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Variability in nicotine conditioned place preference, stress-induced reinstatement, and effects of guanfacine in male and female mice

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Abstract

Relapse to smoking occurs at higher rates in women compared to men, especially when triggered by stress. Studies suggest that sex-specific interactions between nicotine reward and stress contribute to these sex differences. Accordingly, novel treatment options targeting stress pathways, such as guanfacine, an α2-adrenergic receptor agonist, may provide sex-sensitive therapeutic effects. Preclinical studies are critical for elucidating neurobiological mechanisms of stress-induced relapse and potential therapies, but rodent models of nicotine addiction are often hindered by large behavioral variability. In this study, we used nicotine conditioned place preference to investigate stress-induced reinstatement of nicotine preference in male and female mice, and the effects of guanfacine on this behavior. Our results showed that overall, nicotine induced significant place preference acquisition and swim stress-induced reinstatement in both male and female mice, but with different nicotine dose-response patterns. In addition, we explored the variability in nicotine-dependent behaviors with median split analyses and found that initial chamber preference in each sex differentially accounted for variability in stress-induced reinstatement. In groups that showed significant stress-induced reinstatement, pretreatment with guanfacine attenuated this behavior. Finally, we evaluated neuronal activation by Arc immunoreactivity in the infralimbic cortex, prelimbic cortex, anterior insula, basolateral amygdala, lateral central amygdala, and nucleus accumbens core and shell. Guanfacine induced sex-dependent changes in Arc immunoreactivity in the infralimbic cortex and anterior insula. This study demonstrates sex-dependent relationships between initial chamber preference and stress-induced reinstatement of nicotine conditioned place preference, and the effects of guanfacine on both behavior and neurobiological mechanisms.

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Authors Contributions

AML, YSM and MRP designed the experiment. AML performed behavioral testing, immunohistochemical staining, microscopic imaging and data analysis. CAC performed microscopic imaging. SAM and YSM contributed to data analysis and interpretation. AML drafted the manuscript. All authors reviewed and edited the manuscript.

Conflict of Interest

The authors have no conflicts of interest to disclose.

Keywords

nicotine; conditioned place preference; sex differences; stress; reinstatement; guanfacine

1 INTRODUCTION

Tobacco smoking remains a major public health problem [1]. Despite the availability of smoking cessation medications, more than 70% of smokers who make a quit attempt relapse within a year [2]. Notably, clinical and epidemiological studies demonstrate significant sex differences in relapse behavior [3]. Women showed 31% greater odds of relapse to smoking over a 30-day period compared to men [4], and 44% greater odds over a 3-year period [5].

Nicotine, the primary psychoactive component in tobacco, is a relatively weak primary reinforcer [6,7]. However, nicotine can also enhance the rewarding properties of nonpharmacological stimuli [6,8,9], enhance cognitive function [10], and interact with stress and anxiety [11–13] to support addiction-related behaviors. Interactions between these various effects of nicotine may account for the well-documented inter-individual variability in nicotine use and relapse observed in both human studies and rodent models [13–18]. Further, individual differences may interact with sex to affect nicotine use. For example, women are less sensitive to the primary reinforcing effects of nicotine than men [19], but are more motivated by nicotine's effects on stress and negative affect [19–21]. Stress is a well-established, sex-divergent factor contributing to relapse to nicotine use [12,22,23]. Greater negative affect is reported by women than men during abstinence from nicotine, and this negative affect is a greater predictor of lapse or relapse in women [24–26]. Women are also more likely to relapse in response to stressful life events [27]. Accordingly, smoking cessation medications also vary in effectiveness between men and women. Nicotine replacement therapies are less effective in women than in men [28], and clonidine, an α2-adrenergic receptor (α2-AR) agonist, is more effective in women than men for achieving smoking cessation [29].

Preclinical studies have demonstrated the role of noradrenaline and α2-ARs in stress-induced drug self-administration [22,30,31] and conditioned place preference (CPP) [32,33]. CPP is one useful model to study the rewarding effects of drugs because it involves a short training period relative to self-administration, allows testing in drug-free states, and is adaptable to various species [34]. Although stress-induced reinstatement of nicotine seeking has been demonstrated in both self-administration [35,36] and CPP [37–39], only self-administration studies have examined either the effects of α2-AR agonists on stress-induced reinstatement of nicotine seeking [35,36], or sex differences in stress-induced reinstatement [13,40]. Further, no preclinical studies using either self-administration or CPP have examined sex differences in the effect of α2-AR agonists on stress-induced reinstatement, despite clinical evidence suggesting its sex-specific mechanisms [29,41,42].

We therefore established a model to investigate sex differences in stress-induced reinstatement of nicotine CPP in male and female mice, and to determine the effects of guanfacine on reinstatement. Other preclinical studies in male rodents have used clonidine, which induces several adverse side effects including sedation and postural hypotension [41].

Guanfacine is an FDA-approved α2-AR agonist that is more specific for the α2A subtype and has less sedative and hypotensive effects than clonidine [43]. Furthermore, guanfacine attenuates stress-precipitated nicotine craving and *ad libitum* smoking in abstinent smokers [44]. C3H/HeJ mice were selected for this study because they exhibit an increase in locomotion after acute, systemic nicotine injection, unlike most other strains [45], and because mice less sensitive to the hypolocomotor effects of an acute nicotine injection exhibit greater nicotine-induced conditioned place preference [46]. We used a counterbalanced CPP design, where nicotine is paired with both initially preferred and non-preferred chambers across animals (as opposed to a biased design, where nicotine is paired only with the initially non-preferred chamber) in order to avoid pitfalls in interpretation and to leverage any variability in the data for more insight into nicotine CPP mechanisms [34,47,48]. We used a forced swim stress to induce reinstatement of nicotine CPP, as has been used previously in male mice [38]. Finally, we used immunohistochemistry for the immediate-early gene Arc [49] to identify brain regions that are potential substrates for sex differences in the effects of nicotine, stress, and guanfacine.

2 METHODS

2.1 Animals

Male and female C3H/HeJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) at 6-10 weeks of age, and tested at 10-12 weeks of age, following at least one week of acclimation. Mice were group-housed, maintained on a 12-hour light-dark cycle (lights on at 7:00 AM), and provided standard chow and water *ad libitum*.

Dose-response, guanfacine, and Arc immunohistochemistry experiments were conducted in independent groups of mice. Sample sizes were determined based on preliminary data, previous studies of sex differences in nicotine CPP [50] and immunohistochemical markers of neuronal activity [51], and a power analysis for behavioral experiments indicating a sample size of approximately 12 mice to detect a moderate effect size ($d = 0.3$, $\beta = 80\%$, $\alpha = 0.05$). All procedures were approved by the Yale University Institutional Animal Care and Use Committee.

2.2 Drugs

Nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline and injected subcutaneously (s.c.) at free base concentrations of 0.5, 0.75, or 1.0 mg/kg at 10 ml/kg. Guanfacine (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline and injected intraperitoneally (0.15 mg/kg injected at 10 ml/kg).

2.3 Apparatus

CPP training and testing were conducted in a three-chamber CPP apparatus (Med Associates, Inc., Fairfax, VT, USA). The two conditioning chambers are distinguished by tactile cues (flooring consisting of either horizontal or vertical steel rods overlaid by a wire grid) and a single visual cue (opaque tape 1.25 cm x 2.5 cm affixed to the door of one chamber); the interiors of both chambers are black. Manual guillotine doors separate the two

conditioning chambers from a neutral, middle, gray chamber with smooth flooring. Med-PC IV software was used to record time spent in each chamber.

2.4 CPP acquisition

All behavioral testing took place in an isolated, dimly lit room with only the experimenter present, between 8 a.m. and 1 p.m. Each day, mice were transferred to the testing room and allowed to acclimate for at least 30 min prior to handling or testing.

On days 1-3, mice were handled as described previously [52], with additional habituation to a syringe (without needle) applied to the back of the neck as if administering a subcutaneous injection. On day 4, the pretest day, each mouse was placed in the middle gray chamber, and after a 5-sec acclimation, the guillotine doors were opened manually. The mice then freely explored all three chambers for 15 min and time spent in each chamber was recorded. Mice that spent > 50% of the time in any of the three chambers were excluded from further evaluation (16/106 males and 14/136 females). Subsequently, conditioning group (control vs. nicotine) and drug-chamber pairings, were pseudo-randomly assigned to achieve a balanced CPP design. On days 5-10, saline or nicotine was injected subcutaneously and the mouse was immediately confined to one conditioning chamber for 30 min. Injection and chamber were alternated between days. Control mice received saline in both conditioning chambers, but one of the saline-paired chambers was arbitrarily labeled as the "drug" chamber solely for data analyses. On day 11, the post-test day, chamber preference was evaluated for 15 min as on the pretest day. Drug preference is expressed as time spent in the drug-paired chamber minus time spent in the saline-paired chamber on test day.

2.5 CPP abstinence, extinction, and reinstatement

Following the post-test, mice were subjected to two weeks of forced abstinence in the home-cage. This abstinence period was first included to reflect the human condition of relapse after abstinence and then empirically tested and found to be necessary for stress-induced reinstatement. Similarly, cue-induced reinstatement of nicotine self-administration has been demonstrated after 7 days of abstinence, but not after 1 day of abstinence [53], and footshock-induced reinstatement of heroin self-administration after 6 and 12 days of abstinence, but not after 1 day of abstinence [54]. After the abstinence period, CPP was extinguished by repeated drug-free testing sessions (15-min free exploration). Drug preference on the first day of extinction was considered the “post-abstinence” score, and the last day of extinction was achieved when average drug preference minus the standard error of the mean was below zero. Twenty-four hours after extinction was achieved, mice were tested for stress-induced reinstatement of nicotine CPP by exposure to a 6-min forced swim stress in 21-22°C water, followed by 30 min of recovery in a paper towel-lined cage, then a 15-min test in the CPP apparatus as above. For the dose-response experiment, no injections preceded the swim stress. In guanfacine experiments, mice in each conditioning group were split into two sub-groups with roughly equivalent pretest, acquisition, post-abstinence, and extinction scores, and each group received an injection of either guanfacine (0.15 mg/kg) or saline i.p. 30 min prior to the stressor. The forced swim session was recorded, and videos were subsequently blinded and scored for time spent immobile during the 6-min swim.

2.6 Estrous phase by visualization

Vaginal openings were visually inspected for width of the opening, swelling of the tissue, tissue color and moistness [55]. Phase was recorded as "proestrous," including proestrous and estrous phases, or "non-proestrous," including diestrous and metestrous phases. We observed a greater proportion of females in non-proestrous phases compared to proestrous phases; this distribution may reflect the Lee-Boot effect, wherein group-housed female mice have been observed to exhibit more irregular estrous cycles with a prolonged diestrous phase [56,57].

2.7 Behavioral conditioning and tissue collection for Arc immunohistochemistry

Separate cohorts of mice were subjected to a parallel behavioral protocol for immunohistochemistry measurements. Briefly, mice were handled for 3 days, then administered alternating injections of nicotine or saline for 6 days, followed immediately by 30 min of isolation in alternating contexts with distinct tactile cues (the smooth floor of an empty mouse cage or a layer of clean bedding). Following conditioning, mice were subjected to 2 weeks of forced abstinence, after which they were administered an injection of guanfacine or saline followed 30 min later by a 6-min forced swim stress, as described above. 90 min after the forced swim stressor, mice were transcardially perfused with ice-cold 1X PBS, then ice-cold 4% paraformaldehyde. Brains were collected and post-fixed in 4% paraformaldehyde overnight, then transferred to 30% sucrose in 1X PBS. Brains were sectioned into 40 μm-thick coronal sections on a freezing sliding microtome (Leica SM 200R) and collected in 1X PBS with 0.02% sodium azide.

2.8 Arc immunohistochemistry

Sections containing the anatomical region of interest were selected from every 6th section of the entire brain for stereological counting and were stained using a standard protocol [58]. Regions selected include the anterior insula (AI), infralimbic cortex (IL), prelimbic cortex (PL), nucleus accumbens (NAc) core and shell, basolateral amygdala (BLA), and lateral nucleus of the central amygdala (CeL) (Figure S3). Primary antibody incubation occurred overnight at room temperature in 1:1000 guinea pig anti-Arc (Synaptic Systems, Goettingen, Germany). Secondary antibody incubation occurred for 2 hr at room temperature in 1:1000 donkey anti-guinea pig AlexaFluor647 (MilliporeSigma, St. Louis, MO) and was followed by a 5 min incubation in 1:1000 DAPI (Thermo Scientific, Germany). Sections were then mounted on SuperFrost Plus (Fisher Scientific) glass slides. Coverslips were applied with Fluoromount-G mounting solution (Electron Microscopy Sciences, Hatfield, PA) and sealed with clear nail polish. Tissue was processed in batches, with all experimental groups represented in each batch, and results were pooled for final analysis.

2.9 Arc cell counting

Immunohistochemically stained brain sections were imaged with an Olympus FluoView FV10i confocal microscope with a 10X objective. Image files were blinded prior to analysis using the Fiji image processing package [59]. A threshold was applied to create a binary image in which the top 2.5% of the signal intensity histogram was considered foreground and the rest background. The Watershed function was applied, separating putatively overlapping

cells with a 1 pixel-wide line. Within each ROI, cells were then counted using the Analyze Particles function with a size restriction of 60-3000 μm^2. The area of each ROI was also measured in order to calculate Arc-immunoreactive cells per mm^2. See Table S1 for n's of each group.

2.10 Statistical Analysis

GraphPad Prism 8.0 software was used for data analysis and graph production. For all data, outlier analyses were performed using GraphPad's ROUT function with the standard Q = 1% [60]. No outliers were identified in any behavioral dataset. For Arc data, one outlier was removed from the CeL dataset (female, nicotine conditioning group, saline pre-treatment) and one from the IL dataset (female, control conditioning group, guanfacine pre-treatment). Subsequently, data were analyzed within sex by mixed 2-way Repeated Measures ANOVAs. For the dose-response experiment, ANOVAs were followed up with Dunnet's tests within nicotine dose in order to compare drug preference on acquisition, post-abstinence, extinction, and reinstatement days to the pretest preference as a baseline. For guanfacine and Arc experiments, significant ($p < 0.05$) interactions were followed by Sidak's multiple comparison tests. For median split analyses, 2-way Repeated Measures ANOVAs were conducted within sex with split subgroup as between-subjects factors and test day as within-subjects factors. Significant interactions were followed by Dunnett's tests to compare test days to the pretest day within split group, and Tukey's post-hoc test to compare the same test days across split groups. Brown-Forsythe tests were also conducted to test for significant differences in variance using the lawstat package in R (https://CRAN.R-project.org/package=lawstat). All graphs show means with standard error of the mean.

3 RESULTS

3.1 Stress-induced reinstatement of nicotine CPP is demonstrated in male and female mice administered 0.5 mg/kg nicotine, but only in female mice administered 0.75 mg/kg nicotine

Male and female C3H/HeJ mice were administered 0, 0.5, 0.75, or 1.0 mg/kg nicotine in the drug-paired chamber to determine the dose response curves for stress-induced reinstatement of nicotine CPP (Figure 1). In males, a two-way mixed ANOVA revealed a significant effect of test day ($F_{(4, 152)} = 4.75$, $p = 0.001$), but no effect of dose ($F_{(3, 38)} = 1.51$, $p = 0.227$) and no interaction ($F_{(12, 152)} = 1.53$, $p = 0.119$; Figure 1b). Planned *post-hoc* analyses with Dunnett's tests comparing preference scores to the pretest preference within dose showed significant stress-induced reinstatement of nicotine CPP only in the 0.5 mg/kg nicotine group ($p = 0.0002$). At this dose, there was a trend for a positive increase in drug preference between pretest and acquisition ($M_{diff} = 101.1$, 95% CI [5.76, 196.5]). A follow-up analysis of the multiple cohorts of male mice tested in Figures 1, 3, and 4 confirmed that the 0.5 mg/kg dose of nicotine produces significant CPP acquisition and post-abstinence nicotine-chamber preference, followed by successful extinction (Figure 2a,b). A two-way mixed ANOVA with conditioning group (saline control vs. 0.5 mg/kg nicotine) as a between-subjects factor and test day as a within-subjects factor showed a significant interaction ($F_{(3, 213)} = 3.58$, $p = 0.015$) and significant main effects of conditioning group ($F_{(1,71)} = 9.02$, $p = 0.0037$) and test day ($F_{(3, 213)} = 6.14$, $p = 0.0005$). Dunnett's *post-hoc* test showed

significant differences between pretest and acquisition preference scores ($p = 0.0020$) and between pretest and post-abstinence ($p < 0.001$) in animals administered 0.5 mg/kg nicotine, but no significant differences in the control group (pretest vs. acquisition: $p = 0.75$; pretest vs. post-abstinence: $p = 0.96$; Figure 2a). Acquisition scores were also examined by cohort to further demonstrate significant CPP acquisition across cohorts, and there was a main effect of conditioning drug ($F_{(1,67)} = 7.53$, $p = 0.0078$) but no effect of cohort ($F_{(2,67)} = 0.30$, $p = 0.74$) or an interaction ($F_{(2,67)} = 1.0$, $p = 0.37$; Figure 2b). Reinstatement data was not pooled across cohorts due to the experimental variable of treatment prior to the forced swim stressor (none for dose-response experiments, i.e. cohort 1, and saline or guanfacine pre-treatment in cohorts 2 and 3).

In female mice, a two-way ANOVA across test days and dose revealed a significant interaction ($F_{(12, 168)} = 3.23$, $p = 0.0003$) and a significant main effect of dose ($F_{(3, 42)} = 3.20$, $p = 0.033$), but no significant main effect of test day ($F_{(4, 168)} = 1.66$, $p = 0.162$; Figure 1c). Dunnett's tests showed significant stress-induced reinstatement of nicotine place preference compared to pretest preference in mice administered 0.5 mg/kg ($p = 0.0004$) and 0.75 mg/kg ($p = 0.041$) nicotine. However, the difference in means between pretest and acquisition was greater in the 0.75 mg/kg cohort ($M_{\text{diff}} = 68.5$ [95% CI: -35.82, 172.9]) than in the 0.5 mg/kg cohort ($M_{\text{diff}} = 11.04$ [95% CI: -113.3, 135.4]). Subsequent cohorts were tested with 0.75 mg/kg nicotine, and follow-up analyses of all cohorts used in our experiments confirmed that treatment with this nicotine dose produced a significant CPP acquisition and post-abstinence preference compared to pretest scores in female mice (Figure 2c,d). A two-way mixed ANOVA showed a significant interaction of test day and conditioning drug ($F_{(3,303)} = 3.70$, $p = 0.012$), and Dunnett's *post-hoc* test showed a significant increase in preference between pretest and acquisition ($p = 0.024$) and pretest and post-abstinence tests ($p = 0.0007$) in the 0.75 mg/kg nicotine group but not in the control conditioning group (pretest vs. acquisition: $p = 0.99$; pretest vs. post-abstinence: $p = 0.97$; Figure 2c). Extinction preference scores were not different from pretest preference in either 0.75 mg/kg nicotine or control conditioning groups. Further comparison of acquisition scores by the various cohorts showed a main effect of conditioning group ($F_{(1,95)} = 5.30$, $p = 0.024$) but no main effect of cohort ($F_{(3,95)} = 0.16$, $p = 0.92$) or an interaction ($F_{(3,95)} = 0.65$, $p = 0.56$; Figure 2d).

Previous studies have shown that female rats and mice display a right-shifted dose-response curve for nicotine CPP [50,61,62]. Thus testing for the effects of guanfacine on stress-induced reinstatement proceeded with the use of 0.5 mg/kg nicotine to train male mice and 0.75 mg/kg nicotine to train female mice.

3.2 The effect of guanfacine is not significant in whole-cohort analyses

Male and female mice were tested for stress-induced reinstatement of nicotine CPP as above, with the addition of saline or guanfacine (0.15 mg/kg, i.p.) treatment 30 min prior to the forced swim stressor. There was a sex difference in the degree of variance in reinstatement preference scores, as shown by the Brown-Forsythe test (male vs. female: test statistic = 23.39, $p < 0.0001$). There were no differences in variance between conditioning

groups (control vs. nicotine: test statistic = 1.015, $p = 0.316$) or pre-stress treatments (saline vs. guanfacine: test statistic = 0.469, $p = 0.495$).

Due to the significant difference in variance and the different nicotine doses used in male (0.5 mg/kg) and female (0.75 mg/kg) mice, separate two-way ANOVAs were conducted within sex. In males, there was a significant interaction between conditioning group and pre-stress treatment ($F_{(1, 44)} = 4.57$, $p = 0.038$), but no main effects of conditioning group ($F_{(1, 44)} = 0.703$, $p = 0.406$) or pre-stress treatment ($F_{(1, 44)} = 0.0056$, $p = 0.941$; Figure 3a). *Post-hoc* analyses with Sidak's multiple comparisons tests showed an almost significant stress-induced reinstatement of nicotine CPP in nicotine vs. control male mice receiving saline pre-treatment ($t_{(44)} = 2.20$, $p = 0.066$), but not in male mice receiving guanfacine pre-treatment ($t_{(44)} = 0.882$, $p = 0.619$). In females, a two-way ANOVA showed no significant main effects of conditioning group ($F_{(1, 44)} = 0.738$, $p = 0.393$) or pre-stress treatment ($F_{(1, 44)} = 0.301$, $p = 0.585$) or an interaction ($F_{(1, 44)} = 1.88$, $p = 0.175$; Figure 3b).

Immobility during the forced swim stressor was analyzed with two-way ANOVAs within sex. Results showed that guanfacine treatment did not change time spent immobile during the forced swim in either males (Figure S1a) or females (Figure S1b), although there was a main effect of nicotine treatment in lowering immobility in males ($F_{(1, 44)} = 17.93$, $p = 0.0001$) but not females.

This initial analysis of the data suggests that guanfacine did not significantly attenuate stress-induced reinstatement in either male or female mice, although there was a significant interaction and a trending post-hoc test in male mice. However, there was a notable degree of variability in individuals' preference scores in the reinstatement test. Subsequently, we further examined this variability to determine whether results might vary based on specific subgroups of mice.

3.3 Initial chamber preference interacts with stress and guanfacine on nicotine CPP reinstatement: male mice

To explore the variability in CPP reinstatement in both male and female mice, we conducted median split analyses, where subjects within each treatment group, comprised of the same sex (M = male, F = female), conditioning group (C = control [saline], N = nicotine), and pre-swim treatment (S = saline, G = guanfacine) were split into two subgroups across the median of preference scores on the pretest day. The "low" subgroup spent less time in, or avoided, the future drug-paired chamber on pretest day (Lo-Pre), and the "high" subgroup demonstrated an initial preference for the future drug-paired chamber (Hi-Pre). A two-way ANOVA was then conducted with median split subgroups across conditioning groups and pre-swim treatments within sex as a factor, and all test days as the second factor. Although the analyses were conducted across all split subgroups within sex, data are presented in Figure 4 as separate for saline and guanfacine pre-treatment subgroups for ease of viewing.

In males, there was a significant interaction between initial chamber preference subgroup and test day ($F_{(28,160)} = 1.93$, $p = 0.0062$) and a significant main effect of subgroup ($F_{(7,40)} = 5.15$, $p = 0.0003$) but not test day ($F_{(4,160)} = 1.92$, $p = 0.11$; Figure 4a,b). The significant interaction was followed by Dunnett's tests to compare the pretest day to each subsequent

test day within subgroup, and Tukey's tests to compare the same test days across subgroups. In the Lo-Pre nicotine-treated subgroups, preference for the nicotine-paired chamber over the saline-paired chamber (preference score) after acquisition was significantly different from pretest in the subgroup that would subsequently receive guanfacine prior to stress-induced reinstatement (MNG Lo-Pre; $p = 0.032$; Figure 4b), and nearly significant in the subgroup that would subsequently receive saline prior to stress-induced reinstatement (MNS Lo-Pre; $p = 0.094$; Figure 4a). Similar, or even greater, average preference score for the nicotine-paired chamber was measured after acquisition in the Hi-Pre MNG and MNS subgroups, but these were not significantly different from pretest, as was expected given the high pretest preference scores. In contrast, Hi-Pre control conditioning subgroups (MCS Hi-Pre and MCG Hi-Pre) show acquisition test preference scores that trend towards zero (Figure 4a,b). Further, the MCG Lo-Pre control group also shows an average preference score on acquisition testing that trends towards zero, and MCS Lo-Pre shows a positive preference score on acquisition testing that is significantly different from pretest ($p = 0.0028$; Figure 4a). This contrast between control and nicotine conditioning groups supports the idea that nicotine-specific processes drive chamber preference. Further, preference for the nicotine-paired chamber is maintained after two weeks of abstinence; in MNS Lo-Pre, post-abstinence preference is even enhanced over the acquisition score and is significantly different from pretest ($p = 0.002$; Figure 4a).

Finally, stress-induced reinstatement was significantly different from pretest only for MNS Hi-Pre ($p = 0.049$; Figure 4a), and this reinstatement was significantly different from reinstatement in MCS Lo-Pre ($p < 0.0001$), MNS Lo-Pre ($p = 0.0046$), and MNG Lo-Pre ($p = 0.0007$). These data show that only male mice that received nicotine in the initially preferred chamber subsequently demonstrate stress-induced reinstatement of nicotine CPP, despite evidence of initial CPP acquisition and post-abstinence maintenance of nicotine-chamber preference in all nicotine-treated mice. Importantly, guanfacine attenuates this stress-induced reinstatement in Hi-Pre nicotine-treated animals (Figure 4a,b; yellow highlight). The raw data for time spent in the saline-paired chamber and the drug-paired chamber are shown in Figure S2 and were divided into the same Lo-Pre and Hi-Pre groups as in Figure 4a,b (i.e. based on pretest difference score). Differences between time spent in the saline-paired chamber and the drug-paired chamber were analyzed by paired two-tailed t-tests with Holm-Sidak corrections for multiple tests across all test days and split subgroups within sex. Analyzing raw chamber times, similar to analyzing difference scores, showed that there was significant reinstatement only in MNS Hi-Pre, and not in MNG Hi-Pre, MNS and MNG Lo-Pre, or any control group (Figure S2a,b).

3.4 Initial chamber preference interacts with stress and guanfacine on nicotine CPP reinstatement: female mice

In contrast to median split analyses by pretest preference in male mice, female mice show an effect of nicotine treatment in the initially non-preferred chamber. Overall, there was a significant interaction between split subgroup and test day ($F_{(28,272)} = 1.59$, $p = 0.033$) and significant main effects of split subgroup ($F_{(7,68)} = 3.45$, $p = 0.0032$) and test day ($F_{(4,272)} = 5.22$, $p = 0.0005$; Figure 4c,d). *Post-hoc* analyses within and across split subgroups show that the effects of initial chamber preference on acquisition and post-abstinence scores were

significant in male but not female mice; there is a similar pattern between pretest, acquisition, and post-abstinence preference scores between female nicotine-treated (FNS and FNG) and male nicotine-treated (MNS and MNG) groups, but no significant *post-hoc* comparisons in the FNS and FNG groups. However, significant stress-induced reinstatement compared to pretest preference was only observed in the FNS Lo-Pre group, i.e. female mice administered nicotine in the initially non-preferred chamber and treated with saline prior to the swim stressor ($p < 0.0001$; Figure 4c). Reinstatement in the FNS Lo-Pre group was also significantly different from reinstatement in FCS Lo-Pre ($p = 0.0088$), FCS Hi-Pre ($p = 0.0002$), and FNS Hi-Pre ($p = 0.0012$) groups. Further, guanfacine significantly attenuated stress-induced reinstatement in the parallel Lo-Pre group (FNG Lo-Pre; $p = 0.0006$ vs. FNS Lo-Pre; Figure 4c,d yellow highlight). Interestingly, there was also trend for guanfacine treatment to increase preferred-chamber preference in both FCG Hi-Pre and FNG Hi-Pre ($p = 0.073$ vs. pretest) groups (Figure 4d). As for males, analyzing raw chamber times for female mice produced similar findings as did analyses by difference scores, i.e. significant reinstatement in FNS Lo-Pre and no significant reinstatement in FNG Lo-Pre groups, and an increase in reinstated drug-chamber preference that was significant in the FCG Hi-Pre group and observable, though not significant, in the FNG Hi-Pre group (Figure S2c,d).

3.5 Estrous cycle did not affect preference scores in female mice

To determine whether estrous phase also contributed to the variability in preference scores observed in female mice, data were reorganized by estrous phase as measured at several points during the CPP procedure. Estrous phase was dichotomized to proestrous and estrous (p&e) or diestrous and metestrous (d&m). Preference scores were then analyzed by two-way ANOVA, with estrous phase and conditioning group as between-subjects factors. There were no significant effects of estrous phase on acquisition scores, based on estrous phase during conditioning or on acquisition test day (Figure 5a,b). There were also no effects of estrous phase during post-abstinence testing or reinstatement testing on post-abstinence or reinstatement preference scores, respectively (Figure 5c,d).

3.6 Guanfacine had varying effects on Arc expression across brain regions in male and female mice

To gain insight into brain areas potentially involved in sex-specific responses to guanfacine in the context of nicotine treatment and stress, we examined Arc expression in separate cohorts of male and female mice. These mice were subjected to a parallel schedule of nicotine and saline injections in distinct contexts as in CPP behavioral cohorts, followed by two weeks of abstinence and a forced swim stress that was preceded by saline or guanfacine pre-treatment. In subcortical areas, guanfacine had similar effects on Arc expression in male and female mice (see Table S2 for all statistical results, and Figure S3 for representative images). In the BLA, 2-way ANOVAs within sex revealed no significant interactions or main effects of conditioning group, although there were trending main effects of pre-stress treatment in the same direction in both sexes (males: $F_{(1, 20)} = 3.86$, $p = 0.064$; females: $F_{(1, 20)} = 2.48$, $p = 0.131$; Figure 6a,b). In the CeL, there were significant main effects of conditioning group (male: $F_{(1,20)} = 5.17$, $p = 0.034$; female: $F_{(1,19)} = 22.11$, $p = 0.0002$) and pre-stress treatment (male: $F_{(1,20)} = 9.14$, $p = 0.007$; female: $F_{(1,19)} = 8.59$, $p = 0.008$) in both male and female mice, such that CeL Arc immunoreactivity was lower in nicotine-

treated compared to control subjects, and higher in guanfacine- vs. saline-treated subjects (Figure 6c,d). There were no significant interactions. In the NAc core, as in the BLA, there were trends toward main effects of conditioning group in the same direction in male and female mice, but neither reached significance (male: $F_{(1,20)} = 2.73$, $p = 0.114$; female: $F_{(1,20)} = 4.26$, $p = 0.052$; Figure 6e,f). In the NAc shell, there were no significant main effects of conditioning group or pre-stress treatment, and no significant interactions in either male or female mice (Figure 6g,h).

In cortical areas, there were sex-convergent as well as sex-divergent effects of guanfacine on Arc-immunoreactivity. In the IL, there was a significant main effect of pre-stress treatment in male mice ($F_{(1,20)} = 6.43$, $p = 0.020$) where guanfacine increased Arc-immunoreactivity (Figure 7a). In contrast, there was a near-significant main effect of pre-stress treatment in female mice in IL ($F_{(1,19)} = 3.69$, $p = 0.070$), where guanfacine decreased Arc-immunoreactivity (Figure 7b). In the PL, there were no significant main effects of conditioning group or pre-stress treatment, and no significant interactions in either male or female mice (Figure 7c,d). In the AI, guanfacine decreased Arc-immunoreactivity in male mice with a near-significant effect ($F_{(1,20)} = 4.27$, $p = 0.052$; Figure 7e), but no effect was seen in female mice ($F_{(1,20)} = 0.26$, $p = 0.614$; Figure 7f).

4 DISCUSSION

In the current study, we examined stress-induced reinstatement of nicotine CPP in male and female mice and found sex-divergent CPP behaviors in nicotine dose response, especially in interaction with initial chamber preference, and sex-divergent patterns of Arc immunoreactivity in the AI and IL areas of frontal cortex.

First, we found that female mice demonstrate CPP acquisition and stress-induced reinstatement when administered a higher nicotine dose (0.75 mg/kg) than male mice (0.5 mg/kg), consistent with previous studies showing right-shifted dose-response curves for CPP acquisition in female mice and rats compared to males [50,61,62]. In our study, CPP acquisition was statistically significant only when cohorts were pooled for analysis (Figure 2). However, the consistent acquisition scores across independently tested cohorts strongly suggests the effect of nicotine is real despite being small (Figure 2b,d). Nicotine is known to be a relatively weak primary reinforcer [6,7] with well-documented variability in the expression of nicotine reward-related behaviors [13,15–18,52,63,64]. While previous studies have reported significant nicotine CPP acquisition with $n < 12$, the small effect size in our study compared to others is likely due to a combination of strain differences [45,52] and the use of counterbalanced vs. biased procedures [65], as discussed further below. Other factors that may contribute are the use of two- vs. three-chamber CPP apparatuses [61], visual vs. textural cues to distinguish the two conditioning compartments [37], and schedule of conditioning sessions [66]. Although female mice also showed significant reinstatement when administered 0.5 mg/kg nicotine, there was no indication of CPP acquisition with this dose (Figure 1c), and male mice did not show significant reinstatement when administered 0.75 mg/kg nicotine (Figure 1b). Furthermore, the degree of CPP acquisition and reinstatement at 0.5 mg/kg for males and 0.75 mg/kg for females was not quantitatively different. Only nicotine self-administration studies have compared male and female rats in stress-induced reinstatement

[13,40], and these studies found no sex differences when footshock [13] or yohimbine [40], an α2-AR antagonist, was used as a stressor.

Our study design produced a rich behavioral dataset in the various days on which CPP was measured and in the variability in preference scores. This variability in response to nicotine has been well-documented [13,15–18,52,63,64]. Our study further explored a potential source of variability with median split analyses based on pretest preference scores. Our balanced CPP design, where nicotine was paired with initially preferred (Hi-Pre) or non-preferred chambers (Lo-Pre) between mice, allowed a virtually even split between two behaviorally meaningful categories. Initial chamber preference is thought to reflect unconditioned anxiogenic responses to the initially non-preferred chamber, such that post-conditioning acquisition of preference for an initially non-preferred chamber may reflect a combination of unconditioned habituation, drug-mediated non-avoidance, and drug-dependent reward or approach [34,48,67]. First, our split analyses showed that habituation does factor into post-conditioning preferences (Figure 4, control Lo-Pre). However, nicotine-specific conditioning effects, beyond habituation alone, were observed in the positive acquisition scores in both nicotine Lo-Pre and Hi-Pre groups vs. in control Lo-Pre groups only, as well as in positive post-abstinence scores for nicotine but not control groups (Figure 4). The difference between acquisition and pretest scores in nicotine Hi-Pre groups are not significant, however, likely due to a ceiling effect [68]. These patterns were similar between males and females, but more robust in males. Overall, these analyses show that a biased CPP design produces stronger CPP acquisition, as previously demonstrated [63,64], and that post-abstinence preference scores can help distinguish specific nicotine effects from habituation.

The split analyses further present novel and striking findings on sex-dependent interactions between initial chamber preference and subsequent stress-induced reinstatement. These data can be interpreted within the framework of previous hypotheses about sex differences in the effect of nicotine on various behavioral processes. For example, regulation of negative affect seems to be a more prominent motivation for smoking and relapse in women [20,21,42]. Our data show that stress-induced reinstatement of nicotine CPP is only observed in female mice when nicotine is paired with the initially non-preferred or anxiogenic chamber, suggesting that nicotine's ability to support addiction-related behaviors in female mice may depend to some extent on its anxiolytic properties. However, studies on nicotine's anxiolytic effects are mixed, showing either increased [69–71] or decreased anxiolysis [72,73] in female rodents compared to males. On the other hand, male mice only show stress-induced reinstatement when nicotine is paired with the initially preferred chamber. These data suggest that nicotine's role as a reinforcement enhancer may be more significant for males [6,8]. In other words, nicotine enhances the rewarding effects that drove initial chamber preference in male mice, such that stress reinstates CPP only to the initially preferred chamber. However, sex differences were not observed in nicotine's reward-enhancing effects in a self-administration paradigm [74], suggesting that this sex difference may specifically interact with stress-induced reinstatement.

Another interesting finding from the split analyses was the dissociation between initial CPP acquisition and subsequent stress-induced reinstatement. In males, nicotine Lo-Pre groups exhibited significant CPP acquisition or post-abstinence nicotine preference scores, but there

was no significant stress-induced reinstatement (Figure 4a, b). On the other hand, CPP acquisition and post-abstinence scores were not statistically significant in MNS Hi-Pre mice, due to the high pretest preference, but there was significant CPP reinstatement. Similarly in female mice, CPP acquisition and post-abstinence scores were not significant in nicotine Lo-Pre groups, despite significant reinstatement. These data not only illustrate considerations related to CPP design, as discussed above, but also suggest there may be distinct neurobiological mechanisms mediating initial reward, maintenance of craving, and stress-induced reinstatement [75,76]. For example, the noradrenergic system is a potential substrate for these distinct mechanisms, and studies with morphine and heroin have shown that the locus coeruleus and medullary noradrenergic nuclei are differentially involved in withdrawal and stress-induced reinstatement [33,75,77]. Also of interest are how these mechanisms interact with sex. Even something as seemingly fundamental as dopamine release in response to nicotine has been shown to differ in nuanced ways between male and female rats [78], and men and women who smoke [79].

When examining responses to guanfacine overall, there was a significant interaction but no significant post-hoc findings in males, and no significant interaction or post-hoc comparisons in females (Figure 3). In contrast to these whole-cohort analyses, our split analyses showed that guanfacine blocked stress-induced reinstatement in both sexes, but only in the sex-specific conditions under which significant reinstatement was observed with saline pre-treatment (i.e. male Hi-Pre, female Lo-Pre; Figure 4 yellow highlights). These results are, to our knowledge, the first demonstration that guanfacine attenuates stress-induced reinstatement of nicotine CPP in male and female mice, however these effects seem to be limited to specific nicotine pairing parameters. Moreover, our results illustrate how a balanced CPP design can mask significant effects of stress and guanfacine on nicotine CPP. Future studies might leverage the sex-specific influence of initial chamber preference to study interactions between stress and nicotine reward.

Surprisingly, guanfacine also seemed to increase preference for the initially preferred chamber in male and female mice. The whole-cohort analyses suggest that this effect occurs in male and female control mice only (Figure 3), yet the split analyses reveal that guanfacine most strikingly increases preference for the initially preferred chamber in female control and nicotine-treated Hi-Pre mice (Figure 4d), and to a lesser extent in male control mice (Figure 4b). The similarity of this effect in male and female control mice, and more notably female control and nicotine Hi-Pre groups, suggests it is independent of nicotine. However, it is unclear why guanfacine would have this effect. Guanfacine also has anxiolytic- and antidepressant-like effects [51,80], and enhances working memory [43]. Perhaps guanfacine enhanced spatial working memory for the initially preferred context, thereby increasing time spent in that chamber [81,82].

There are some limitations to our use of guanfacine. For example, clinical studies use chronic guanfacine treatment (2-3 weeks minimum, including a dose escalation period [44,83]), whereas we tested an acute injection. Further, the dose 0.15 mg/kg was chosen because it reduced immobility on the second day of a two-day forced swim test in male and female mice [51], but different doses may reveal sex differences in guanfacine sensitivity. In the present study, guanfacine did not decrease immobility in either male or female mice (Figure

S1), likely because we used a single 6-min swim vs. a two-day procedure, and C3H/HeJ vs. C57BL/6J mice. Finally, although the focus of this study was to demonstrate stress-induced reinstatement of nicotine CPP and modulate it with guanfacine, further investigations without a stressor might provide more information on the effect of guanfacine itself.

We found no significant effect of estrous phase on CPP (Figure 5), which is consistent with previous studies of intact, freely-cycling female rats [40,84]. However, other studies associate higher estradiol levels with greater nicotine reward in rats [85,86], and higher progesterone levels with protective effects against nicotine reward [87] and relapse [88,89] in human subjects. Our study was insufficiently powered to examine estrous phase within the split subgroups, but it is possible that in the Lo-Pre subgroups where significant stress-induced reinstatement was found, there may be significant effects of estrous phase.

Neuronal activation was measured by the immediate early gene Arc [49] following a swim stress (which was preceded by saline or guanfacine treatment) but without subsequent exposure to the nicotine-paired context in order to reflect how the brain is primed by the stressor with or without guanfacine, without being confounded by drug-associated contextual cues or drug-seeking behavior. The Arc experiments are conducted in independent cohorts of mice from the behavioral studies, and thus rather than correlating with behavior, these findings represent an initial survey of brain regions with potential sex-specific responses to guanfacine in the context of nicotine CPP and stress following nicotine abstinence. These experiments did not test the effects of nicotine, stress, or abstinence *per se* on the expression of immediate early genes, which have been examined in previous studies [90–96].

In the BLA we did not find a significant effect of nicotine on Arc immunoreactivity (Figure 6a,b), although chemogenetic inactivation of BLA CaMKIIα neurons has been shown to induce reinstatement of nicotine CPP in mice [37]. There was a near-significant decrease in BLA Arc immunoreactivity after guanfacine, which is consistent with previous research using *in vivo* electrophysiology and clonidine in the BLA [97]. The CeA has a well-established role in stress-induced behaviors [22,36], and the CeL but not the CeM mediates relapse to methamphetamine self-administration [98]. Guanfacine increased CeL Arc immunoreactivity (Figure 6c,d), as seen previously [99], but nicotine-treated groups showed decreased CeL activity overall. Previous studies have shown enhanced c-fos immunoreactivity in the CeA after incubation of nicotine self-administration [93] and after footshock which followed nicotine self-administration training [96]. However, this enhancement may depend on exposure to drug-paired contexts following incubation or in conjunction with footshock [100]. In contrast, our mice were not re-exposed to nicotine-paired contexts following stress and prior to Arc immunohistochemistry. The NAc shell is activated in response to nicotine [101] and nicotine-associated cues [102], while the NAc core has been implicated in nicotine aversion [103] and drug-stress interactions for alcohol [104] and cocaine [105]. We found low NAc Arc expression overall, likely because mice were not re-exposed to nicotine or nicotine-paired contexts prior to perfusion. In all of the above regions, males and females showed similar Arc expression patterns, suggesting that these brain regions did not contribute to the observed sex differences in behavior.

There were sex-dependent effects of guanfacine in the IL and AI cortical regions. In the IL, guanfacine increased Arc immunoreactivity in male mice, and decreased Arc immunoreactivity in female mice, although the latter effect did not reach significance (Figure 7a,b). The IL is an important node for both stress-[106,107] and drug-related behaviors [108,109], with demonstrated sex differences in stress response [107]. Although the PL and not the IL is implicated in footshock-induced reinstatement of cocaine self-administration [110], we found no significant differences in PL Arc immunoreactivity (Figure 7c, d). Lack of re-exposure to the drug-paired context, and/or different neurobiological mechanisms between stress-induced reinstatement of self-administration vs. CPP [34,75], may account for this discrepancy. In the AI, guanfacine decreased Arc immunoreactivity in male mice, but had no effect in female mice (Figure 7e,f). The insula has been associated with increased smoking lapse in humans [111] as well as cue-induced reinstatement of nicotine seeking in rats [53]. Future investigations could determine if modulating the activity of the IL or AI drives sex-dependent responses to stress-induced reinstatement and guanfacine.

In conclusion, the current study presents a novel application of nicotine CPP to study sex differences in stress-induced reinstatement. Using this procedure, we demonstrate that initial chamber preference interacts with stress-induced reinstatement but in opposite directions for male and female mice, implying sex-dependent contributions of nicotine's anxiolytic and reward-enhancing effects to nicotine addiction. Further, acutely-administered guanfacine attenuates reinstatement in both male and female mice, but only in the nicotine-pairing conditions under which significant stress-induced reinstatement was observed for that sex. Finally, Arc immunohistochemical experiments suggest guanfacine has sex-dependent effects on neuronal activity in AI and IL, identifying these brain regions for future investigations into their potential roles in stress-induced reinstatement of nicotine CPP in male vs. female subjects. Overall, this study establishes a preclinical model for studying sex differences in stress-induced nicotine CPP and furthers our knowledge of sex-dependent effects of guanfacine on behavior and patterns of neural activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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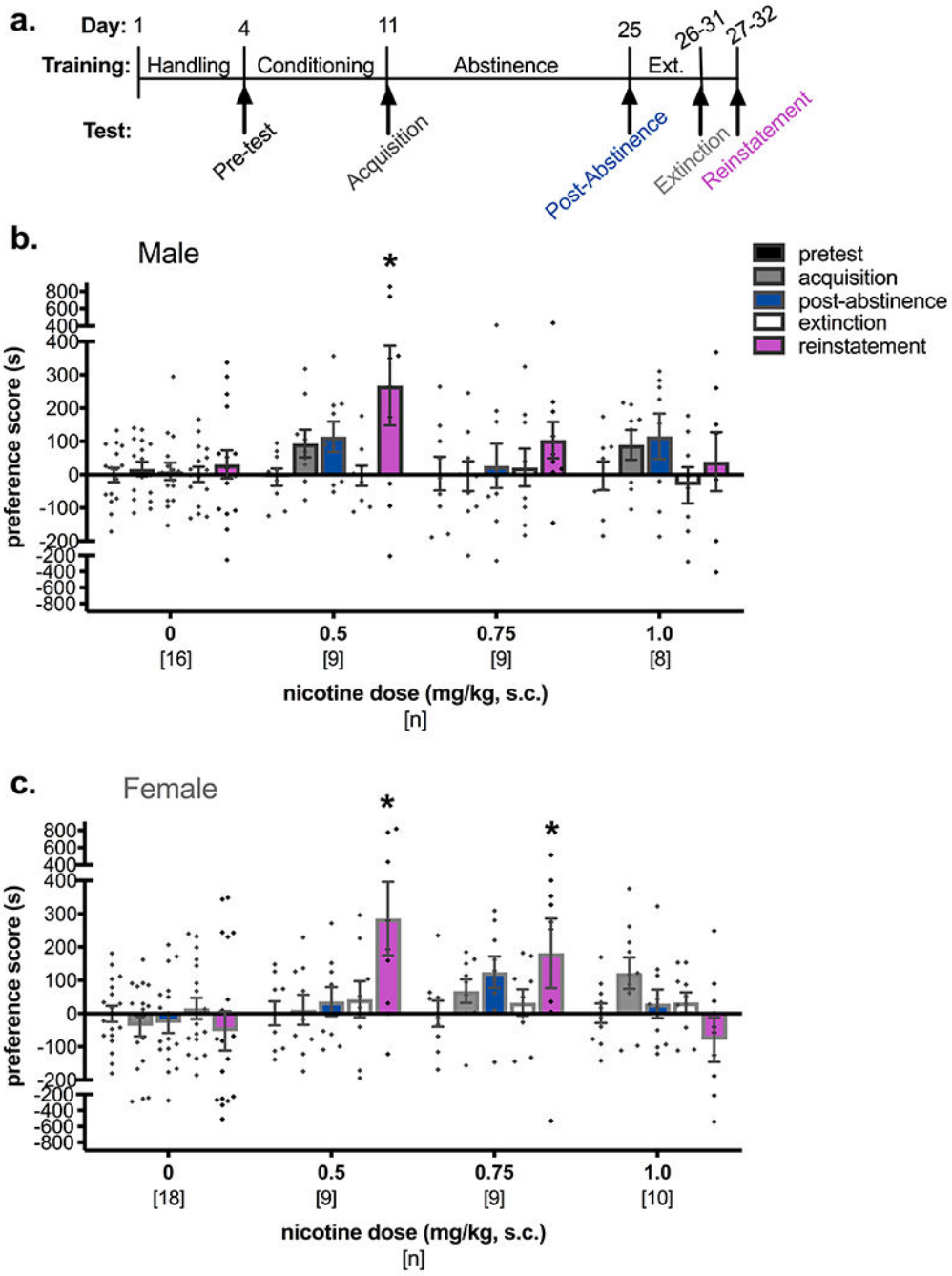

Figure 1. Dose-response relationships for stress-induced reinstatement of nicotine CPP in male and female mice.

(a) Timeline for CPP conditioning, acquisition, post-abstinence, extinction, and stress-induced reinstatement. Arrows indicate test days for which data are represented in (b) and (c) for male and female mice, respectively. Significant stress-induced reinstatement of nicotine CPP is seen for male mice trained with 0.5 mg/kg nicotine and for female mice trained with 0.5 mg/kg and 0.75 mg/kg nicotine. *$p < 0.05$ compared to pretest day within nicotine dose using Dunnett's tests.

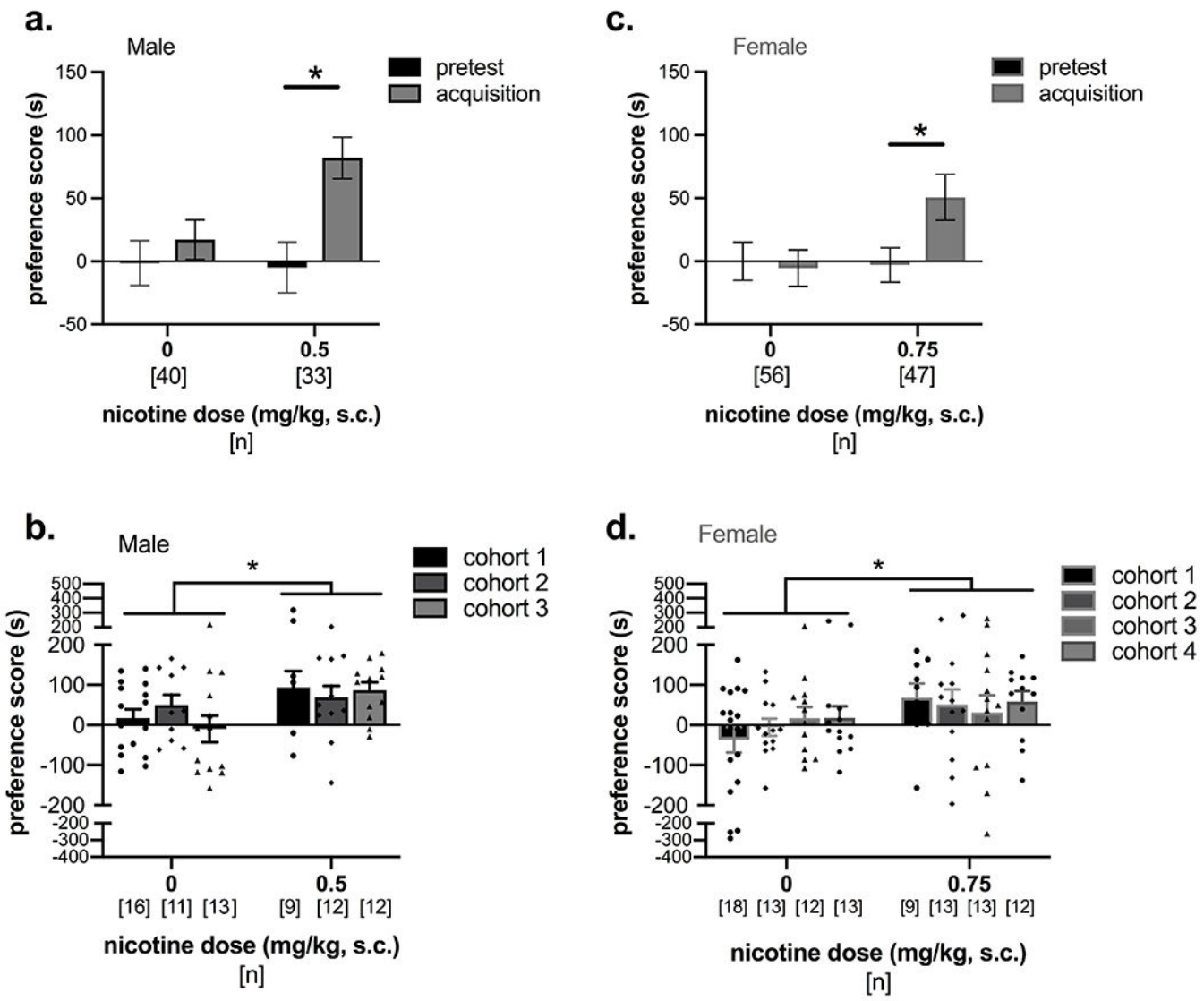

Figure 2. Male and female mice demonstrate significant acquisition of nicotine CPP.

(a) Male mice from all experimental cohorts are combined to demonstrate significant nicotine CPP acquisition and post-abstinence preference, followed by extinction when administered 0.5 mg/kg nicotine. (b) Acquisition scores are shown separately for the control and nicotine conditioning groups of the three independent cohorts of male mice that were combined in (a). Cohort 1 of males is the same as the 0.5 mg/kg group and control mice in Figure 1b, and cohorts 2 and 3 are the same male mice in Figures 3a and 4a-b. There was a significant effect of conditioning group on acquisition score, but no differences by cohort. (c) Female mice from all experimental cohorts are combined to demonstrate significant nicotine CPP acquisition when administered 0.75 mg/kg nicotine. (d) Acquisition scores for control and nicotine groups of the four independent cohorts of female mice combined in (c) show a significant effect of conditioning group, but no differences by cohort. Cohort 1 of females is the same as the 0.75 mg/kg group and control mice in Figure 1c, and cohorts 2-4 are the same mice represented in Figures 3b, 4c-d, and 5. *$p < 0.05$ compared to pretest day

within conditioning group using Dunnett's tests for (a) and (b), *$p < 0.05$ main effect of nicotine dose by two-way ANOVA for (c) and (d)

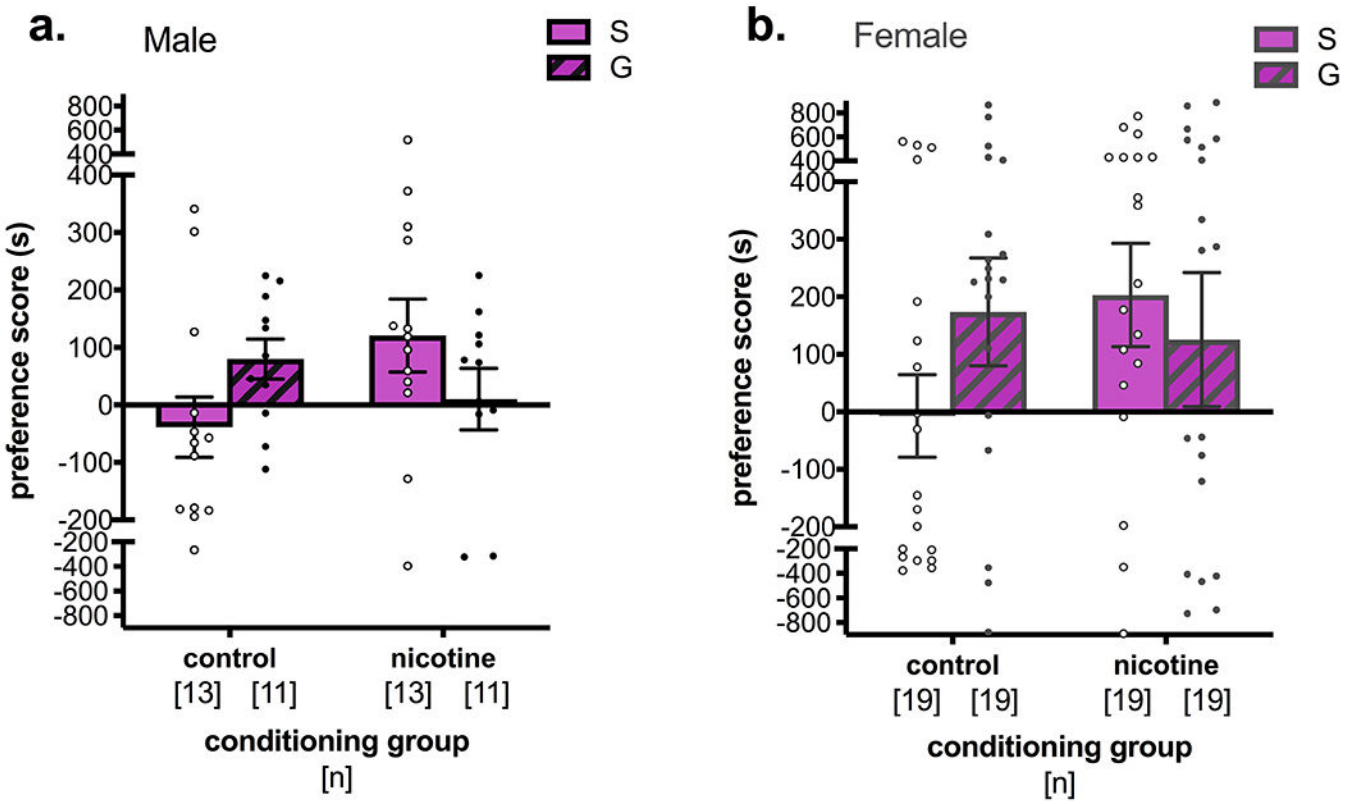

Figure 3. Guanfacine produced variable responses to stress-induced reinstatement in male and female mice.

Separate two-way ANOVAs within sex showed (a) a significant interaction of conditioning group and pre-stress treatment in male mice, and a near-significant difference between control- and nicotine-conditioned mice receiving saline pre-treatment. (b) No significant main effects or interactions were observed in female mice. S = saline, G = guanfacine

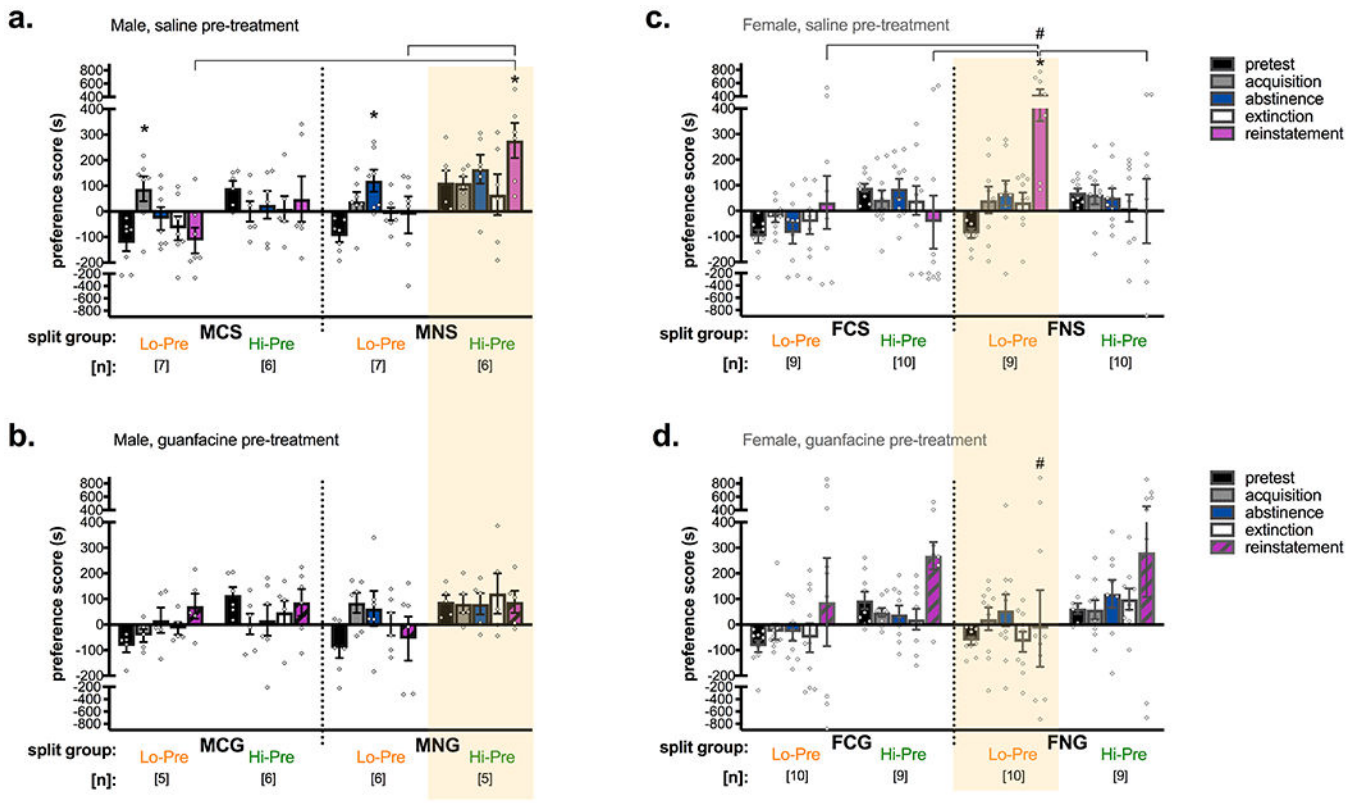

Figure 4. Median split analyses demonstrate significant interactions between initial chamber preference and subsequent CPP acquisition and stress-induced reinstatement, and significant effects of guanfacine.

Two-way ANOVAs conducted within sex between split subgroup and test day found significant interactions in both male and female mice. Data were analyzed by split subgroups across pre-treatment conditions, but are presented for male (a,b) and female (c,d) mice separately for saline (a,c) and guanfacine (b,d) pre-treatment for ease of viewing. Further, the yellow box highlights the significant findings regarding stress-induced reinstatement. (a) In male mice, only nicotine treatment in the initially preferred chamber subsequently leads to significant stress-induced reinstatement of nicotine CPP when the swim stress is preceded by a control saline injection. (b) This reinstatement is not significant when male mice in the same split subgroup (i.e. nicotine treatment in the initially preferred chamber) receive guanfacine (0.15 mg/kg, i.p.) prior to the swim stress. (c) In contrast, only female mice with nicotine treatment in the initially non-preferred chamber demonstrate significant stress-induced reinstatement of nicotine CPP with a saline pre-swim treatment, (d) and not with a guanfacine pre-swim treatment. Additionally, these two reinstatement preference scores (FNS Lo-Pre vs. FNG Lo-Pre) are significantly different from each other. *$p < 0.05$ compared to pretest day within nicotine dose using Dunnett's tests. Brackets indicate $p < 0.05$ compared to same test day in different split group using Tukey's multiple comparison test. #$p < 0.05$ for comparison to corresponding day, also marked by #, across saline and guanfacine pre-treatment groups by Tukey's test. MCS = male, control conditioning group, saline pre-treatment prior to stress. MNS = male, nicotine (0.5 mg/kg, s.c.) conditioning group, saline pre-treatment. MCG = male, control conditioning group, guanfacine (0.15

mg/kg, i.p.) pre-treatment. MNG = male, nicotine (0.5 mg/kg, s.c.) conditioning group, guanfacine pre-treatment. FCS = female, control conditioning group, saline pre-treatment. FNS = female, nicotine (0.75 mg/kg, s.c.) conditioning group, saline pre-treatment. FCG = female, control conditioning group, guanfacine (0.15 mg/kg, i.p.) pre-treatment. FNG = female, nicotine (0.75 mg/kg, s.c.) conditioning group, guanfacine pre-treatment. Lo-Pre = initial non-preference for future drug-chamber, Hi-Pre = initial preference for future drug-chamber.

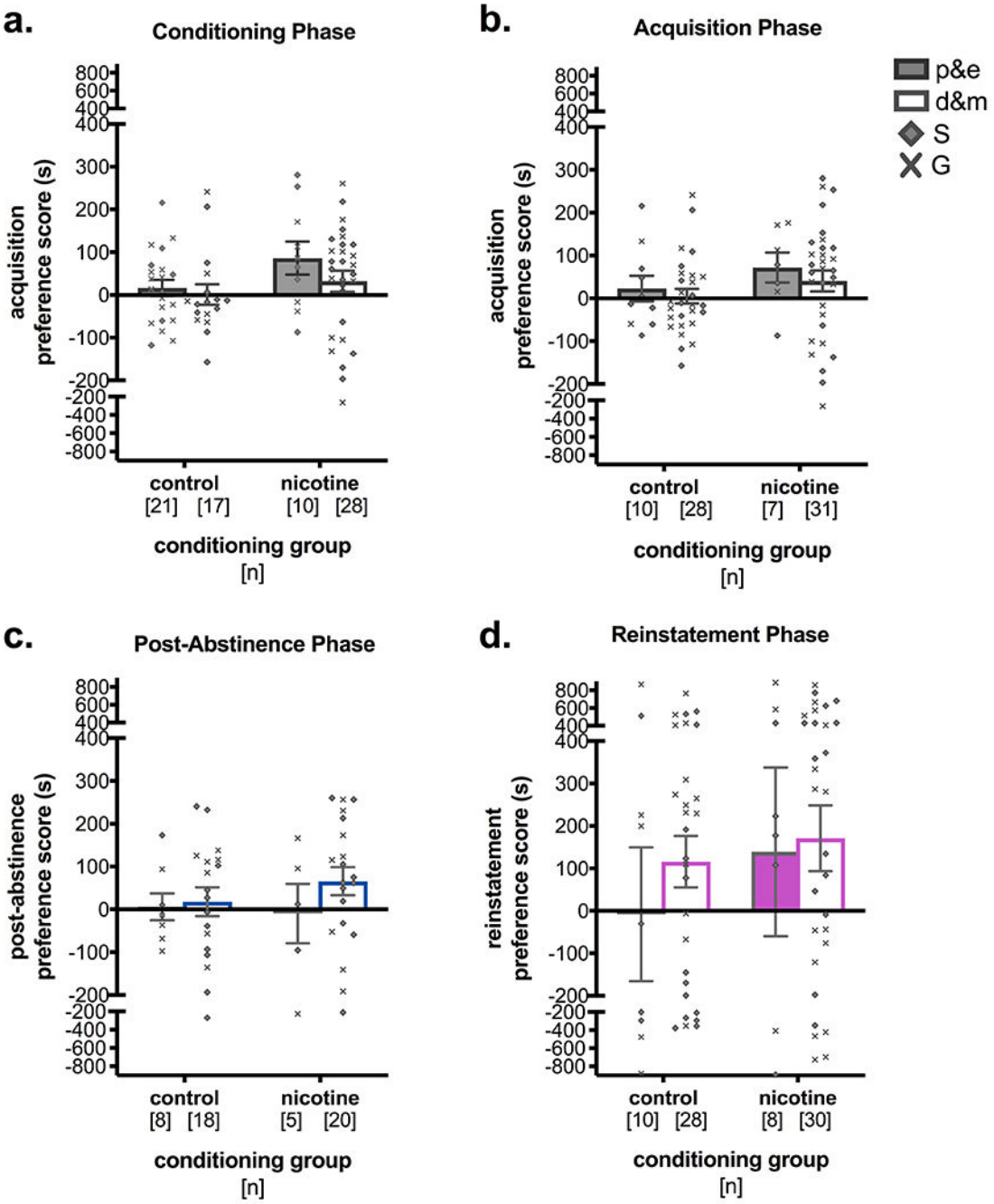

Figure 5. Estrous phase did not affect acquisition, post-abstinence, or reinstatement preference scores.

Estrous phase in female mice was measured at key points in the CPP procedure and its relationship to CPP behaviors was examined. (a) Estrous phase was measured at the midpoint of conditioning, the morning prior to conditioning day 4 of 6, and was found to have no influence on CPP acquisition in either control or nicotine (0.75 mg/kg, s.c.) conditioning groups. (b) Similarly, estrous phase on acquisition test day also did not affect CPP preference scores. (c) Estrous phase was also measured after 2 weeks of abstinence, and did not have a significant effect on post-abstinence CPP preference scores. (d) Finally,

estrous phase as measured on reinstatement test day did not have a significant effect on stress-induced reinstatement of nicotine CPP. There was also no effect of saline vs. guanfacine pre-treatment on the relationship between estrous phase and reinstatement scores. p&e = proestrous and estrous, d&m = diestrous and metestrous, S = saline, G = guanfacine. *$p < 0.05$

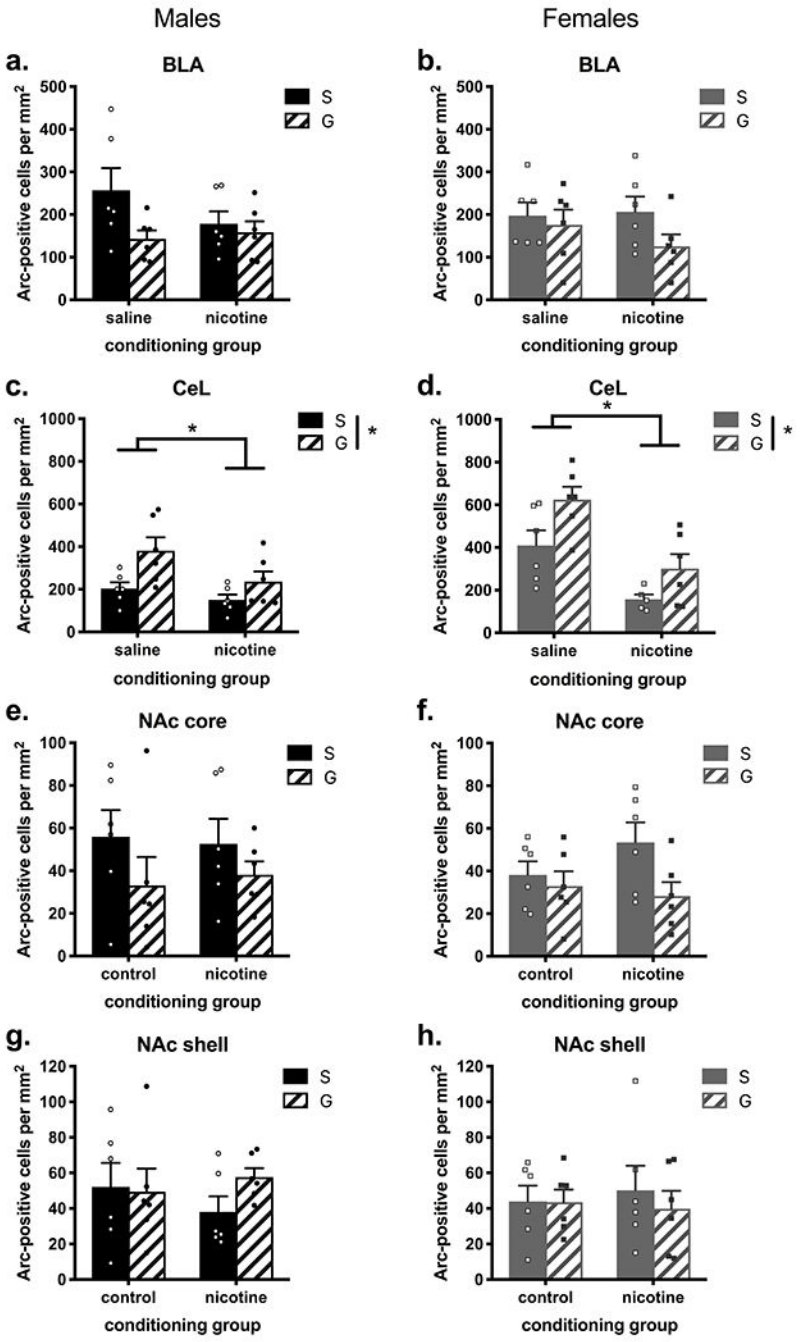

Figure 6. Arc immunoreactivity in subcortical areas of nicotine- or saline-treated mice with or without guanfacine administration before a swim stress.

Guanfacine appeared to reduce Arc-immunoreactivity in the BLA of (a) male mice and (b) female mice overall, but neither effect was significant. In the CeL of both (c) male and (d) female mice, nicotine significantly decreased Arc-immunoreactivity, and guanfacine significantly increased Arc-immunoreactivity. In the NAc core, similar to the BLA, guanfacine appeared to decrease mean Arc-immunoreactivity in (e) male mice and (f) female mice, but neither effect was significant. (g, h) There were no main effects of

conditioning group or pre-stress treatment and no interaction effects in the NAc shell of male and female mice. n = 5-6 per group. S = saline, G = guanfacine, *$p < 0.05$

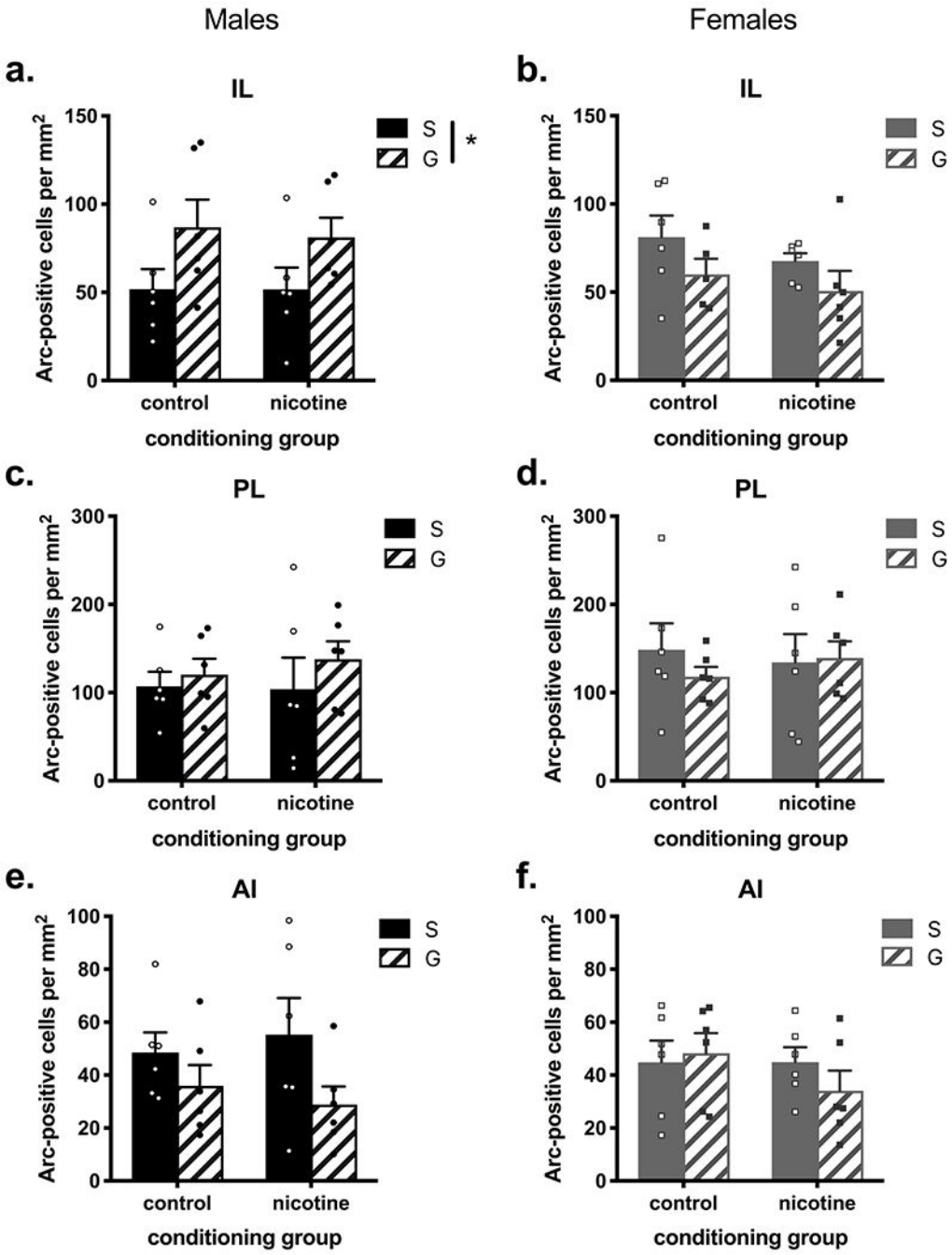

Figure 7. Arc immunoreactivity in cortical areas of nicotine- or saline-treated mice with or without guanfacine administration before a swim stress.

(a) Arc-immunoreactivity in the IL showed a main effect of pre-stress treatment in male mice, and a near-significant effect in (b) female mice but in the opposite direction. (c, d) In the PL, no significant main effects or interactions of conditioning group and pre-stress treatment were found in either sex. (e) In the AI, there was a near-significant main effect of pre-stress treatment in male mice, (f) but no main effect of either conditioning group or pre-

stress treatment and no interaction effect in female mice. n = 5-6 per group. S = saline, G = guanfacine, $*p < 0.05$