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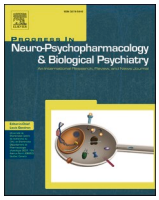

Pharmacological modulation of Kv3 voltage-gated potassium channels regulates fear discrimination and expression in a response-dependent manner

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ABSTRACT

Various psychiatric diseases are characterized by aberrant cognition and emotional regulation. This includes inappropriately attributing affective salience to innocuous cues, which can be investigated using translationally relevant preclinical models of fear discrimination. Activity in the underpinning corticolimbic circuitry is governed by parvalbumin-expressing GABAergic interneurons, which also regulate fear discrimination. Kv3 voltage-gated potassium channels are highly expressed in these neurons and are important for controlling their activity, suggesting that pharmacological Kv3 modulation may regulate fear discrimination. We determined the effect of the positive Kv3 modulator AUT00206 given systemically to female rats undergoing limited or extended auditory fear discrimination training, which we have previously shown results in more discrimination or generalization, respectively, based on freezing at retrieval. We also characterized darting and other active fear-related responses. We found that limited training resulted in more discrimination based on freezing, which was unaffected by AUT00206. In contrast, extended training resulted in more generalization based on freezing and the emergence of discrimination based on darting during training and, to a lesser extent, at retrieval. Importantly, AUT00206 given before extended training had dissociable effects on fear discrimination and expression at retrieval depending on the response examined. While AUT00206 mitigated generalization without affecting expression based on freezing, it reduced expression without affecting discrimination based on darting, although darting levels were low overall. These results indicate that pharmacological Kv3 modulation regulates fear discrimination and expression in a response-dependent manner. They also raise the possibility that targeting Kv3 channels may ameliorate perturbed cognition and emotional regulation in psychiatric disease.

1. Introduction

Disturbances in cognition and emotional regulation are features of various psychiatric diseases [Anticevic and Corlett, 2012; Mogg and Bradley, 2018]. This includes the misattribution of affective salience to innocuous stimuli [Kapur, 2003; Christianson et al., 2012], which can be investigated by examining fear discrimination using translationally relevant preclinical models. During fear discrimination learning, one cue (CS+) predicts threat through its association with an aversive unconditioned stimulus (US), while another cue (CS-) predicts the non-occurrence of the US to signal safety. More discrimination is displayed if the CS+ elicits higher levels of fear-related behavior than the CS-, whereas more generalization occurs if the CS+ and CS- elicit similar fear levels [Dunsmoor and Paz, 2015]. During fear discrimination learning the CS- signals safety to inhibit fear, which is impaired with fear over-generalization in psychiatric disease [Jensen et al., 2008; Lissek et al., 2010; Kaczkurkin et al., 2017]. Understanding the neurobiological basis of fear discrimination may therefore help to identify novel

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pharmacological targets for treating certain features of psychiatric disease.

Fear discrimination is mediated by a network of inter-connected corticolimbic areas that includes the medial prefrontal cortex (mPFC), primary auditory cortex (AC), amygdala, and hippocampus. Oscillatory activity in and synchrony between these areas have been implicated in fear discrimination [Headley and Weinberger, 2011; Likhtik et al., 2014; Concina et al., 2018; Tzovara et al., 2019; Day et al., 2020; Stujenske et al., 2022]. Parvalbumin-expressing GABAergic interneurons (PV interneurons) are crucial for orchestrating corticolimbic activity and functional connectivity by modulating synchronized firing in pyramidal cells and oscillatory dynamics between inter-connected areas [Klausberger, 2009; Sohal, 2012; Lucas and Clem, 2018]. Recent studies have shown that corticolimbic PV interneurons are involved in regulating fear discrimination [Aizenberg et al., 2015; Guo et al., 2018; Yan et al., 2019; Stujenske et al., 2022]. The voltage-gated potassium channels Kv3.1 and Kv3.2 are highly expressed in PV interneurons and pharmacological modulators of these channels regulate the fast-spiking phenotype of PV interneurons [Rosato-Siri et al., 2015; Kaczmarek and Zhang, 2017]. Preliminary evidence indicates that the positive Kv3.1/3.2 modulator AUT00206 influences gamma oscillations in mPFC [Neill et al., 2015], suggesting that pharmacological Kv3.1/3.2 modulation may also regulate fear discrimination.

In this study we examined the effects of systemic AUT00206 administration on auditory fear discrimination learning and memory retrieval. We have shown in female rats that limited training results in more fear discrimination at retrieval, whereas extended training leads to more fear generalization [Day et al., 2016, 2020]. We took advantage of these training-dependent phenotypes by determining the effects of AUT00206 on fear discrimination with limited or extended training, respectively. Our previous findings were based on freezing as a prototypical fear response [Fendt and Fanselow, 1999], but active fear responding in the form of darting can also be expressed during fear conditioning and discrimination learning [Gruene et al., 2015; Greiner et al., 2019; Colom-Lapetina et al., 2019; Morena et al., 2021; Mitchell et al., 2022; Demars et al., 2022; Trott et al., 2022]. Thus darting and other active fear-related responses were also characterized here. Finally, we determined the effects of AUT00206 on anxiety-like behavior, locomotor activity, and shock sensitivity to assess if any drug effects on fear discrimination and expression were attributable to non-specific effects on these behavioral measures.

2. Materials and methods

2.1. Animals

Naturally cycling adult female Lister hooded rats (Charles River, UK) weighing 165–245 g at the beginning of the experiments were used in this study. We have shown in female, but not male, rats that one day or three days of discrimination training results in more fear discrimination or generalization, respectively, based on cue-induced freezing at retrieval [Day et al., 2016, 2020]. Therefore only females were used to determine the effects of AUT00206 on fear discrimination learning and memory retrieval. Group sizes (see below) were based on these previous studies. Rats were group housed (4/cage) in individually ventilated cages on a 12 h light/dark cycle (lights on at 8:00) with free access to food and water. All experimental procedures were conducted with ethical approval from the University of Nottingham Animal Welfare and Ethical Review Body and in accordance with the Animals (Scientific Procedures) Act 1986, UK (Home Office Project Licence 30/3230). Separate cohorts of rats were used in Experiments 1–4 (Fig. 1). All behavioral testing occurred during the animals' light cycle.

2.2. Drug administration

AUT00206 (5,5-dimethyl-3-[2-(7-methylspiro[2H-benzofuran-3,1′-cyclopropane]-4-yl)oxypyrimidin-5-yl]imidazolidine-2,4-dione) was suspended in vehicle (12.5% captisol (*w/v*), 0.5% hydroxypropyl methylcellulose (*w/v*), 0.1% Tween 80 (*v/v*) in distilled water) and injected at 30 mg/kg (i.p. in 0.2 mL/100 g). This dose was based on preliminary evidence that a comparable oral dose ameliorated cognitive deficits induced by subchronic phencyclidine administration [Neill et al., 2015].

2.3. Experiment 1: Effect of AUT00206 on auditory fear discrimination with limited training

The paradigm used for fear discrimination learning and memory testing with limited training was adapted from our previous study [Day et al., 2016] and is illustrated in Fig. 1A. Two behavioral testing chambers were used and the apparatus has been described elsewhere [Stevenson et al., 2009]. On Day 1, animals were habituated to two contexts (A and B), where they received two presentations each of 2 and 9 kHz tones (30 s, 80 dB, 2 min inter-trial interval (ITI)). On Day 2,

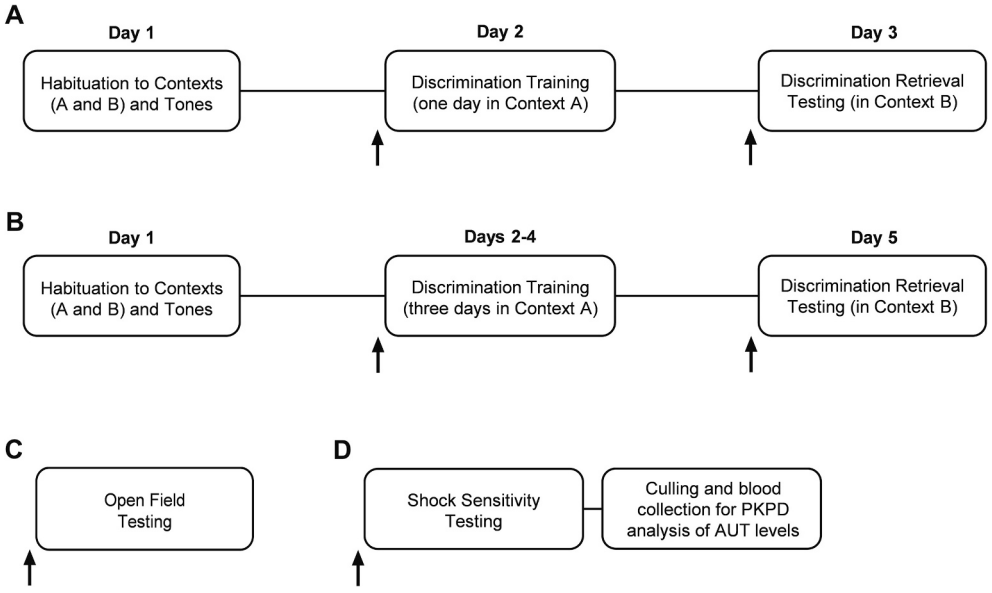

Fig. 1. Schematic representation of the behavioral testing procedures used in Experiments 1–4. A) The limited fear discrimination training paradigm used in Experiment 1. B) The extended fear discrimination training paradigm used in Experiment 2. C) Animals underwent open field testing in Experiment 3. D) Animals underwent shock sensitivity testing and immediately afterwards were culled and blood was collected for the later pharmacokinetic/pharmacodynamic analysis of plasma AUT00206 (AUT) concentration in Experiment 4. AUT or vehicle was injected 30 min before behavioral testing (indicated by the arrows).

animals underwent discrimination training in context A, consisting of five pairings of one tone (CS+; 30 s, 80 dB, 2 min ITI) with a footshock US (0.5 s, 0.4 mA, ending at tone offset) and five presentations of the other tone alone (CS-; 30 s, 80 dB, 2 min ITI), with each CS+/US pairing being followed by a CS- presentation. The tones used for the CS+ or CS- were counterbalanced between animals. On Day 3, animals received two presentations each of the CS+ and CS- in context B to assess discrimination memory retrieval. Each cue was followed by presentation of the other cue and the order in which the CS+ and CS− were first presented was counterbalanced between rats. Tone and footshock presentations were controlled by a PC running MED-PC IV software (Med Associates, US). Animals were injected with AUT00206 or vehicle 30 min before training (Day 2) and/or retrieval (Day 3), resulting in four groups: vehicle given before training and retrieval (VEH/VEH), vehicle given before training and AUT00206 given before retrieval (VEH/AUT), AUT00206 given before training and vehicle given before retrieval (AUT/VEH), and AUT00206 given before training and retrieval (AUT/AUT). Animals were randomly allocated to one of the four treatment groups (n = 10 per group). Animals were tested at approximately the same time of day on each day and behavior on Days 2–3 was recorded for later data analysis.

2.4. Experiment 2: Effect of AUT00206 on auditory fear discrimination with extended training

The paradigm used for discrimination learning and retrieval with extended training was based on our previous studies [Day et al., 2016, 2020] and is depicted in Fig. 1B. On Day 1, animals underwent context and tone habituation as in Experiment 1. On Days 2–4, animals underwent once-daily training sessions in context A, consisting of five CS+/US pairings and five CS- presentations as in Experiment 1, except that 0.5 mA footshocks were used. On Day 5, animals underwent retrieval testing in context B as in Experiment 1. Animals were injected with AUT00206 or vehicle 30 min before training (Days 2–4) and/or testing (Day 5), resulting in the four groups as in Experiment 1. Animals were randomly allocated to one of the four treatment groups (n = 10 per group). Animals were tested at approximately the same time of day on each day and behavior on Days 2–5 was recorded for later data analysis.

2.5. Experiment 3: Effect of AUT00206 on behavior during open field testing

The effect of AUT00206 on behavior during open field testing (Fig. 1C) was examined as we have described elsewhere [Day et al., 2016]. Rats were injected with AUT00206 or vehicle 30 min before being placed in the open field for 10 min. Animals were randomly allocated to one of the two treatment groups (n = 10 per group). Behavior was recorded during testing for later data analysis.

2.6. Experiment 4: Effects of AUT00206 on shock sensitivity and blood AUT00206 levels

The effect of AUT00206 on shock sensitivity (Fig. 1D) was examined as we have previously described [Day et al., 2016]. Animals were injected with AUT00206 or vehicle 30 min before receiving 10 footshocks of increasing intensity (0.05–0.5 mA, 0.5 s, 1 min ITI). Animals were randomly allocated to one of the two treatment groups (n = 10 per group). Behavior was recorded during testing for data analysis. Upon completion of shock sensitivity testing, animals treated with AUT00206 were deeply anesthetized with isoflurane before blood was collected via cardiac puncture, immediately after which they were culled. The time from AUT00206 injection to blood collection was 45–60 min. For each animal, two 70 μL blood samples were placed in two tubes both containing 130 μL of 0.1 M HEPES buffered saline and stored at −20 °C. AUT00206 levels were later determined in these samples using liquid chromatography-mass spectrometry as we have previously described [Anderson et al., 2018].

2.7. Data analysis

In Experiments 1–2, freezing (i.e. absence of movement except in relation to breathing) was quantified in response to cue presentations during fear discrimination training and retrieval testing. Freezing was scored manually by two observers blind to drug treatment. Freezing was scored at 3 s intervals and the cumulative duration of freezing during the 30 s cue presentations was calculated and expressed as a percentage of the cue duration. Discrimination during training was inferred from freezing during each CS+/US pairing and CS- presentation. Discrimination at retrieval was inferred from mean freezing in response to the two CS+ and two CS- presentations. A discrimination ratio calculated from mean freezing in response to the CS+ and CS- at retrieval was also determined (ratio = (CS+) / (CS+ + CS-); [Robinson, 2017]) to account for inter-individual variability in and potential drug effects on absolute freezing levels. Discrimination ratio values towards 1 or 0.5 reflected more fear discrimination or generalization, respectively. A discrimination ratio value of 0.5 was assigned where no freezing occurred in response to either cue. Baseline fear during retrieval was inferred from freezing in the 2 min period before cue presentations and quantified as above. For training, freezing data from the VEH/VEH and VEH/AUT groups were combined and data from the AUT/VEH and AUT/AUT groups were combined. During training, differences between the VEH and AUT groups in response to CS+/US pairings and CS- presentations were analyzed using three-way (Experiment 1) or four-way (Experiment 2) analysis of variance (ANOVA), with treatment as the between-subject factor and cue and trial (and day for Experiment 2) as within-subject factors. During retrieval testing, group differences in response to the CS+ and CS- were analyzed using three-way ANOVA, with treatment before training and treatment before retrieval as between-subject factors and cue as the within-subject factor. Discrimination ratio data were subjected to arcsine square root transformation to stabilize the variance [Sokal and Rohlf, 1995] and group differences were analyzed using two-way ANOVA, with treatment before training and treatment before retrieval as between-subject factors. Group differences in baseline fear were also analyzed using two-way ANOVA, with treatment before training and treatment before retrieval as between-subject factors. In Experiment 1, a statistical outlier in the VEH/AUT group was identified based on freezing before cue presentations during retrieval (Grubbs test; $\alpha = 0.05$) and all data from this rat was omitted from the analysis. In Experiment 2, one statistical outlier in each of the VEH/VEH and AUT/VEH groups was also identified based on freezing before cue presentations during retrieval (Grubbs test; $\alpha = 0.05$) and all data from these two rats was omitted from the analysis. One rat in the VEH/AUT group did not receive footshock with CS+/US pairings on the first day of discrimination training, therefore all data from this rat was also omitted from the analysis.

Recent studies have shown that females can also display darting as an active fear response during fear conditioning and discrimination learning under conditions in which males typically express freezing [Gruene et al., 2015; Greiner et al., 2019]. Therefore we also characterized active fear responding during fear discrimination training and at retrieval, initially by quantifying various active behaviors in response to cue presentations in the VEH/VEH controls in Experiments 1–2. We observed three behaviors - jumping, climbing, and darting - that we classified as potential active fear responses. Each behavior was scored manually by two observers at 3 s intervals and their cumulative durations during the 30 s cue presentations were calculated and expressed as percentages of the cue duration, as for freezing. Although darting has previously been expressed as the rate of darting or number of darts [Gruene et al., 2015; Greiner et al., 2019], we expressed darting and the other active behaviors as a percentage of cue duration to facilitate direct comparisons with freezing. During training, differences in response to CS+/US pairings vs CS- presentations were analyzed separately for each

behavior using two-way (Experiment 1) or three-way (Experiment 2) ANOVA, with cue and trial (and day for Experiment 2) as within-subject factors. During retrieval testing, differences in response to the CS+ and CS- were analyzed separately for each behavior using paired *t*-tests. In Experiment 1, we found very low levels of each behavior and low discrimination during limited training and retrieval (Fig. 2). In Experiment 2, we also found low levels of jumping and climbing overall and low discrimination during extended training and retrieval. However, darting emerged over the course of extended training and we found more discrimination during training based on this measure (Fig. 3). Therefore we also examined AUT00206 effects on darting as an active fear response during fear discrimination training and at retrieval in Experiments 1–2, as for freezing. Previous studies have shown that only a proportion of females express cue-induced darting during a single session of fear conditioning, which led to the characterization of distinct 'darter' and 'non-darter' subpopulations [Gruene et al., 2015; Colom-Lapetina et al., 2019]. However, we found that all animals in the VEH/VEH and VEH/AUT groups expressed at least some darting in response to CS+/US pairings by the third day of extended discrimination training in Experiment 2. As a result, we did not consider the darting data in relation to separate darter and non-darter subpopulations, which is consistent with a previous fear discrimination study that also used repeated fear discrimination training sessions [Greiner et al., 2019].

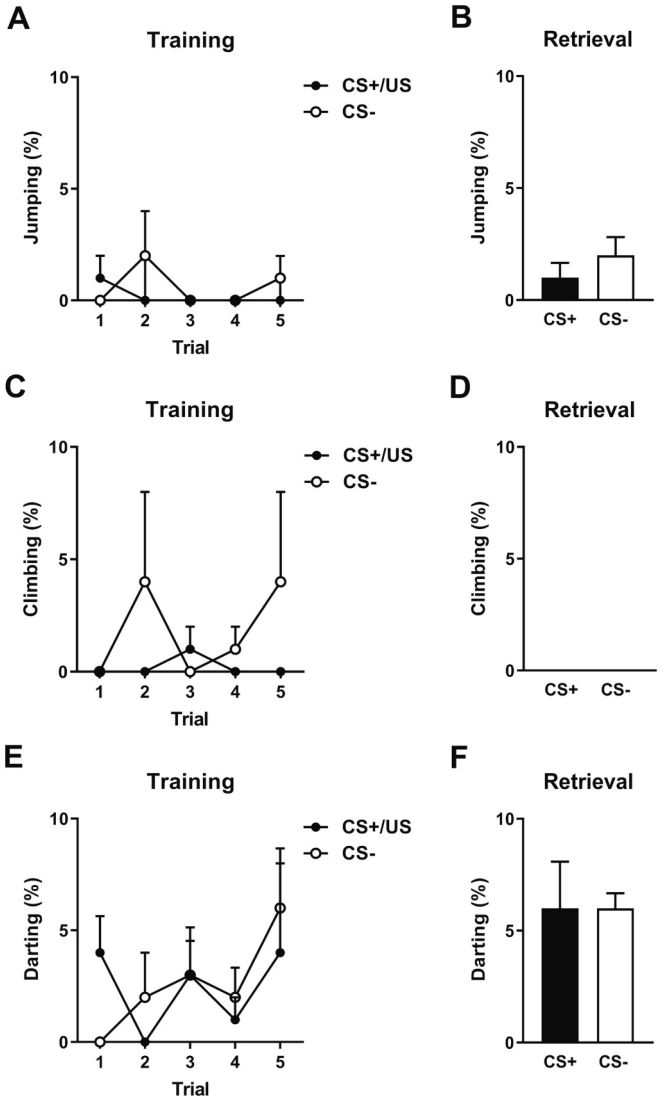

Fig. 2. Active fear-related behaviors in response to the cues during limited fear discrimination training and retrieval in the VEH/VEH controls. Very low levels of jumping (A-B), climbing (C-D), and darting (*E*-F) occurred, with low discrimination shown during training (CS+/US pairings vs CS-) and retrieval (CS+ vs CS-) based on these measures.

In Experiment 3, behavior during open field testing was analyzed using Ethovision software (Noldus, Netherlands). The percentage of time spent in, the number of entries into, and the latency to enter the inner zone of the open field were quantified as measures of anxiety-like behavior. The horizontal distance moved during the open field test was also quantified as a measure of locomotor activity. Group differences on these measures were analyzed using unpaired *t*-tests.

In Experiment 4, the threshold current eliciting flinch and vocalization responses during shock sensitivity testing were scored manually by two observers blind to treatment. Group differences on these measures were analyzed using two-way ANOVA, with treatment as the between-subject factor and response as the within-subject factor. Blood AUT00206 levels 45–60 min after AUT00206 injection were also quantified. One rat treated with AUT00206 was found to have no detectable blood levels of AUT00206, therefore the data from this rat was omitted from the shock sensitivity analysis.

All data are presented as the mean + SEM. The level of significance for all comparisons was set at $P < 0.05$. Follow up analyses on the significant main effects and interactions resulting from the ANOVA tests were conducted using further ANOVA testing and/or post-hoc comparisons (Tukey's test). Partial eta squared (η_p^2) and Cohen's d values were also reported as indices of effect sizes for all statistically significant effects resulting from the ANOVAs and t-tests, respectively.

3. Results

3.1. Darting emerges as an active fear response during extended fear discrimination training in controls

The different active fear-related behaviors that were initially characterized during limited discrimination training and at retrieval in the VEH/VEH controls ($n = 10$) are shown in Fig. 2. We found very low levels of each behavior and low discrimination during training and retrieval. Two-way ANOVA on jumping during the one day of training (Fig. 2A) found no main effect of cue ($F_{(1, 9)} = 0.64$, $P = 0.44$) or cue x trial interaction ($F_{(4, 36)} = 1.09$, $P = 0.37$). There was also no difference in jumping between the CS+ and CS- at retrieval ($t_9 = 1.00$, $P = 0.34$; Fig. 2B). Similarly, two-way ANOVA on climbing during training (Fig. 2C) found no main effect of cue ($F_{(1, 9)} = 2.25$, $P = 0.17$) or cue x trial interaction ($F_{(4, 36)} = 0.75$, $P = 0.56$). No climbing was observed in response to either cue during retrieval (Fig. 2D). Finally, two-way ANOVA on darting during training (Fig. 2E) found no main effect of cue ($F_{(1, 9)} = 0.027$, $P = 0.87$) or cue x trial interaction ($F_{(4, 36)} = 1.02$, $P = 0.41$), and there was no difference in darting between the CS+ and CS- at retrieval ($t_9 = 0$, $P > 0.99$; Fig. 2F).

Characterization of the active fear-related behaviors during extended discrimination training and at retrieval in the VEH/VEH controls ($n = 9$) is shown in Fig. 3. Again, we found low levels of jumping and climbing and low discrimination during training and retrieval based on these measures. Three-way ANOVA on jumping during the three days of training (Fig. 3A) found no main effect of cue ($F_{(1, 8)} = 0.22$, $P = 0.65$) or any interactions involving cue (not shown). There was also no difference in jumping between the CS+ and CS- during retrieval ($t_8 = 1.51$, $P = 0.17$; Fig. 3B). Similarly, three-way ANOVA on climbing during training (Fig. 3C) found no main effect of cue ($F_{(1, 8)} = 1.36$, $P = 0.28$) or any interactions involving cue (not shown), and there was no difference in climbing between the CS+ and CS- at retrieval ($t_8 = 1.00$, $P = 0.35$; Fig. 3D). However, darting emerged over the course of training and we found more discrimination on Day 3 based on this measure. Three-way ANOVA on darting during training (Fig. 3E) revealed a significant main effect of cue ($F_{(1, 8)} = 8.85$, $P = 0.018$, $\eta_p^2 = 0.53$) and a significant cue x day interaction ($F_{(2, 16)} = 12.94$, $P < 0.001$, $\eta_p^2 = 0.62$), but no other

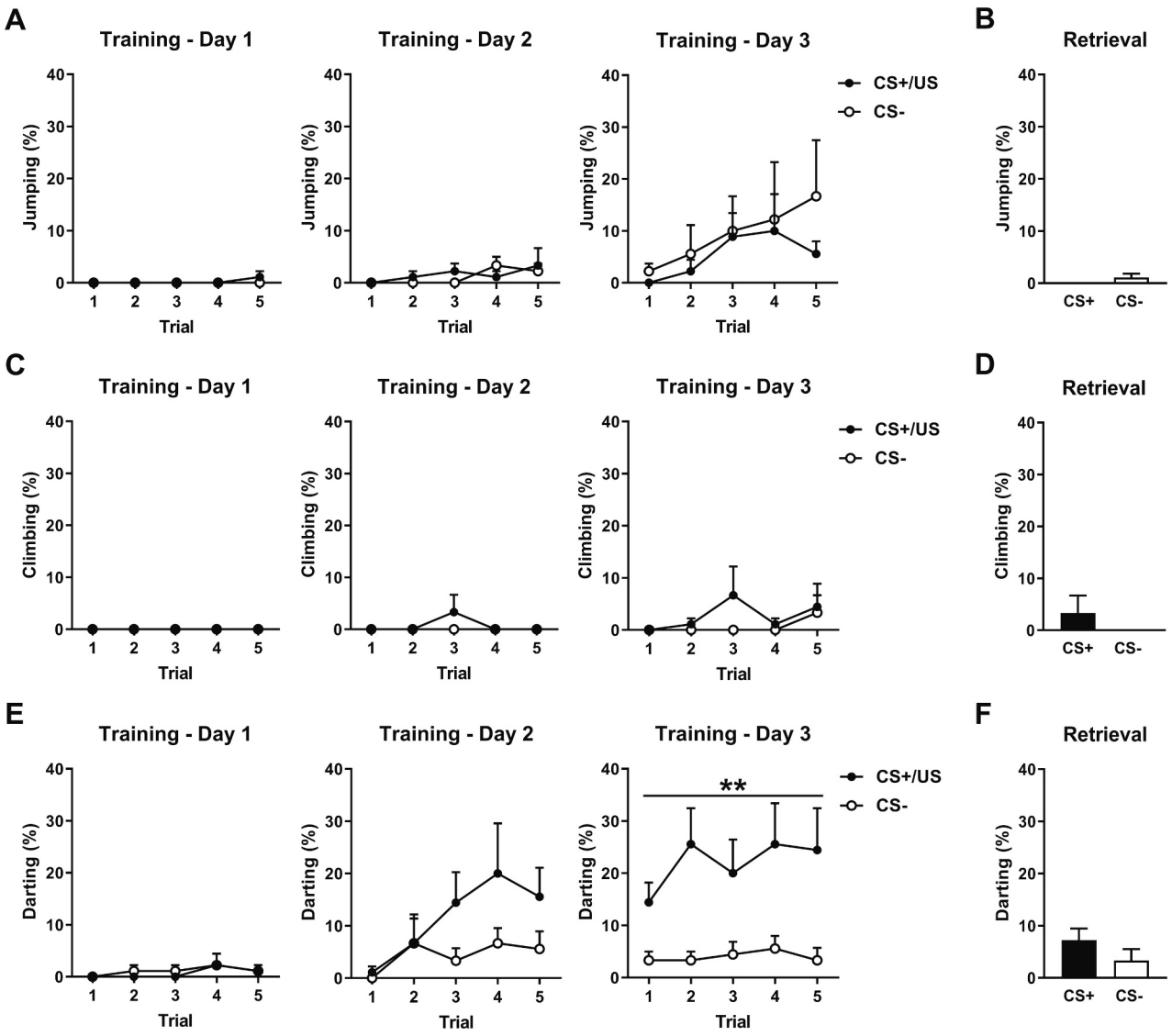

Fig. 3. Active fear-related behaviors in response to the cues during extended fear discrimination training and retrieval in the VEH/VEH controls. Low levels of jumping (A-B) and climbing (C-D) occurred, with low discrimination shown during training (CS+/US pairings vs CS-) and retrieval (CS+ vs CS-) based on these measures. E) For darting, low levels occurred during training on Day 1; however, darting levels increased on Day 2 and by Day 3 discrimination between the cues reached significance (** CS+/US pairings > CS-; $P < 0.01$). F) Darting levels and discrimination based on this measure were lower at retrieval (CS+ vs CS-).

interactions involving cue (not shown). Further analysis indicated that while there were no differences in darting between the CS+/US pairings and CS- presentations on Days 1 (main effect of cue: $F_{(1, 8)} = 2.29$, $P = 0.17$; cue x trial interaction: $F_{(4, 32)} = 0.73$, $P = 0.58$) or 2 (main effect of cue: $F_{(1, 8)} = 3.73$, $P = 0.09$; cue x trial interaction: $F_{(4, 32)} = 1.56$, $P = 0.21$), darting was significantly increased across CS+/US pairings, compared to CS- presentations, on Day 3 (main effect of cue: $F_{(1, 8)} = 14.36$, $P = 0.0053$, $\eta_p^2 = 0.64$; cue x trial interaction: $F_{(4, 32)} = 0.71$, $P = 0.59$). During retrieval (Fig. 3F), we found much lower levels of darting in response to the CS+ and there was no difference between the CS+ and CS- ($t_8 = 1.14$, $P = 0.29$). Although less discrimination occurred at retrieval based on darting, more discrimination was observed later on during extended training based on this measure in the VEH/VEH controls. Therefore we went on to examine the effects of AUT00206 on fear discrimination learning and memory with limited or extended training based on both freezing and darting behavior in Experiments 1–2.

3.2. AUT00206 has no effect on fear discrimination with limited training

The effects of AUT00206 on freezing during limited discrimination training and at retrieval are shown in Fig. 4A-D. Three-way ANOVA on freezing during the one day of training revealed significant main effects of treatment ($F_{(1, 37)} = 5.93$, $P = 0.02$, $\eta_p^2 = 0.14$) and trial ($F_{(4, 148)} = 10.44$, $P < 0.001$, $\eta_p^2 = 0.22$) but not cue ($F_{(1, 37)} = 0.19$, $P = 0.67$). It also revealed a significant cue x trial interaction ($F_{(4, 148)} = 4.24$, $P = 0.003$, $\eta_p^2 = 0.10$) but no other interactions (not shown). Post-hoc analysis showed that, compared to vehicle ($n = 19$), AUT00206 ($n = 20$) significantly increased freezing during training across all trials and both cues ($P < 0.05$; Fig. 4A). Two-way ANOVA on freezing before cue presentations at retrieval showed no main effects of treatment before training ($F_{(1, 35)} = 1.71$, $P = 0.20$) or treatment before retrieval ($F_{(1, 35)} = 3.49$, $P = 0.07$), and no interaction between these factors ($F_{(1, 35)} = 0.0025$, $P = 0.96$), indicating a lack of effect of AUT00206 on baseline freezing (Fig. 4B). Three-way ANOVA on freezing during cue presentations at retrieval revealed significant main effects of treatment before retrieval ($F_{(1, 35)} = 7.66$, $P = 0.009$, $\eta_p^2 = 0.18$) and cue ($F_{(1, 35)} = 20.26$, $P < 0.001$, $\eta_p^2 = 0.37$), but not treatment before training ($F_{(1, 35)} = 1.22$, $P = 0.28$). There were no interactions between any of the factors (not shown). Post-hoc analysis showed that freezing was significantly

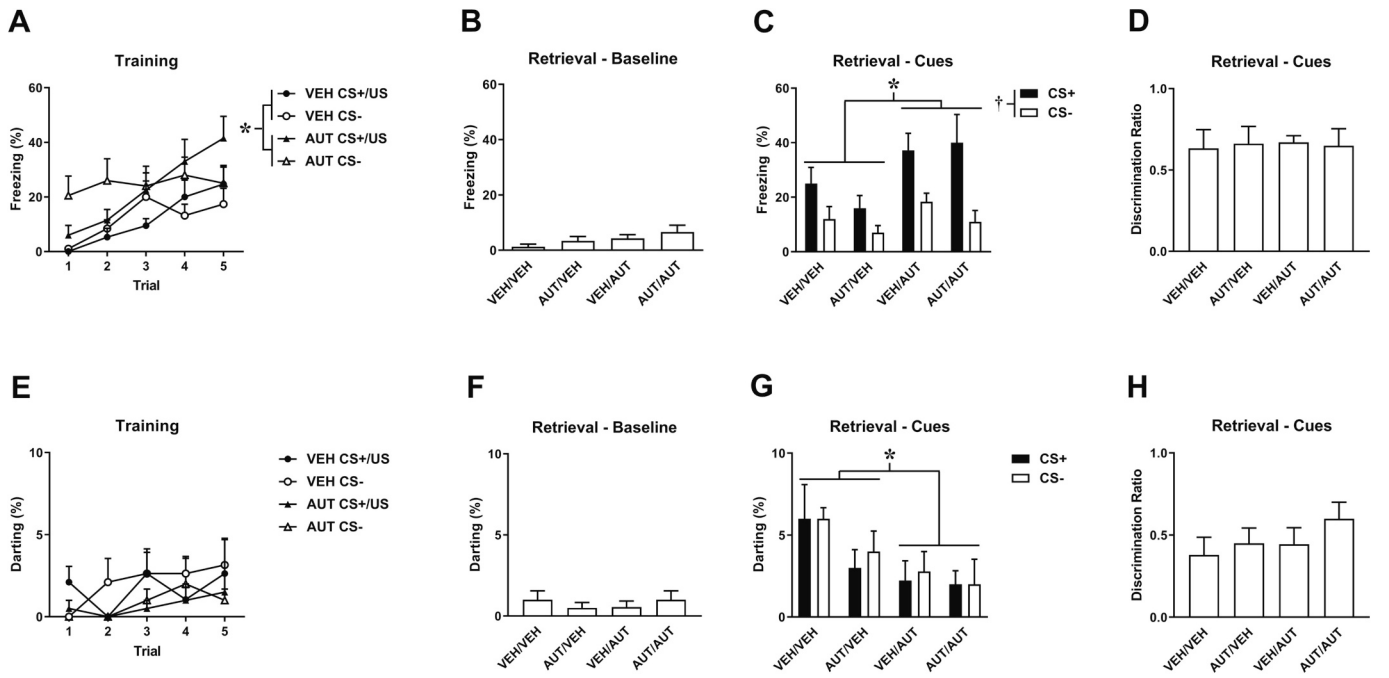

Fig. 4. Effects of AUT00206 (AUT) given before fear discrimination training and/or retrieval with limited discrimination training, based on freezing (A-D) and darting (E-H). A) Freezing in response to CS+/US pairings and CS- presentations during training. Freezing was increased by AUT, compared to vehicle (VEH), across CS+/US pairings and CS- presentations (* $P < 0.05$). B) Freezing before cue presentations at retrieval was unaffected by AUT. C) Freezing in response to the CS+ and CS- at retrieval. Freezing was increased during the CS+, compared to the CS-, across all groups († $P < 0.05$). Freezing was also increased in the VEH/AUT and AUT/AUT, compared to the VEH/VEH and AUT/VEH, groups across both cues (* $P < 0.05$). D) The discrimination ratio, based on freezing during the CS+ and CS- at retrieval, was unaffected by AUT. E) Darting in response to CS+/US pairings and CS- presentations during training. AUT had no effect on darting during training. F) Darting before cue presentations during retrieval was unaffected by AUT. G) Darting in response to the CS+ and CS- at retrieval. There were no differences in darting during the CS+, compared to the CS-, across all groups. Darting was decreased in the VEH/AUT and AUT/AUT, compared to the VEH/VEH and AUT/VEH, groups across both cues (* $P < 0.05$). H) The discrimination ratio, based on darting during the CS+ and CS- at retrieval, was unaffected by AUT.

increased during the CS+, compared to the CS-, across all groups ($P < 0.05$), indicating more discrimination. Freezing was significantly increased in the VEH/AUT ($n = 9$) and AUT/AUT ($n = 10$), compared to the VEH/VEH (n = 10) and AUT/VEH (n = 10), groups across both cues ($P < 0.05$), indicating that AUT00206 given before retrieval enhanced freezing in response to the cues at retrieval (Fig. 4C). Two-way ANOVA on the discrimination ratio based on freezing showed no main effects of treatment before training ($F_{(1, 35)} = 0.044$, $P = 0.83$) or treatment before retrieval ($F_{(1, 35)} = 0.0083$, $P = 0.93$), and no interaction between these factors ($F_{(1, 35)} = 0.0002$, $P = 0.99$), indicating that AUT00206 had no effect on discrimination (Fig. 4D).

The effects of AUT00206 on darting during limited training and at retrieval are shown in Fig. 4E-H. The levels of darting were very low throughout in comparison to freezing. Three-way ANOVA on darting during the one day of training showed no main effects of treatment ($F_{(1, 37)} = 2.39$, $P = 0.13$), cue ($F_{(1, 37)} = 0.56$, $P = 0.46$) or trial ($F_{(4, 148)} = 1.61$, $P = 0.17$), and no interactions between any of these factors (not shown), indicating a lack of effect of AUT00206 on darting during training (Fig. 4E). Two-way ANOVA on darting before cue presentations at retrieval showed no main effects of treatment before training ($F_{(1, 35)} = 0.0035$, $P = 0.95$) or treatment before retrieval ($F_{(1, 35)} = 0.0035$, P = 0.95), and no interaction between these factors ($F_{(1, 35)} = 1.02$, $P = 0.32$), indicating a lack of effect of AUT00206 on baseline darting (Fig. 4F). Three-way ANOVA on darting during cue presentations at retrieval revealed a significant main effect of treatment before retrieval ($F_{(1, 35)} = 8.03$, $P = 0.008$, $\eta_p^2 = 0.19$) but no main effects of treatment before training ($F_{(1, 35)} = 2.89$, $P = 0.10$) or cue ($F_{(1, 35)} = 0.16$, $P = 0.69$), and no interactions between any of the factors (not shown). Despite the low levels observed, post-hoc analysis showed that darting was significantly decreased in the VEH/AUT and AUT/AUT, compared to the VEH/VEH and AUT/VEH, groups ($P < 0.05$), indicating that AUT00206 given before retrieval reduced darting in response to the cues at retrieval (Fig. 4G). Two-way ANOVA on the discrimination ratio based on darting showed no main effects of treatment before training ($F_{(1, 35)} = 1.68$, $P = 0.20$) or treatment before retrieval ($F_{(1, 35)} = 1.54$, $P = 0.22$), and no interaction between these factors ($F_{(1, 35)} = 0.09$, $P = 0.77$), indicating that AUT00206 had no effect on discrimination (Fig. 4H).

3.3. AUT00206 has dissociable effects on fear discrimination and expression with extended training in a response-dependent manner

The effects of AUT00206 on freezing during extended discrimination training and at retrieval are shown in Fig. 5A-F. Four-way ANOVA on freezing during the three days of training revealed significant main effects of treatment ($F_{(1, 35)} = 5.63$, $P = 0.023$, $\eta_p^2 = 0.14$), trial ($F_{(4, 140)} = 8.00$, $P < 0.001$, $\eta_p^2 = 0.19$), and day ($F_{(2, 70)} = 4.91$, $P = 0.010$, $\eta_p^2 = 0.12$) but not cue ($F_{(1, 35)} = 0.56$, P = 0.46). It also revealed significant cue x trial ($F_{(4, 140)} = 8.06$, $P < 0.001$, $\eta_p^2 = 0.19$) and trial x day ($F_{(8, 280)} = 21.76$, $P < 0.001$, $\eta_p^2 = 0.38$) interactions. There were no other interactions between the factors (not shown). Post-hoc analysis showed that AUT00206 ($n = 19$) significantly increased freezing, compared to vehicle ($n = 18$), across all days, trials, and both cues ($P < 0.05$; Fig. 5A-C). Two-way ANOVA on freezing before cue presentations at retrieval revealed a significant main effect of treatment before training ($F_{(1,33)} = 5.85$, $P = 0.021$, $\eta_p^2 = 0.15$) but no main effect of treatment before retrieval ($F_{(1, 33)} = 0.0091$, $P = 0.92$) or interaction between these factors ($F_{(1, 33)} = 0.46$, $P = 0.50$). Post-hoc analysis showed that freezing was significantly decreased in the AUT/VEH ($n = 9$) and AUT/AUT ($n = 10$), compared to the VEH/VEH (n = 9) and VEH/AUT (n = 9), groups ($P < 0.05$), indicating that AUT00206 given before training reduced

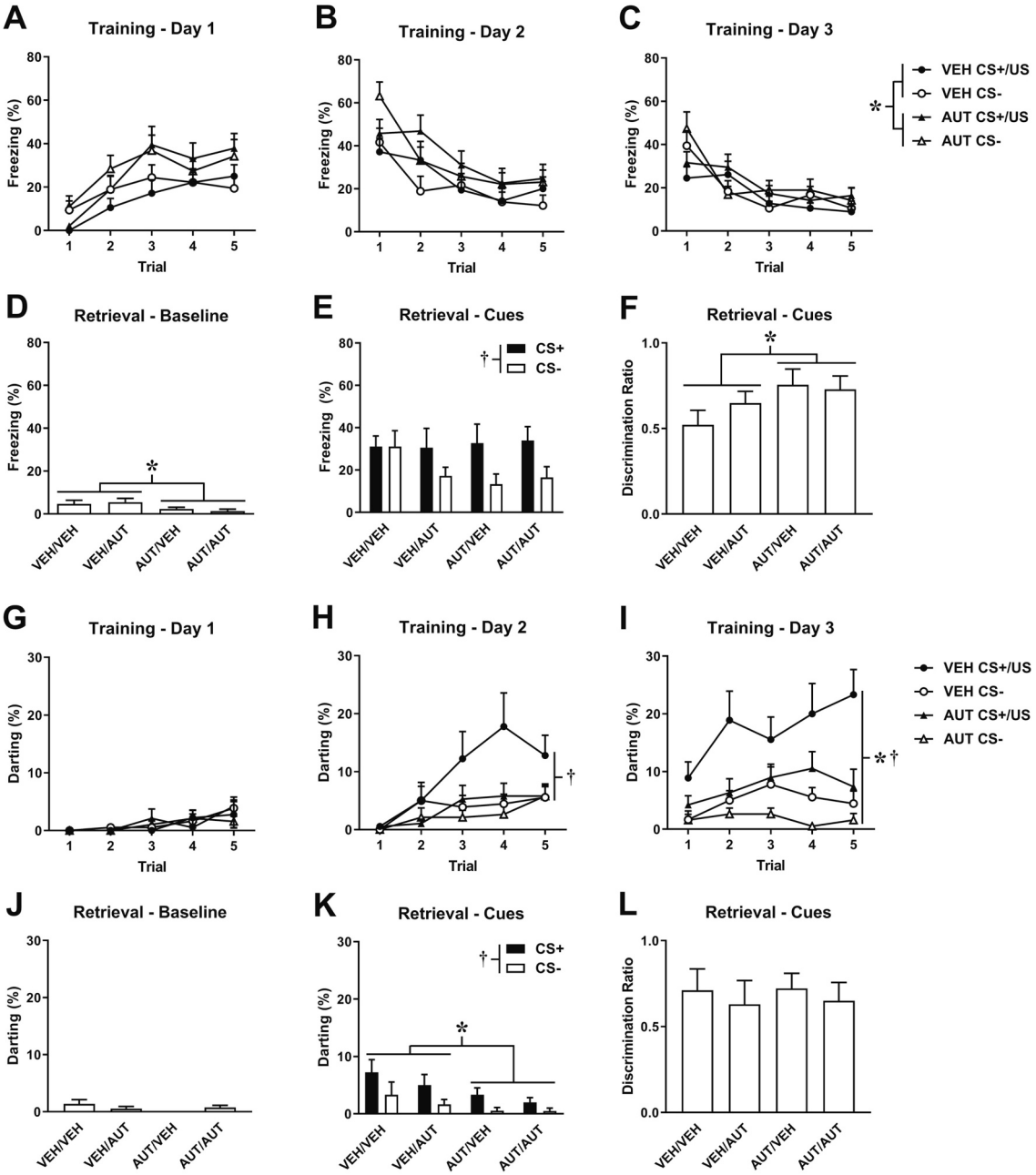

Fig. 5. Effects of AUT00206 (AUT) given before fear discrimination training and/or retrieval with extended discrimination training, based on freezing (A-F) and darting (G-L). A-C) Freezing in response to CS+/US pairings and CS- presentations on Day 1 (A), Day 2 (B), and Day 3 (C) of training. Freezing was increased by AUT, compared to vehicle (VEH), across all days and both cues during training (* $P < 0.05$). D) Freezing before cue presentations during retrieval was decreased in the AUT/VEH and AUT/AUT, compared to the VEH/VEH and VEH/AUT, groups (* $P < 0.05$). E) Freezing in response to the CS+ and CS- at retrieval. Freezing was increased during the CS+, compared to the CS-, across all groups († $P < 0.05$). F) The discrimination ratio, based on freezing during the CS+ and CS- at retrieval, was increased in the AUT/VEH and AUT/AUT, compared to the VEH/VEH and VEH/AUT, groups (* $P < 0.05$). G-I) Darting in response to CS+/US pairings and CS- presentations on Day 1 (G), Day 2 (H), and Day 3 (I) of training. G) AUT had no effect on darting on Day 1 of training. H) Darting was increased in response to CS+/US pairings, compared to CS- presentations, in the VEH († $P < 0.05$), but not the AUT, group on Day 2 of training. I) On Day 3 of training, darting was increased in response to CS+/US pairings, compared to CS- presentations, in the VEH and AUT groups († $P < 0.05$). Darting was also decreased by AUT, compared to VEH, in response to CS+/US pairings and CS- presentations (* $P < 0.05$). J) Darting before cue presentations during retrieval was unaffected by AUT. K) Darting in response to the CS+ and CS- at retrieval. Darting was increased in response to the CS+, compared to the CS-, across all groups († $P < 0.05$). Darting was also decreased in the AUT/VEH and AUT/AUT, compared to the VEH/VEH and VEH/AUT, groups across both cues (* $P < 0.05$). L) The discrimination ratio, based on darting during the CS+ and CS- at retrieval, was unaffected by AUT.

baseline freezing at retrieval (Fig. 5D). Three-way ANOVA on freezing during cue presentations at retrieval revealed a significant main effect of cue ($F_{(1, 33)} = 10.14$, $P = 0.003$, $\eta_p^2 = 0.24$) but no main effects of treatment before training ($F_{(1, 35)} = 0.40$, $P = 0.53$) or treatment before retrieval ($F_{(1, 35)} = 0.23$, $P = 0.64$), and no interactions between any of the factors (not shown). Post-hoc analysis showed that freezing was significantly increased during the CS+, compared to the CS-, across all groups ($P < 0.05$), indicating more discrimination. Despite the lack of significant interactions, this appeared to be driven by differences between the CS+ and CS- with AUT00206 treatment since VEH/VEH

controls seemed to generalize more, which was confirmed by a direct comparison showing no difference in freezing between the CS+ and CS- (paired *t*-test: $t_8 = 0$, $P > 0.99$) in this group (Fig. 5E). Two-way ANOVA on the discrimination ratio based on freezing revealed a significant main effect of treatment before training ($F_{(1, 33)} = 4.70$, $P = 0.038$, $\eta_p^2 = 0.13$) but no main effect of treatment before retrieval ($F_{(1, 33)} = 0.16$, $P = 0.69$) or interaction between these factors ($F_{(1, 33)} = 0.92$, $P = 0.34$). Post-hoc analysis showed that the discrimination ratio was significantly increased in the AUT/VEH and AUT/AUT, compared to the VEH/VEH and VEH/AUT, groups ($P < 0.05$), indicating that AUT00206 given before training enhanced discrimination at retrieval based on freezing (Fig. 5F).

The effects of AUT00206 on darting during extended training and at retrieval are shown in Fig. 5G-L. Four-way ANOVA on darting during the three days of training revealed significant main effects of treatment ($F_{(1, 35)} = 4.66$, $P = 0.038$, $\eta_p^2 = 0.12$), cue ($F_{(1, 35)} = 19.85$, $P < 0.001$, $\eta_p^2 = 0.36$), trial ($F_{(4, 140)} = 12.83$, $P < 0.001$, $\eta_p^2 = 0.27$), and day ($F_{(2, 70)} = 30.23$, $P < 0.001$, $\eta_p^2 = 0.46$). It also revealed significant treatment x day ($F_{(2, 70)} = 7.03$, $P = 0.002$, $\eta_p^2 = 0.17$), cue x trial ($F_{(4, 140)} = 4.80$, $P = 0.001$, $\eta_p^2 = 0.12$), cue x day ($F_{(2, 70)} = 23.33$, $P < 0.001$, $\eta_p^2 = 0.40$), trial x day ($F_{(8, 280)} = 2.28$, $P = 0.022$, $\eta_p^2 = 0.061$), treatment x cue x day ($F_{(2, 70)} = 4.26$, $P = 0.018$, $\eta_p^2 = 0.11$), and cue x trial x day ($F_{(8, 280)} = 2.34$, $P = 0.019$, $\eta_p^2 = 0.063$) interactions. There were no other interactions between the factors (not shown). On Day 1 (Fig. 5G), darting levels were very low overall and further analysis revealed no differences between the CS+/US pairings and CS- presentations (main effect of cue: $F_{(1, 35)} = 0.006$, $P = 0.94$) or any effect of AUT00206 (main effect of treatment: $F_{(1, 35)} = 0$, $P = 0.99$). On Day 2 (Fig. 5H), further analysis revealed that darting was significantly increased in response to the CS+/US pairings, compared to the CS-, in the vehicle (main effect of cue: $F_{(1, 17)} = 6.62$, $P = 0.020$, $\eta_p^2 = 0.28$) but not the AUT00206 (main effect of cue: $F_{(1, 18)} = 1.42$, $P = 0.25$) group. On Day 3 (Fig. 5I), darting was significantly increased in response to the CS+/US pairings, compared to the CS-, in both the vehicle (main effect of cue: $F_{(1, 17)} = 19.07$, $P < 0.001$, $\eta_p^2 = 0.53$) and AUT00206 (main effect of cue: $F_{(1, 18)} = 11.49$, $P = 0.003$, $\eta_p^2 = 0.39$) groups. Darting was also significantly decreased in the AUT00206, compared to the vehicle, group in response to both the CS+/US pairings and CS- (main effect of treatment: $F_{(1, 35)} = 7.59$, $P = 0.009$, $\eta_p^2 = 0.18$). Darting levels before cue presentations at retrieval were very low and two-way ANOVA showed no main effects of treatment before training ($F_{(1, 33)} = 1.76$, $P = 0.19$) or treatment before retrieval ($F_{(1, 33)} = 0.0086$, $P = 0.93$), and no interaction between these factors ($F_{(1, 33)} = 3.09$, $P = 0.088$), indicating that AUT00206 had no effect on baseline darting before retrieval (Fig. 5J). Three-way ANOVA on darting during cue presentations at retrieval revealed significant main effects of treatment before training ($F_{(1, 33)} = 8.73$, $P = 0.006$, $\eta_p^2 = 0.21$) and cue ($F_{(1, 33)} = 7.21$, $P = 0.011$, $\eta_p^2 = 0.18$) but not treatment before retrieval ($F_{(1, 33)} = 2.07$, $P = 0.16$), and there were no interactions between any of the factors (not shown). Post-hoc analysis showed that darting was significantly increased during the CS+, compared to the CS-, across all groups ($P < 0.05$), indicating more discrimination. Despite the lower levels observed in comparison to Days 2–3 of training, darting was also significantly decreased in the AUT/VEH and AUT/AUT, compared to the VEH/VEH and VEH/AUT, groups across both cues ($P < 0.05$), indicating that AUT00206 given before training reduced darting at retrieval (Fig. 5K). Two-way ANOVA on the discrimination ratio based on darting at retrieval showed no main effects of treatment before training ($F_{(1, 33)} = 0.022$, $P = 0.88$) or treatment before retrieval ($F_{(1, 33)} = 0.51$, $P = 0.48$), and no interaction between these factors ($F_{(1, 33)} = 0.0073$, $P = 0.93$), indicating that AUT00206 had no effect on discrimination based on this measure (Fig. 5L).

3.4. AUT00206 reduces locomotor activity in the open field test without affecting shock sensitivity

The effects of AUT00206 on behavior during open field testing are shown in Fig. 6A-D. Compared to vehicle ($n = 10$), AUT00206 ($n = 10$) significantly decreased the time spent in ($t_{18} = 2.79$, $P = 0.012$, Cohen's $d = 1.25$; Fig. 6A) and number of entries into ($t_{18} = 2.66$, $P = 0.016$, Cohen's $d = 1.19$; Fig. 6B), but not latency to enter ($t_{18} = 0.067$, $P = 0.95$; Fig. 6C), the inner zone of the open field. AUT00206 also significantly decreased the horizontal distance moved ($t_{18} = 2.88$, $P = 0.01$, Cohen's $d = 1.29$; Fig. 6D), compared to vehicle, indicating that its effects were likely due to reduced locomotion rather than enhanced anxiety-like behavior. The effects of AUT00206 on shock sensitivity are shown in Fig. 6E. Two-way ANOVA revealed no main effect of treatment ($F_{(1, 17)} = 0.20$, $P = 0.66$) or treatment x response interaction ($F_{(1, 17)} = 2.44$, $P = 0.14$), indicating that there were no differences in the threshold current eliciting flinch or vocalization responses between AUT00206 ($n = 9$) and vehicle ($n = 10$) treatment. Mean AUT00206 concentration in the blood 45–60 min after AUT00206 treatment was 7.85 ± 1.28 μg/mL (range = 1.1–13.4 μg/mL).

4. Discussion

In this study we found more fear discrimination at retrieval after limited training based on freezing, while darting expression and discrimination based on this measure were both low. AUT00206 increased freezing and decreased darting acutely but had no effect on freezing-based discrimination with limited training. In contrast, with extended training we found more generalization at retrieval in relation to freezing, whereas discrimination emerged during training based on darting. AUT00206 also increased freezing and decreased darting acutely during extended training. Importantly, AUT00206 given before extended training had dissociable effects on fear discrimination and expression at retrieval in a response-dependent manner. AUT00206 reduced baseline fear before cue presentations and enhanced fear discrimination between the CS+ and CS- without affecting fear expression in response to the CS+ in relation to freezing. In contrast, AUT00206 reduced cue-induced fear expression without affecting fear discrimination based on darting, although absolute darting levels were low overall. AUT00206 had no effect on shock sensitivity and reduced locomotor activity during open field testing, indicating that increased freezing and decreased darting with acute AUT00206 treatment may have involved non-specific effects on locomotion. However, this cannot explain the lasting effects of AUT00206 given before extended training on behavior at retrieval. Our results instead suggest that AUT00206 mitigated fear generalization based on freezing and reduced fear expression based on darting that resulted from extended discrimination training. These findings provide evidence that pharmacological modulation of Kv3.1/3.2 channels regulates fear discrimination and expression in a response-dependent manner.

We found previously that whether females show more discrimination or generalization at retrieval depends on the extent of fear discrimination training received [Day et al., 2016, 2020]. The present results confirm these previous findings showing more discrimination with limited training and more generalization with extended training, based on cue-induced freezing at retrieval. We also examined active fear responding since darting has been reported during fear conditioning and discrimination learning in several [Gruene et al., 2015; Greiner et al., 2019; Colom-Lapetina et al., 2019; Morena et al., 2021; Mitchell et al., 2022; Trott et al., 2022], but not all [Foilb et al., 2017; Blume et al., 2017; Colon et al., 2018; Totty et al., 2021; Tryon et al., 2021], recent studies. With limited training we found very low levels of darting and low discrimination in relation to this measure. However, darting levels increased and discrimination based on darting arose over the course of extended training, in agreement with a recent study [Greiner et al., 2019]. As was the case in these previous studies, we observed darting

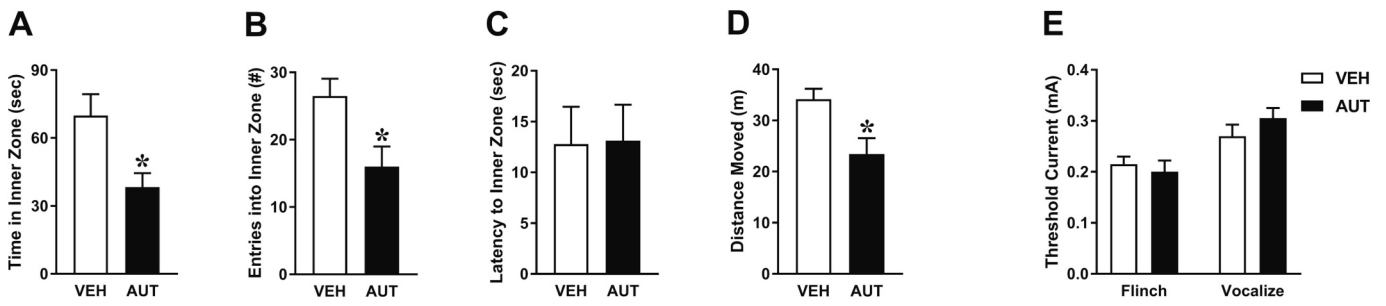

Fig. 6. Effects of AUT00206 (AUT) on behavior in the open field test and on shock sensitivity. A-B) Compared to vehicle (VEH), AUT decreased the time spent in (A) and number of entries into (B) the inner zone of the open field (* $P < 0.05$). C) AUT had no effect on the latency to enter the inner zone of the open field. D) AUT decreased the horizontal distance moved in the open field (* $P < 0.05$). E) There was no difference in the threshold current eliciting flinch responses or audible vocalizations between AUT and VEH treatment.

without accounting for the estrus cycle phase in naturally cycling females, although other studies have demonstrated a role for gonadal hormones in regulating fear discrimination in females [Toufexis et al., 2007; Trask et al., 2020]. Differences in darting between limited and extended training may reflect a switch from freezing to darting behavior with greater threat imminence, as modelled by the increased number of CS+/US pairings received [Greiner et al., 2019; Mitchell et al., 2022]. Furthermore, a shift back towards freezing in the absence of the US might explain why darting returned to lower levels at retrieval [Perusini and Fanselow, 2015]. Other studies have shown that active fear responding is only expressed in the training context [Fadok et al., 2017; Totty et al., 2021], which could also explain the lower levels of darting expressed in the retrieval context. However, more recent evidence indicates that higher darting levels can occur outside of the conditioning context with testing in a larger environment, which may facilitate the expression of this active fear behavior [Mitchell et al., 2022; Demars et al., 2022].

During limited discrimination training AUT00206 increased freezing and had no effect on darting, although this may have involved a floor effect since darting levels were very low. During limited training we found low discrimination between the cues in relation to either response, but limited training did result in more discrimination at later retrieval based on freezing. AUT00206 increased freezing at retrieval but had no effect on discrimination based on this measure. AUT00206 also decreased darting at retrieval despite the low levels displayed, although it had no effect on discrimination based on darting. However, these acute effects of AUT00206 on freezing and darting may have involved non-specific drug effects on locomotion since we also found that AUT00206 reduced locomotor activity in the open field test. This is supported by evidence indicating that Kv3.1 channel knockout results in hyperactivity [Parekh et al., 2018; Bee et al., 2021].

During extended discrimination training AUT00206 increased freezing across the sessions and decreased darting on Day 3. We found low discrimination between the cues during training based on freezing and this was unaffected by AUT00206. However, discrimination based on darting emerged on Day 2 of training, which was influenced by AUT00206. More discrimination was observed with vehicle but not AUT00206 treatment on Day 2, whereas both treatment groups showed more discrimination on Day 3, suggesting that AUT00206 delayed discrimination learning based on darting. Again, these acute drug effects on freezing and darting during extended training may have involved non-specific effects on locomotion. However, AUT00206 given before extended training also had enduring effects on freezing and darting during later retrieval. In terms of freezing, AUT00206 resulted in reduced baseline fear before cue presentations and enhanced discrimination between the cues, indicating the amelioration of contextual and cued fear generalization, respectively, without affecting cue-induced fear expression. In contrast, AUT00206 resulted in decreased darting in response to both cues, indicating reduced fear expression, without affecting baseline fear or discrimination based on this measure. One interpretation of these results when considered in relation to the threat imminence continuum [Perusini and Fanselow, 2015] is that AUT00206 given before extended training reduced the switch from freezing to darting and allowed for more discrimination based on freezing to occur at retrieval.

A limitation of this study is that the neural basis of these behavioral effects of AUT00206 with extended discrimination training remain to be elucidated since drug was given systemically. Positive Kv3.1/3.2 modulation regulates the fast-spiking phenotype of PV interneurons [Rosato-Siri et al., 2015], which are important for governing the firing pattern of pyramidal cell populations and oscillatory dynamics in various corticolimbic areas [Klausberger, 2009; Sohal, 2012; Lucas and Clem, 2018]. Preliminary evidence indicates that AUT00206 influences prefrontal gamma oscillations in vitro, which is consistent with Kv3.1/3.2 modulation in PV interneurons [Neill et al., 2015]. Recent studies have shown that PV interneurons in mPFC, AC, amygdala, and hippocampus are involved in regulating fear discrimination based on freezing [Aizenberg et al., 2015; Guo et al., 2018; Yan et al., 2019; Stujenske et al., 2022]. Gamma and theta oscillations in these inter-connected areas have also been implicated in fear discrimination based on freezing [Headley and Weinberger, 2011; Likhtik et al., 2014; Concina et al., 2018; Tzovara et al., 2019; Day et al., 2020; Stujenske et al., 2022]. It is therefore tempting to speculate that AUT00206 regulates fear discrimination based on freezing by modulating Kv3.1/3.2 channels on PV interneurons and, in turn, oscillatory activity in this corticolimbic circuitry. However, this interpretation should be considered with caution since Kv3.1 and Kv3.2 channels are also expressed in other neuronal subtypes and brain areas [Kaczmarek and Zhang, 2017]. For example, AUT00206 acting on midbrain dopaminergic and basal ganglia neurons may have been involved in the acute locomotor effects reported here [Parekh et al., 2018]. Interestingly, the activation of parvalbumin-expressing projection neurons in different corticothalamic, corticostriatal, and tectofugal circuits has been shown to mediate active fear behavior [Lee et al., 2014; Shang et al., 2015, 2018; Dong et al., 2019; Wang et al., 2019]. However, we found that AUT00206 reduced darting during extended training and had no acute effect at retrieval, suggesting that other circuit mechanisms underpin AUT00206 regulation of darting. Further research is therefore needed to determine the neural basis of AUT00206 regulation of fear discrimination and expression based on freezing and darting, respectively.

To our knowledge this is the first study to show that pharmacological modulation of Kv3.1/3.2 channels regulates fear discrimination and expression. Although AUT00206 had non-specific locomotor effects acutely, when given before extended discrimination training it had enduring effects during later retrieval in a response-dependent manner. Our results add support to the idea that Kv3.1/3.2 channels might be novel targets for treating certain features of psychiatric disease [Pratt et al., 2008; Yanagi et al., 2014; Neill et al., 2015; Medrihan et al., 2020;

Sagi et al., 2020], such as the inappropriate attribution of affective salience to innocuous cues. This study also highlights the utility of characterizing both freezing and darting to better understand fear discrimination and expression. However, more research is needed to determine the adaptive significance and neurobiological basis of darting as an active fear response. Although a recent study found that active darting and flight responses during fear conditioning result largely from non-associative processes [Trott et al., 2022], other studies provide evidence for the associative nature of darting as a conditioned fear response [Gruene et al., 2015; Mitchell et al., 2022; Demars et al., 2022]. A recent study in males showed that the response expressed during early extinction predicted later fear renewal based on freezing, such that darting was associated with more fear renewal than freezing [Demars et al., 2022]. This is broadly in line with our results in females showing that darting during extended discrimination training is linked to more fear generalization based on freezing at retrieval. Moreover, Demars et al. (2022) found that freezing and darting were associated with differences in the expression of genes involved in GABAergic signalling in mPFC, which may also be involved in the behavioral phenotype resulting from extended discrimination training and its mitigation by positive Kv3.1/3.2 modulation as reported here. Future studies are therefore needed to examine the neural substrates underlying AUT00206 regulation of fear discrimination and expression in both females and males.

Ethical statement

All experimental procedures were conducted with ethical approval from the University of Nottingham Animal Welfare and Ethical Review Body and in accordance with the Animals (Scientific Procedures) Act 1986, UK (Home Office Project Licence 30/3230).

CRediT authorship contribution statement

Christine Stubbendorff: Data curation, Formal analysis, Investigation, Supervision, Writing – review & editing. **Ed Hale:** Formal analysis, Investigation, Supervision. **Harriet L.L. Day:** Formal analysis. **Jessica Smith:** Formal analysis, Funding acquisition. **Giuseppe S. Alvaro:** Resources. **Charles H. Large:** Conceptualization, Project administration, Resources, Writing – review & editing. **Carl W. Stevenson:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

This study was funded by Autifony Therapeutics. GSA and CHL are shareholders and full-time employees of Autifony Therapeutics. The other authors declare no competing interests.

Data availability

Data will be made available on request.

Acknowledgments

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