotype effect and interaction, p's>0.33). Concomitant with an increase in active nose-poking was a modest, but significant, increase in the number of inactive nose pokes emitted during demand-response testing (Fig.3B) [Schedule effect: $F(2,164)=4.55$, $p=0.01$]. However, no group difference was apparent for this measure (Phenotype effect and interaction, p's>0.55). A Phenotype by Schedule interaction was detected for the response allocation towards the active hole (Fig.3C) [Schedule effect: $F(2,164)=4.77$, $p=0.01$; Phenotype effect, $p=0.51$; interaction, $F(2,164)=2.38$, $p=0.05$]. However, deconstruction of this interaction along the Schedule factor failed to indicate significant group differences at any of the reinforcement schedules (univariate ANOVAs, for FR1, $p=0.93$; for FR2: $p=0.07$; for FR5: $p=0.44$). As observed in males (Szumlinski et al. 2017), MA intake declined as a function of increasing response requirement, but there was no phenotypic difference in MA intake during this phase of testing (Fig.3D) [Schedule effect: $F(2,164)=127.23$, $p<0.0001$; other p's>0.25].

3.4 Dose-Response Testing.

Lastly, the female mice underwent dose-response testing under an FR1 (+ 20-sec time-out) schedule of reinforcement (Fig.4). Although the dose-response function for active nose-poking by CPP mice appeared to be shifted below that of the other two groups (particularly at the higher MA concentrations; Fig.4A), the group difference in active nose-poking was not statistically significant [Dose effect: $F(8,656)=3.92$, $p<0.0001$; Phenotype effect and interaction, p's>0.09]. No group differences were apparent for inactive hole-poking at any MA concentration tested (Fig.4B) [Dose effect: $F(8,656)=2.03$, $p=0.04$; Phenotype effect and interaction, p's>45] or for dose-response allocation function, which was flat and remained well above the 70% criterion (Fig.4C; Phenotype X Dose ANOVA, all p's>0.20). In stark contrast to our prior findings for males (Szumlinski et al. 2017), MA intake increased linearly as a function of dose in female [Dose effect: $F(8,656)=202.41$, $p<0.0001$] and we detected a significant Phenotype X Dose interaction (Fig.4D) [Phenotype effect:

p=0.38; interaction: $F_{(16,656)}=2.36$, p=0.002]. While this interaction appeared to reflect less MA intake by CPP versus CPA mice at higher MA doses (Fig.4C), deconstruction of the data along the Dose factor failed to detect significant group differences at any of the MA concentrations tested (one-way ANOVAs for 5–200 mg/L, all p's>0.30; for 400 mg/L, p=0.09). As inspection of Fig.4C strongly suggested a difference in the intake of 400 mg/L between CPP and CPA mice and given the significant interaction detected by omnibus ANOVA, a planned comparison was conducted between these groups for this concentration and revealed significantly higher intake in the CPA versus CPP females (p=0.03). Further, correlational analyses conducted between CPP Score and MA intake at each concentration during dose-response testing indicated an inverse relationship between these variables, which was statistically reliable at the 160 mg/L and 400 mg/L concentration, with a strong trend observed at the 200 mg/L concentration (see Table 1).

4. Discussion

Previously, we showed that MA-induced place-conditioning procedures can be applied to commercially available male, isogenic, B6J mice to identify individual differences in the propensity for subsequent MA-taking behavior under operant-conditioning procedures as a complementary approach to selectively bred lines to study the neurobiology of MA addiction vulnerability (Szumlinski et al. 2017). As our prior work focused exclusively on males, the present study extended our investigation to female subjects with the hypotheses that MA-induced place-conditioning procedures would be similarly effective at identifying "addiction-vulnerable" and "addiction-resilient" females, with a greater proportion of females initially exhibiting MA-preference versus that previously reported for males. While our prior results showed that individual differences in place-conditioning positively correlate with measures of oral MA reinforcement and intake under operant-conditioning procedures in males (Szumlinski et al. 2017), for females, we detected an opposite relationship. Below, we discuss the significance of these findings for the validation of MA-induced place-conditioning as a procedurally simple tool for identifying individuals that model idiopathic MA addiction vulnerability, as well as resiliency and for the study of sex differences in MA reward and reinforcement.

4.1. Biological sex does not impact the relative motivational/affective valence of 2 mg/kg MA

Similar to B6J males (Szumlinski et al., 2017), the present findings indicate that there is substantial variation in the motivational valence impact of 2 mg/kg MA across B6J females. However, contrary to our hypothesis, the proportion of B6J females categorized as CPP, Neutral and CPA was nearly identical to that reported previously in B6J males (Szumlinski et al., 2017). Such findings argue against a role for genetic/chromosomal, organizational or activational factors related to biological sex in the early perception of 2 mg/kg MA as appetitive or aversive. Whether or not the relative proportion of mice exhibiting CPP vs. neutrality/CPA varies with the MA-conditioning dose or genetic background are important research questions that should be targeted in future work. However, it is interesting to note that when 2 mg/kg MA is employed to induce place-conditioning, neither the magnitude of MA's acute locomotor response (Fig.1B), nor the magnitude of locomotor sensitization

produced by repeated MA-pairing (Fig.1C), is in any way related to place-conditioning phenotype of B6J females. This contrasts with our prior study of B6J males in which the magnitude of MA-induced locomotor sensitization during the conditioning phase of the study inversely related to the degree of place-preference exhibited in a drug-free state (Szumlinski et al., 2017). Thus, in contrast to B6J males (Szumlinski et al., 2017), individual differences in sensitivity to MA's psychomotor-activating or -sensitizing effects do not predict the affective motivational valence of the drug in B6J females.

As female rodents and humans tend to be more sensitive to the psychomotor-activating effects of MA (Johnson et al., 2000; Mayo et al., 2019; Milesi-Hallé et al. 2007; Ohia-Nwoko et al. 2017; Schindler et al., 2002), the lack of any overt relationship between CPP Score and MA-induced locomotor sensitization in female subjects might reflect a ceiling effect upon behavior. The dose-response function for MA-induced locomotor activity is an inverted U-shape, with higher doses eliciting focused, stereotyped, behaviors that are physically incompatible with horizontal locomotion. Moreover, the time of onset, frequency and intensity of stereotyped behaviors increases upon repeated exposure to stimulant-type drugs (e.g., Segal et al., 1981; Segal and Kuczenski, 1994). As our video-tracking system is only capable of recording the displacement of the animals' center of gravity (i.e., movement along the horizontal plane), it remains to be determined how CPP Score relates to other measures of MA-induced motor hyperactivity (e.g., rearing, focused sniffing) or changes in such behaviors with repeated MA-pairings of relevance to understanding the psychobiological factors driving initial MA preference/aversion in laboratory animal models.

4.2. Oral MA is reinforcing in a population of female B6J mice

Humans self-administer amphetamines by various routes, and while the preferred route of administration varies with geographical region, the oral route is common in initial drug abuse (Comchai and Comchai, 2015; Courtney and Ray, 2014; Panenka et al. 2013; see also www.nida.nih.org). This observation lends face validity to oral MA self-administration methods for studying abuse- and addiction-related traits in laboratory animals (e.g., Kamens et al. 2005; Reed et al., 2020; Shabani et al., 2019; Wheeler et al. 2009). In contrast to traditional intravenous (IV) self-administration methods, oral self-administration methods are procedurally simple, technically facile and non-invasive, which renders them preferable for large-scale studies, particularly in mice. While B6J mice are reported to orally consume less MA than the DBA/2J mouse strain (Wheeler et al., 2009), oral MA is reinforcing in male B6J mice (Szumlinski et al. 2017), as well as male and female mice on a mixed B6J-DBA/2J background (Shabani et al. 2012a). Here, we replicate our findings for B6J males in B6J females by providing evidence that B6J females will respond for, and consume, MA solutions within the range of 5 to 400 mg/L under an FR1 reinforcement schedule. As reported for B6J males (Szumlinski et al., 2017), B6J females can be trained to nose-poke for a relatively low concentration of MA (20 mg/L) in the absence of any food/fluid restriction, prior response training with a palatable reinforcer or adulteration of the MA solution - procedural variables that can confound data interpretation (see Sanchis-Segura and Spanagel 2006). However, akin to B6J males (Szumlinski et al., 2017), B6J females are highly sensitive to response demand, with MA intake dropping precipitously when mice are

required to emit more than 2 nose-pokes for reinforcement by the 20 mg/L MA solution (Fig. 3D). Whether or not the drop in MA intake with increasing response demand varies as a function of MA concentration, duration of the self-administration session or operadum employed (i.e., nose-poke hole vs. lever) are important procedural variables that will be systematically evaluated as we move forward developing our mouse model.

4.3.   Apparent sex differences in oral MA reinforcement and intake in B6J mice.

Consistent with prior evidence that B6J mice tend to exhibit low oral MA intake (Wheeler et al., 2009), we reported that the dose-response function for MA intake by male B6J mice under operant-conditioning procedures exhibits an inverted U-shape over a relatively narrow dose-range (5–40 mg/L), at least when mice are trained at the 20 mg/L concentration as in the present study. Morever, 40 mg/L MA was found to lie on the descending limb of the MA dose-intake function in male B6J mice - an observation that prompted us at the time to forego testing of higher MA concentrations (Szumlinski et al., 2017). Inverted U-shaped dose-intake functions can be interpreted as reflecting either drug satiation or self-restraint over intake to avoid the aversive effects associated with higher drug doses (Lynch 2001). Typically, it is difficult to delineate between these possibilities when animals are tested under operant self-administration procedures alone. However, as we had characterized both MA's locomotor and affective/motivational properties prior to operant-conditioning procedures, we were able to begin to dissect relationships between these properties and subsequent drug-taking and concluded that, in B6J males, both early MA reinforcement and subsequent MA intake relates inversely to the drug's aversive, psychomotor-activating effects (Szumlinski et al., 2017) - an interpretation consistent with reports from the laboratory of T.J. Phillips indicating an inverse relationship between MA intake and aversion sensitivity in B6J-DBA2/J hybrid mice (Harkness et al., 2015; Shabani et al. 2011, 2012a, 2012b; Wheeler et al. 2009) and aligning with results from human studies (e.g., Chait 1993; Davidson et al. 1993; de Wit et al. 1986; DiFranza et al. 2004; Fergusson et al. 2003; Schuckit et al. 1997).

In stark contrast to male B6J mice (Szumlinski et al., 2017), the dose-response function for MA intake increased linearly across a 100-fold dose-range in B6J females, with 400 mg/L MA lying on the ascending limb of the function (Fig.4D). At the time of the female study, 400 mg/L MA was the highest concentration authorized for study by the UCSB IACUC, which prevented the testing of higher MA concentrations and a more complete characterization of the dose-intake function in our female mice. Nevertheless, it is interesting to note that the peak MA intake observed in our prior study of B6J males occurred at 20 mg/L and was approximately 0.15 mg/kg (Szumlinski et al., 2017). In contrast, the intake of the 20 mg/L solution by female B6J mice was approximately 4 times that amount, with their intakes of 200 mg/L MA nearly 100 times the peak intake exhibited by B6J males (Fig.4D). Thus, unlike B6J males (Szumlinski et al., 2017), B6J females are capable of orally self-administering very high doses of MA over a relatively short time-period (>15 mg/kg in 1h), suggesting neither drug satiation nor self-restraint over intake. Such findings extend the results of studies of IV MA self-administration in rats (Cox et al. 2013; Reichel et al., 2012; Ruda-Kucerova et al. 2015) by indicating that a sex difference appears to exist with respect to both the initial reinforcing properties of oral, low-dose, MA,

as well as the dose-sensitivity of oral MA intake in mice with established self-administration behavior.

To the best of our knowledge, prior studies of oral MA self-administration in mice have not reported sex differences in drug intake under either home-cage (e.g., Harkness et al., 2015; Shabani et al., 2011, 2012b; Stafford et al., 2020; Wheeler et al., 2009) or operant-conditioning procedures (Ruan et al., 2020; Shabani et al., 2012a). Indeed, a more recent operant-conditioning study by our group, in which male and female congenic B6J mice were trained and tested concurrently, failed to detect a sex difference in MA intake across an 80–400 mg/L dose-range, with MA intake peaking at 160 mg/L and concentrations greater than 200 mg/L MA lying on the descending limb of the dose-response function (Ruan et al., 2020). In this more recent study, we trained mice to self-administer 80 mg/L MA (*in lieu* of the 20mg/L concentration employed in our study of isogenic B6J mice) and no sex difference in any of our acquisition parameters (e.g., days to reach acquisition criterion, MA intake, active hole-poking or response allocation) were detected (Ruan et al., 2020). Taken altogether, the limited data available from studies of operant-conditioning for oral MA reinforcement in B6J mice (Ruan et al., 2020; Szumlinski et al., 2017; present study) suggest a dose by sex interaction with respect to both the initial acquisition of MA self-administration and the dose-sensitivity of intake following the establishment of stable drug-taking behavior, of key relevance to the design of future studies examining how biological sex might influence early MA abuse and the transition to addiction.

Interestingly, a sex by dose interaction is reported for MA metabolism, at least in rats infused IV with MA (Miliesi-Hallé et al., 2015). While it is tempting to speculate that the apparent sex differences in low-dose oral MA intake between B6J males and females (Szumlinski et al., 2017 and present study) might relate to differences in MA pharmacokinetics, females metabolize MA more slowly and less completely than males when administered doses > 1 mg/kg (Milesi-Hallé et al., 2005). At doses less than 3 mg/kg (IV), no sex difference is observed for MA metabolism (e.g., Milesi-Hallé et al., 2005; 2015). Whether or not sex differences exist for blood or brain levels of MA when the drug is orally self-administered is not known and is an important consideration for future work, both with respect to sex, as well as individual, differences in MA reward and reinforcement. However, if the sex differences reported for MA metabolism in rat (Milesi-Hallé et al., 2005; 2015) extend to the oral route of administration and to mice, it is difficult to reconcile a slower drug metabolism/excretion in female mice with a *greater* capacity to consume drug. A sex difference is also reported with respect to high dose MA-induced neurotoxicity (females < males), which relates to more efficient uptake of dopamine by DAT and VMAT in female vs. male subjects (Bhatt and Dluzen, 2005; Dluzen et al., 2008; Ji et al., 2007; Morissette and Di Paolo, 1993; Walker et al., 2000). Further, the estrous cycle is reported to affect MA-induced dopamine toxicity in female mice; however, estrous regulation of MA-induced dopamine depletion appears to be strain-dependent and the severity of MA-induced neurotoxicity is reported not to vary with estrous cycle in the C57BL/6J strain employed herein (Yu and Liao, 2000). To the best of our knowledge, the precise relevance of sex differences in dopamine uptake or in other measures of dopamine neurotransmission (e.g., D1 or D2 receptor function; see Yoest et al., 2014 for review) for MA self-administration behavior has yet to be explored in any systematic manner. Although the capacity of MA to

reinstate drug-seeking behavior does not appear to vary with estrous cycle in female rats (Cox et al., 2013), we do not know how circulating ovarian hormones influence MA intake or reinforcement in female laboratory rodents to even speculate on whether or not (let alone, how) ovarian hormones might impact initial MA preference/motivational valence, early MA self-administration or the dose-dependency of MA-taking behavior.

4.3. Apparent sex differences in the predictive relationship between place- and operant-conditioning phenotype in B6J mice

We hypothesized at the outset of this study that the place-conditioning phenotype exhibited by female B6J mice following conditioning with 2 mg/kg MA predicts subsequent MA self-administration, as reported previously in B6J males (Szumlinski et al., 2017). Contrary to our hypothesis, place-conditioning phenotype did not generalize to measures of MA reinforcement or intake during either early or later self-administration in B6J females. In males, differences in MA reinforcement were observed between CPP, CPA and Neutral mice as early as the first 5 days of training, with the majority of CPP mice meeting the criterion for operant-response acquisition within this early phase of training and some CPA and Neutral mice failing to reach the acquisition criteria by the end of the 14-day training period (Szumlinski et al., 2017). In contrast, females from all three place-conditioning phenotypes met the criterion for response acquisition by the 5th day of training and there was no significant difference in the rate of acquisition or the amount of MA consumed during the early phase of training (Fig. 3C). While such findings are consistent with a greater tendency for females to acquire MA self-administration more readily, and to consume more MA, than males (Cox et al., 2013; Reichel et al., 2012), they do not support the predictive validity of MA-induced place-conditioning for the initiation of MA self-administration behavior in females, at least when 20 mg/L MA serves as the initial reinforcer and 2 mg/kg MA is employed as the place-conditioning dose. Such data suggest that, in contrast to males, the expression of MA-aversion is context-dependent in female B6 mice and/or may simply relate to self-control over drug exposure. Whether or not a clearer relationship between CPP Score and early responding for MA would manifest in female B6J mice if behavior was reinforced by a different MA concentration is a research question that we intend to address in future parametric study. From the extant data suggesting a sex by reinforcer dose interaction (Ruan et al., 2020; Szumlinski et al., 2017; present study), we argue that the initial MA concentration available during the acquisition phase of operant-conditioning is indeed a major procedural factor affecting the ability to detect individual differences in both initial and subsequent MA-taking behavior.

As discussed above, in our initial study of isogenic B6J males, the MA dose-intake function was inverted U-shaped across a very narrow dose-range (5–40 mg/L). Nevertheless, the entire MA dose-intake function was shifted upwards in CPP males, relative to their Neutral and CPA counterparts, and CPP Score was positively correlated with a number of MA self-administration measures. Together, these data from B6J males supported the predictive validity of place-conditioning procedures for subsequent MA-taking (Szumlinski et al., 2017). In contrast to males, the place-conditioning phenotype expressed by B6J females in the present study had no obvious relationship with MA reinforcement or intake at doses 200 mg/L during dose-response testing; indices of MA reinforcement and intake were

comparable between CPP, Neutral and CPA females. Interestingly, an *inverse* relationship was detected between CPP Score and the intake of the highest MA concentration tested in this study (400 mg/L), with female CPP mice consuming significantly *less* 400 mg/L MA than their CPA counterparts (Fig.4D). As we were not permitted to assay for group differences beyond the 400 mg/L concentration, it remains to be determined whether or not the magnitude or direction of the group difference in MA intake observed at 400 mg/L MA in female B6J mice would change with higher MA concentrations. Thus, based on the present data, we conclude that B6J females can self-administer large amounts of MA irrespective (or in spite) of the affective/motivational valence of their initial drug experience.

The significant inverse correlation detected between CPP Score and the intake of 400 mg/L MA in female B6J mice was a very unexpected result that cannot readily be explained by individual differences in MA-induced psychomotor activation (as indexed during place-conditioning) or preservative responding in the operant-chamber (as indexed by total responding or response allocation), with the caveat that stereotypy was not assayed during either place- or operant-conditioning procedures. The result also does not align with the dogma that drug-taking behavior tends to inversely correlate with initial sensitivity to a drug's aversive properties. At the present time, we do not know why CPA females consume more 400 mg/L MA than CPP mice nor do we know if the effect is replicable. It does seem peculiar that a CPP-CPA difference is not detected in females until the mice are highly MA-experienced. It is not likely that the higher intake of 400 mg/L MA exhibited by CPA mice reflects the development of tolerance to the drug's aversive effects as CPA females exhibited no signs of MA-aversion at any time during the operant-conditioning phase of the study. Curiously, the CPA females were the only group that failed to habituate their locomotor activity in response to daily saline injections and/or the repeated exposure to the entire place-conditioning apparatus during testing (Fig.1C). While initial behavioral hyper-reactivity to a novel environment can predict subsequent low-dose MA self-administration in male rats (Ganarcz et al., 2011), a predictive relationship between novelty-induced locomotor hyperactivity and subsequent MA self-administration has not been observed in mice (Shabani et al. 2011; Szumlinski et al., 2017; Wheeler et al., 2009; Fig.1B). As mice underwent two conditioning sessions per day (saline in the morning, MA in the later afternoon), a failure of CPA females to habituate during the saline-conditioning sessions may reflect a growing anticipation of the behaviorally non-contingent, presumably anxiogenic, forthcoming MA injection. Indeed, anxiety-like behavior is reported to correlate with genetic vulnerability to high MA consumption in both male and female mice and with the severity of MA dependence in humans (Huckans et al., 2017). However, a higher basal anxiety-like state in CPA mice would be predicted to impact MA-taking from the outset of operant-conditioning, which was not observed in the present study.

Alternatively, MA is reported to impair the ability to habituate to a neutral stimulus (Lloyd et al., 2014). To the best of our knowledge, a predictive relationship between habituation failure, the subjective effects of MA, MA reinforcement, and intake has not been explored in either humans or laboratory animals. The fact that no obvious relationship exists between locomotor habituation and MA preference or taking in B6J males (Szumlinski et al., 2017) argues that any such relationships may be sex-dependent. In neuropsychiatric conditions, such as schizophrenia, autism spectrum disorder, Fragile X syndrome, Parkinson's Disease,

Huntington's Disease, Attention Deficit Hyperactivity Disorder and Tourette's syndrome, symptom severity correlates with the degree of impaired habituation (cf., McDiarmid et al., 2017). This raises the intriguing possibility that MA addiction severity might relate to individual differences in sensorimotor habituation, specifically in females, worthy of further exploration at the preclinical and clinical levels.

Finally, it is possible that CPA female mice may be more sensitive to MA-induced neuroplasticity driving an escalation of drug-taking behavior. While we have yet to obtain a sufficient amount of brain tissue to examine the biochemical correlates of a MA-preferring vs. -avoiding phenotype in B6J females or to examine the effects of repeated MA exposure upon mesocorticolimbic neurotransmission in female mice, CPP Score is correlated with a number of glutamate abnormalities within both the nucleus accumbens and prefrontal cortex of B6J and B6J-DBA/2J hybrid males that are reminiscent of the effects of repeated MA exposure upon glutamate function in these regions (Lominac et al., 2016; Szumlinski et al., 2017). Indeed, a sex difference exists with respect to the effects of long-access IV MA self-administration upon both basal and evoked excitability of neurons within the prelimbic cortex of rats, with females exhibiting greater MA-induced neuroexcitability than males, which may contribute to the higher addiction vulnerability reported in females (Pena-Bravo et al., 2019). How individual differences in MA-taking (both within and between sex) relate to drug-induced adaptations within neurocircuits gating the affective/motivational and reinforcing properties of MA is one of the penultimate research questions facing scientists trying to understand the neurobiology of addiction vulnerability.

4.4 Conclusions.

The results of the present experiment indicate that while the proportions of a population of female mice exhibiting MA-preference, -ambivalence and -aversion under place-conditioning procedures are comparable to males, an apparent sex difference exists in the predictive validity of MA-induced place-conditioning procedure as a tool for studying neuropsychological underpinnings of idiopathic MA addiction vulnerability/resiliency in an isogenic mouse strain. In contrast to male B6 mice, the affective/motivational valence of behaviorally non-contingent MA exposure is inversely correlated with both locomotor habituation expressed in a drug-free state during place-conditioning and subsequent high-dose MA intake in B6J females. The present findings highlight a role for sex-related factors in a murine model of oral MA self-administration. Further, the present results indicate that sex-related factors influence the predictive relationship between the initial perception of MA's subjective effects as appetitive versus aversive and subsequent drug-taking behavior of relevance to understanding individual differences in MA addiction vulnerability/resiliency.

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Highlights

- Methamphetamine (MA) addiction is again reaching epidemic proportions requiring the development of validated high through-put screens of addiction vulnerability to study underlying neuropsychology.

- In attempt to generalize prior results from male isogenic mice, female C57BL/6J mice underwent place-conditioning procedures, followed by operant-conditioning for oral MA reinforcement.

- A positive correlation was observed between locomotor habituation during place-conditioning and subsequent MA-taking in female mice.

- An inverse relationship was observed between MA's affective/motivational valence and subsequent MA-taking in female mice.

- Together with past findings, the present study indicates a sex difference exists with respect to the predictive relationship between place- and operant-conditioning phenotype in isogenic C57BL/6J mice.
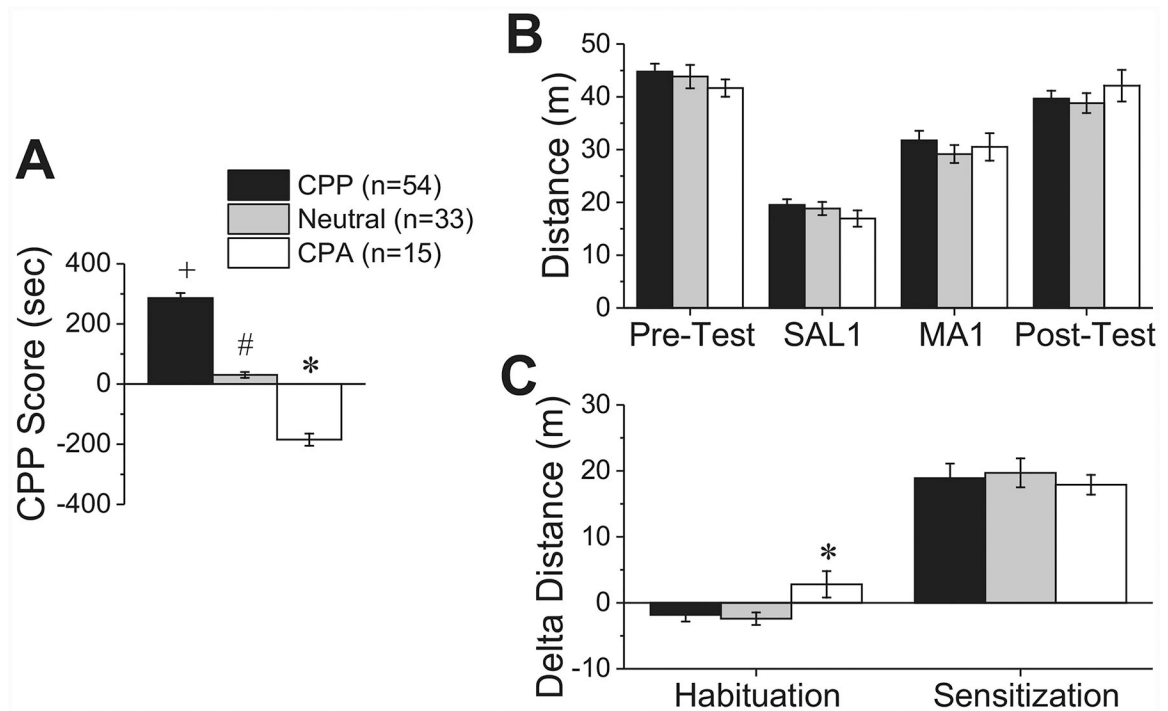
Figure 1: Summary of group differences in MA-induced place-conditioning, spontaneous and MA-induced locomotion between CPP, CPA and Neutral female B6 mice.
(A) Summary of the difference in the time spent in the MA-paired vs. SAL-paired compartments (CPP Score) exhibited by isogenic female B6 mice phenotyped as CPP and CPA during the post-conditioning test. **(B)** Summary of the average distance traveled by the mice during the 15-min pre- and post-conditioning tests when mice had access to both compartments of the place-conditioning apparatus in a drug-free state (Pre-Test and Post-Test) and during their first saline (SAL1) and 2 mg/kg methamphetamine (MA1) conditioning session. **(C)** Summary of the average change in distance traveled (Detla Distance) between the first and fourth saline-conditioning (Habituation) and methamphetamine-conditioning session (Sensitization). The data represent the means ± SEMs of the number of animals indicated in parentheses in pane. A. *p<0.05 vs. CPP and Neutral; +p<0.05 vs. Neutral and CPA; #p<0.05 vs. CPP and CPA (LSD post-hoc tests).
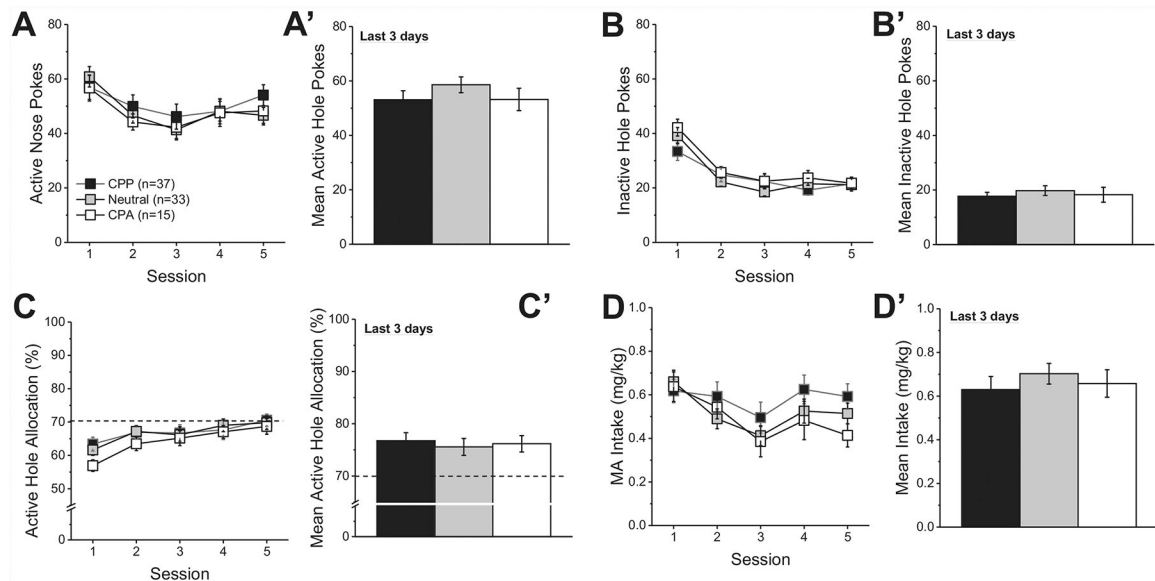
Figure 2: Place-conditioning phenotype does not transfer to operant behavior expressed during early training to response for reinforcement by 20 mg/L MA self-administration.
Summary of the total number of active nose-pokes emitted by the 3 different place-conditioning phenotypes during **(A)** the first 5 days of operant training (1 h sessions; FR1 reinforcement schedule) and **(A')** averaged across the last 3 days of the 2 weeks of training. Summary of the total number of inactive hole pokes **(B & B')**, the relative number of nose-pokes directed at the MA-appropriate, active, hole **(C & C')** and the total MA intake **(D & D')** exhibited during early operant-conditioning with a 20 mg/L MA reinforcer. The data represent the means ± SEMs of the number of animals indicated in parentheses in Panel A.
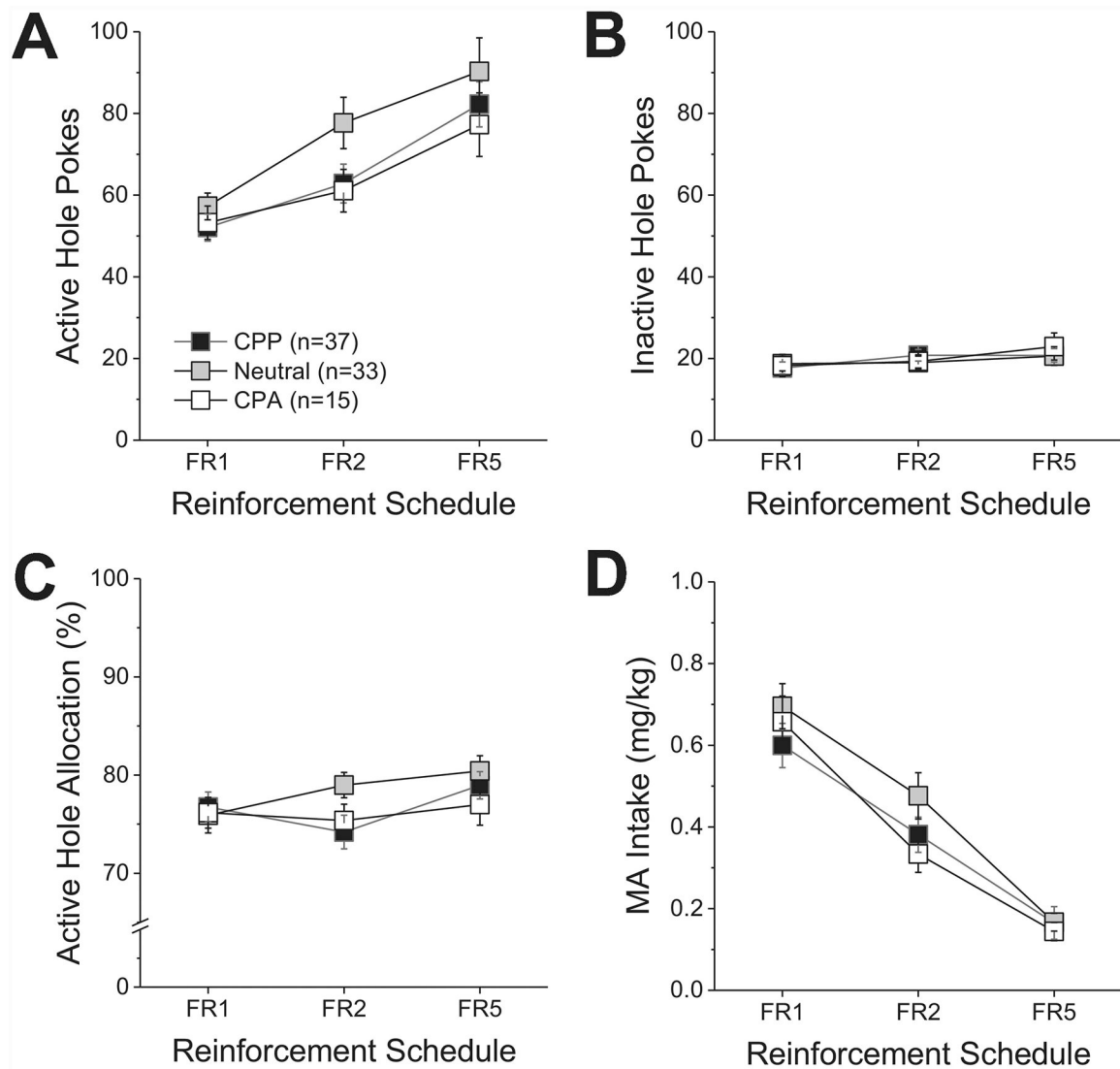
Figure 3: Place-conditioning phenotype does not transfer to operant behavior expressed during demand-response testing, reinforced by 20 mg/L MA.

Summary of the total number of active nose-pokes (**A**), inactive nose-pokes (**B**), response allocation (**C**) and MA intake (**D**) expressed by CPP, Neutral and CPA female mice when the response requirement for 20 mg/L MA reinforcement was increased from FR1, to FR2 to FR5. The data represent the means ± SEMs of the number of animals indicated in parentheses in Panel A.
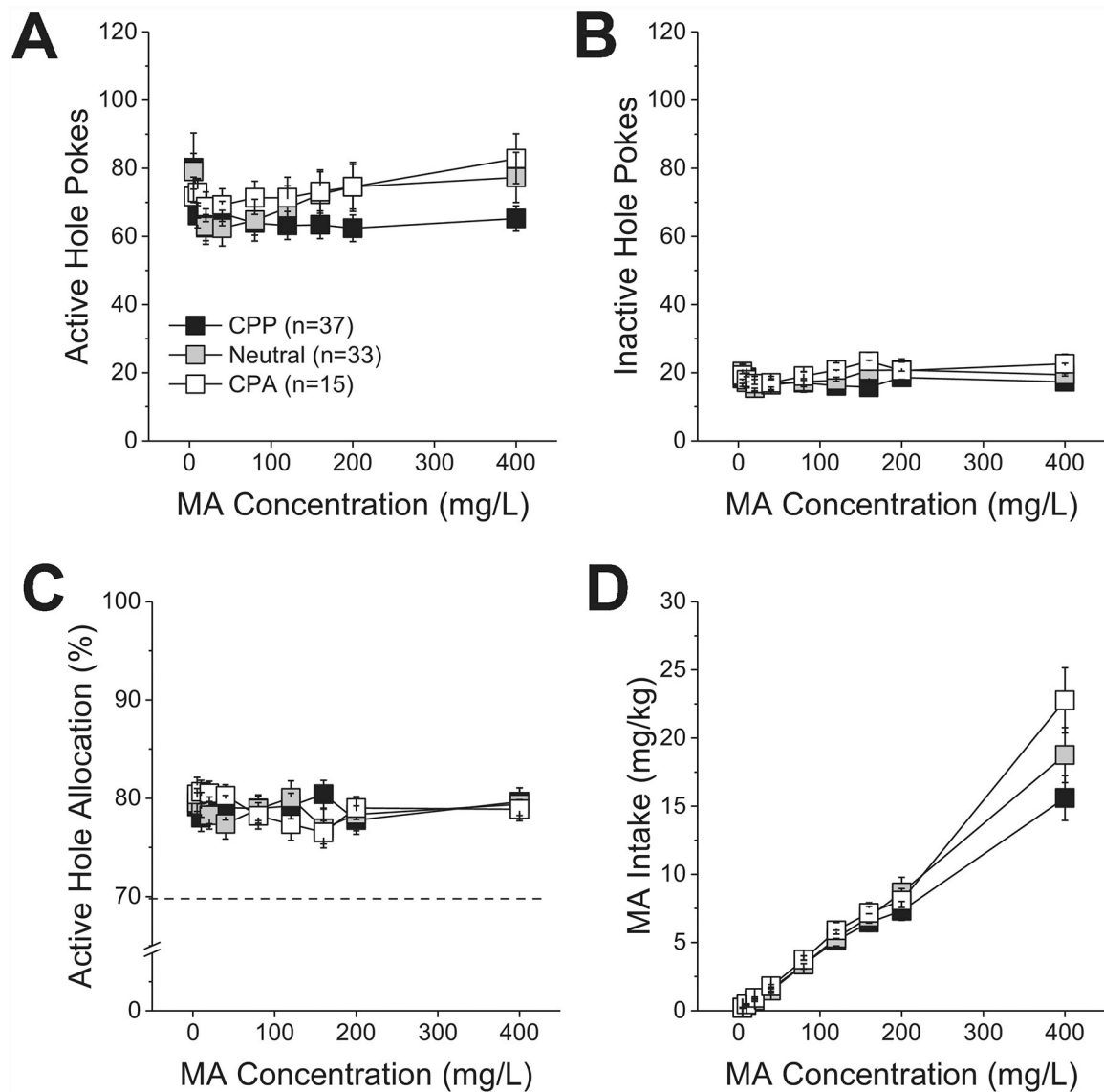
Figure 4. Place-conditioning phenotype does not transfer to operant behavior expressed during dose-response testing across a 100-fold MA dose-range.

Summary of the total number of active nose-pokes (**A**), inactive nose-pokes (**B**), response allocation (**C**) and MA intake (**D**) expressed by CPP, Neutral and CPA female mice as a function of the MA concentration serving as the reinforcer (5–400 mg/L). The data represent the means ± SEMs of the number of animals indicated in parentheses in Panel A.

Table 1:

Summary of the results of correlational analyses conducted between the CPP Score derived from place-conditioning and the average intake of each MA concentration during dose-response testing. N=85 mice.

MA Concentration	Statistic
5 mg/L	r= 0.74, p=0.61
10 mg/L	r= −0.10, p=0.48
20 mg/L	r= −0.07, p=0.59
40 mg/L	r= −0.09, p=0.53
80 mg/L	r= −0.07, p=0.66
120 mg/L	r= −0.26, p=0.14
160 mg/L	r= −0.35, p=0.048 [*]
200 mg/L	r= −0.32, p=0.07
400 mg/L	r= −0.35, p=0.04 [*]

[*]
p<0.05