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Reducing shock imminence eliminates poor avoidance in rats

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Abstract

In the signaled active avoidance (SigAA) paradigm, rats learn to suppress Pavlovian reactions (e.g. freezing) and emit instrumental actions (e.g. shuttling) to escape threats and prevent pain. This paradigm is critical for understanding aversively-motivated instrumental learning and both maladaptive and adaptive coping strategies in human anxiety. However, with standard protocols approximately 25% of rats exhibit high freezing and never master the task (poor avoiders). This has dampened enthusiasm for the paradigm and stalled progress. Here we demonstrate that lowering shock imminence with long-duration warning signals leads to greater freezing suppression and perfect avoidance in all subjects. This suggests that instrumental SigAA mechanisms evolved to cope with temporally distant/uncertain harm and standard protocols that promote inflexible Pavlovian reactions are poorly-designed to study avoidance.

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In the signaled active avoidance paradigm (SigAA), rats learn to suppress Pavlovian reactions (e.g. freezing) and emit instrumental actions (e.g. shuttling) to escape warning signals (WSs) and prevent painful unconditioned stimuli (USs, typically footshocks). Understanding the psychological and neural mechanisms of SigAA is critical for several reasons. First, SigAA is the prototypical paradigm for studying aversively-motivated instrumental actions (Rescorla and Solomon, 1967). Second, maladaptive or excessive avoidance responses (ARs) contribute to every major anxiety disorder (APA, 2013). Third, adaptive ARs reduce emotional reactions and give subjects control over environmental threats (Boeke et al., 2017; Choi et al., 2010; Kamin et al., 1963). Thus, although “avoidance” has a generally negative connotation, SigAA mechanisms may also mediate adaptive proactive coping behaviors and resilience in humans (Collins et al., 2014; van der Kolk, 2006).

Despite its importance as a fundamental learning mechanism with clear relevance to human anxiety, SigAA research has lagged far behind research on Pavlovian threats and instrumental learning with appetitive outcomes (Cain, 2019; Krypotos et al., 2015; LeDoux et al., 2017). The phenomenon of “poor avoidance” in the laboratory has been one major obstacle to progress. Avoidance learning is typically slower than Pavlovian conditioning, but most animals learn to prevent greater than 80% of scheduled shocks. However, a significant subset of animals exhibit high freezing and only rarely emit ARs (approximately 10-30%, depending on the task) (Galatzer-Levy et al., 2014). From a practical standpoint, avoidance studies are more costly and time-consuming because additional animals must be trained to replace poor avoiders that are excluded from final analyses. Pretraining loss-of-function studies are also ill-advised with SigAA, since there is currently no reliable way to predict which

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animals will fail to acquire the task. Finally, the poor avoidance phenomenon raises serious questions about whether instrumental AR learning is a major component of defense worthy of study (Bolles, 1975; Fanselow, 1997; 2018). In the real world, animals evolved defensive learning mechanisms to cope with predators, not shocks, and it is difficult to see how a trial-and-error learning mechanism that fails so often could have evolved under predatory pressure.

One simple explanation for poor avoidance is that researchers have used less than optimal protocols for studying the phenomenon in the laboratory. Avoidance is typically evaluated in small chambers with short-duration warning signals and high-density shock protocols. These conditions are ideal for modelling a state of high predatory imminence that triggers hard-wired, stereotyped fear-like reactions (e.g. freezing) that are incompatible with ARs (Fanselow and Lester, 1988). However, prey animals spend much more time in a state of low predatory imminence where encounters with predators are temporally distant or uncertain. Perhaps instrumental avoidance mechanisms evolved to deal with these anxiety-like states, where animals must balance the need for exploration/procuring rewards with the need to prevent predation (Cain, 2019; Gray and McNaughton, 2000). Under these “pre-encounter” conditions, less rigid defensive behaviors may not interfere with AR learning.

In an effort to solve the poor avoidance problem and optimize avoidance training, we designed two experiments to evaluate AR learning while systematically varying threat intensity. In the first, WS parameters were held constant, but WS-US contingency was varied to test how US certainty affects AR learning. In the second, WS-US contingency was held constant, but WS duration was varied to test how US imminence affects AR learning. In Pavlovian studies, reducing US certainty or imminence appears to promote anxiety over fear; freezing reactions are diminished and more flexible antipredator strategies increase (Blanchard et al., 1989; Cain

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et al., 2005; Goode et al., 2019; Helmstetter and Fanselow, 1993; Kim and Jung, 2018; Mobbs et al., 2007; Rescorla, 1968; Waddell et al., 2006). Also, lesions that impair freezing rescue ARs in poor avoiders, suggesting that freezing reactions interfere with avoidance (Choi et al., 2010; Lazaro-Munoz et al., 2010; Moscarello and LeDoux, 2013). Pavlovian threat reactions also impair avoidance performance in humans (Rigoli et al., 2012). Thus, we predicted that both methods of reducing threat intensity would decrease Pavlovian freezing and improve AR learning.

Experiments were conducted on adult male and female Sprague-Dawley rats (Hilltop Lab Animals) weighing 300-350g on arrival. Rats were pair-housed by sex, had ad lib access to food and water and were tested during the light phase of a 12:12-hour light:dark schedule. All procedures were approved by the NKI Institutional Animal Care and Use Committee.

In all experiments, rats received 10 days of two-way SigAA training in standard shuttleboxes equipped with speakers, houselights, cameras, grid floors and infrared beam arrays to detect shuttling (Coulbourn Instruments). Each session consisted of a 5-minute acclimation followed by 15 trials where warning stimuli (80dB white noise) preceded scrambled 0.5s inescapable footshocks (1.0mA for males, 0.7mA for females). The first trial of Session 1 for all experiments was a Pavlovian trial regardless of the subject's behavior. This forced at least one WS-shock pairing and ensured that all subsequent WS-shuttles occurred during threat of shock. For all subsequent trials, shuttling to the opposite chamber side terminated the WS, produced a feedback stimulus (5s, 5kHz, 80dB tone), and cancelled the upcoming shock (if scheduled). Shuttling was automatically recorded by Graphic State software (Coulbourn Instruments) and behavior was recorded to video files for off-line analysis of freezing. Intertrial intervals (ITIs) were 2-minutes unless otherwise stated. Avoidance percentage was calculated

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by dividing the number of shuttles during a WS by the number of avoidance trials and multiplying by 100. Avoidance latency reflects the time from WS onset to emission of a shuttle response, with failures recorded as the full WS duration. Freezing, defined as the absence of all non-respiratory movement, was scored during the WS for select sessions by two different experienced raters blind to treatment condition (Pearson inter-rater reliability correlation >0.9). To facilitate comparisons of freezing suppression between the studies, Session 10 freezing was also analyzed as a percentage of Session 1 freezing (calculated for individuals and then averaged).

In Experiment 1, rats received identical SigAA training with a 15s warning signal except the likelihood of receiving a shock on failure trials (no AR emitted) was varied. Failure to emit an AR resulted in shock on 100%, 50% or 25% of trials (n=8/group). Avoidance acquisition is depicted in Figures 1A-B. Two-way ANOVAs (GraphPad Prism v8) indicate differences in rate of acquisition between the groups for both the AR% and AR latency measures (Group x Session interactions: $F_{(18,189)}=1.8$, $p=0.02$; $F_{(18,189)}=2.0$, $p=0.01$), however, reducing WS-US contingency did not improve learning. These differences appear to be driven mainly by a deficit in the 25% group, where rats shuttled on average more slowly and less frequently during the WS. Mean freezing during the WSs is depicted in Figures 1C for sessions 1, 6 and 10. On average, freezing declined across avoidance training but there were no significant differences between the groups. A two-way ANOVA revealed a significant effect for Session ($F_{(2,42)}=21.6$, $p<0.01$), but not for Group ($F_{(2,21)}=0.26$) or the Group x Session interaction ($F_{(4,42)}=1.7$). Similarly, Session 10 freezing was lower on average than Session 1 but behavior was highly variable and no group differences were observed (Figure 1D; One-way ANOVA: $F_{(2,21)}=2.1$).

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In Experiment 2, rats received identical avoidance training with 100% WS-US contingency (shock delivered on every failure trial), but the warning signal duration was systematically varied (15s, 60s or 240s, n=8/group). Avoidance acquisition is depicted in Figures 2A-B, and statistical analyses were identical to Experiment 1. AR% increased across training (Session: $F_{(9,189)}$=21.1, p<0.01) and there was a significant effect of WS duration (Group: $F_{(2,21)}$=4.7, p=0.02), however the pattern of change over time did not differ between groups (Group x Session: $F_{(18,189)}$=0.9). The effect of WS duration was driven mainly by the 240s group, where AR% was higher than the 15s group for sessions 2-5 (Dunnett's tests). Remarkably, every rat in the 240s group showed perfect avoidance from session 3 until the end of training (no failures). As expected for different WS durations, there were large differences in AR latency across training (Group x Session: $F_{(18,189)}$=21.9, p<0.01). These differences are not very informative early in training when failures were common and WS duration determined AR latency. However, it is interesting to note that AR latencies decreased across training for all groups and were very similar by the end of training, even though rats in the 60s and 240s groups had much more time to emit ARs (Figure 2B, inset). Large group differences in freezing were also apparent across training (Figure 3C; Groups x Session: $F_{(4,42)}$=37.7, p<0.01). Again, this may be partly explained by the different WS durations. Rats in all groups froze for most of the WS early in training and freezing declined to similarly low levels as ARs were acquired. Dunnett's post tests revealed that rats in the 240s and 60s groups froze more than rats in the 15s group during Session 1 only (p values <0.01). Interestingly, compared to Session 1, rats in the 240s group showed the strongest suppression of freezing by Session 10 (Figure 2D; one-way ANOVA: $F_{(2,21)}$=3.4, p=0.05; Dunnett's test vs. 15s group: p<0.05). This appears to be more than a simple reflection of the programmed differences in

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WS duration; unlike the other groups, there were no rats in the 240s group that maintained or increased their freezing across training (as occurs in poor avoiders; Lazaro-Munoz et al., 2010).

One potential criticism of the long 240s WS is that apparent ARs simply reflect locomotor activity not instrumental shuttling. To address this, we replicated AR training with the 240s WS (N=8; 5 females, 3 males) and included a Yoked control group (n=8; 4 females, 4 males) that received the same stimuli as master rats. All rats in the 240s-WS group again attained perfect avoidance, but not until Session 6 (data not shown). Yoked controls shuttled far less frequently during the WSs – less than 0.2 shuttles/trial on average by the end of training. A two-way ANOVA comparing WS-shuttles across training revealed a highly significant Group x Session interaction ($F_{(9,126)} = 8.3$, $p<0.01$) and Yoked rats shuttled significantly less than Master rats from Sessions 4-10 (Sidak tests). Considered with results from Experiment 2, showing high WS freezing early in training and very low AR latencies late in training, these data support the notion that WS shuttles represent instrumental ARs even with WS durations as high as 240s.

Experiment 2 was designed to test the effect of US imminence on avoidance learning and competing freezing reactions. However, because the WS duration was varied while the ITI was held constant, another explanation is possible. In Pavlovian studies, the conditioned stimulus (CS) to intertrial interval (ITI) ratio has a strong impact on performance of Pavlovian reactions (reviewed in Balsam et al., 2010). Specifically, higher CS:ITI ratios weaken responding, perhaps because the signal loses informational value relative to the background context (Gibbon and Balsam, 1989). Thus, it is possible that the long 240s WS enhanced avoidance because it raised the WS:ITI ratio and weakened competing freezing reactions. To

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address this, we evaluated avoidance learning and freezing with WS:ITI ratios of 2:1, 1:1 and 1:2 using two different WS durations (60s and 240s). We trained four new groups of rats: 60s-WS:30s-ITI (2:1), 60s-WS:60s-ITI (1:1), 240s-WS:480s-ITI (1:2), and 240s-WS:240s-ITI (1:1). The remaining groups for the analysis came from Experiment 2: 60s-WS:120s-ITI (1:2) and 240s-WS:120s-ITI (2:1). Figure 3A depicts total (cumulative) ARs across 10 sessions of training. A two-way ANOVA revealed a significant effect of WS-duration ($F_{(1,42)}$=12.9, p<0.01) and a non-significant trend towards a WS-duration x Ratio interaction ($F_{(2,42)}$=2.6, p=0.09). The main effect for Ratio was not significant ($F_{(2,42)}$=1.3). Thus, reducing the WS:ITI ratio failed to impair AR acquisition. This was especially clear for the 240s groups – perfect avoidance was achieved for all rats in every 240s WS group tested. The same manipulation may reduce avoidance with a 60s WS; total ARs declined as the WS:ITI ratio dropped for the 60s WS. Further, in the 2:1 condition, rats in the 60s group avoided less than rats in the 240s group (planned post-hoc Sidak's comparison). Suppression of freezing was more sensitive to the WS:ITI ratio (Figure 3B). Session 10 freezing increased as the WS:ITI ratio dropped (Ratio: $F_{(2,42)}$=0.03, p=0.03), but this effect was not modulated by WS-duration (WS-duration: $F_{(1,42)}$=0.09; WS-duration x Ratio: $F_{(2,42)}$=1.4). Together these data suggest that the WS:ITI ratio is not a major determinate of AR acquisition when the WS duration is 240s. However, there are indications that reducing this ratio promotes freezing and impairs AR learning, especially with the shorter 60s WS. The reverse also appears true; using a WS:ITI ratio of 2:1 led to very low Session 10 freezing and perfect avoidance for 7 of the 8 rats trained with the 60s WS. A more thorough analysis exploring a wider range of WS:ITI ratios is needed to clarify these findings.

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Lastly, all experiments in this report included both female and male subjects. Sex differences were difficult to evaluate for groups that included poor avoiders, as the likelihood of becoming a poor avoider appears random (equally likely in both sexes). However, since all rats in the 240s-WS condition achieved perfect avoidance we were able to evaluate potential sex differences for these groups. Combining rats from Experiment 2 and the Yoked control experiment (240s WS condition, 120s ITI) resulted in group sizes of N=9 (females) and N=7 (males). Avoidance learning was nearly identical between the sexes as measured by AR% (Session: $F_{(9,126)}=41.0$, $p<0.01$, Sex: $F_{(1,14)}=2.3$, Session x Sex: $F_{(9,126)}=1.0$) and AR latency (Session: $F_{(9,126)}=39.3$, $p<0.01$, Sex: $F_{(1,14)}=3.6$, Session x Sex: $F_{(9,126)}=0.6$). Freezing during WSs across Sessions 1, 6 and 10 was also very similar between the sexes (Session: $F_{(2,28)}=81.5$, $p<0.01$, Sex: $F_{(1,14)}=0.29$, Session x Sex: $F_{(2,28)}=0.08$)

The major finding of these experiments is that reducing US imminence by extending WS duration greatly facilitates SigAA learning. In four separate groups trained with the long-duration (240s) WS, every rat learned and performed the task perfectly (no subsequent failures), sometimes in fewer than 30 trials (Figure 2A). The benefits of the long-duration WS also resisted manipulations of the WS:ITI ratio that promote competing freezing reactions and impair avoidance (Figure 3A). Several observations also argue against the concern that shuttling during long-duration WSs reflects exploration rather than instrumental ARs. First, exploration was severely depressed early in training where rats froze for more than 60% of the WS (Figure 2C). Second, once the response was acquired, ARs were emitted with short latencies (usually <15s), similar to rats trained with the 15s WS. Third, yoked controls shuttled during the WS at a far lower rate than master rats.

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What might explain the enhanced efficiency of SigAA with long-duration WSs? Though there are some reports of improved SigAA learning with longer or more complex WSs (Archer et al., 1984; Coll-Andreu et al., 1993; Levis and Stampfl, 1972; Satorra-Marin et al., 2001), this has not been systematically studied. There are far more studies of US imminence using Pavlovian paradigms. These studies suggest that conditioned stimuli (CSs) activate different components of the survival circuit depending on proximity to harm (modeled by CS-US, delay)(Davis, 1998; Goode et al., 2020; Mobbs et al., 2009; Mobbs et al., 2007; Sullivan et al., 2004; Waddell et al., 2006; Walker and Davis, 1997). Short-duration CSs recruit basolateral and central amygdala, and periaqueductal gray to emit short-latency, inflexible, hard-wired responses that function to prevent threat escalation (e.g. freezing, a post-encounter response) or escape harm (e.g. flight, a circa-strike response). Long-duration CSs recruit bed nucleus of the stria terminalis and prefrontal cortex to flexibly reorganize behavior (e.g. thigmotaxis, altered meal-patterns), presumably to prevent threat escalation and prepare the organism to defend against distant or uncertain harm. Importantly, high US-imminence severely constrains behavior to species-specific defense responses (SSDRs) whereas low US-imminence appears to balance defense with other non-defensive behaviors like exploration and reward procurement (Fanselow, 2018; Mobbs et al., 2015). Thus, long-duration WSs likely trigger less intense SSDRs and more opportunity to emit active responses like shuttling. This is consistent with the pattern of freezing data obtained in our experiments; though 240s-WS rats froze significantly early in training (~62% of the WS; Figure 2C), they had considerably more time to emit the AR and experience the instrumental contingency than rats in the 60s and 15s groups. Freezing appeared to be more easily suppressed in this condition too (Figure 2D). Lastly, trial-and-error SigAA learning mechanisms may have evolved to be optimal under low threat

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conditions, where errors (failures to emit the AR) lead to more intense threats but not necessarily contact with harm.

Though our hypothesis about lowering threat intensity to improve avoidance was supported by the long-duration WS experiment, it was not supported by reducing the WS-US contingency. Rats receiving shocks on only 25% of failure trials did not perform better than rats in the 100% contingency condition. We see two possible explanations for this. First, SigAA learning likely depends, at least in part, on omission of expected US presentations (Bolles et al., 1966; Cain, 2019; Kamin, 1956). So even if 25% WS-US contingency reduces certainty and competing freezing reactions, this may have been offset by degradation of an important reinforcement signal. Second, 10 sessions of SigAA training may have been too few to observe the benefit of reduced WS-US contingency. Though rats in the 25% condition emitted fewer ARs across training, performance was steadily improving and could conceivable surpass performance of rats in the 100% condition with more training. Additional work is needed to clarify these points.

Interestingly, our follow-up experiment (Figure 3) suggests another possible way to improve SigAA efficiency: increase the WS:ITI ratio. Pavlovian studies show that increasing the CS:ITI ratio impairs Pavlovian reactions (Balsam et al., 2010; Delamater and Holland, 2008; Stein et al., 1958). This is likely a result of the CS losing informational value relative to the background context (Gibbon and Balsam, 1989). As a consequence, the CS is a weaker threat. We see a similar pattern in freezing suppression during SigAA training; increasing the WS:ITI ratio produced weaker Session 10 freezing and near-perfect avoidance with the shorter 60s-WS. If replicated, this protocol could ensure good avoidance in all subjects with a significantly shorter session duration.

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In summary, these experiments describe two simple procedural methods to eliminate poor avoidance and improve SigAA learning in rats. This removes a major obstacle to SigAA research that has dampened enthusiasm for the paradigm over decades. Experiments requiring pre-training manipulations can be used with confidence if controls reliably learn and perform the AR. The explanation for enhanced SigAA behavior with low-intensity threats also aligns with functional behavior systems theories of defensive behavior and Pavlovian studies of US-imminence. This work may also help explain how strong avoidance responses may be acquired in human anxiety even when harm is not imminent.

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Figure Captions

Figure 1. Reducing WS-US contingency does not improve avoidance. A) Mean percent avoidance by session. **B)** Mean avoidance response (AR) latency by session. **C)** Mean seconds freezing during warning signals for Sessions 1, 6 and 10. **D)** Mean Session 10 freezing expressed as a percentage of Session 1 freezing. Squares represent individuals. N=8/group (4 females, 4 males). Error bars = S.E.M. *p<0.05 vs. 100% WS-US contingency group

Figure 2. Reducing US imminence leads to perfect avoidance. A) Mean percent avoidance by session. **B)** Mean avoidance response (AR) latency by session. ***inset*:** mean AR latency for individuals during Session 10. **C)** Mean seconds freezing during warning signals for sessions 1, 6 and 10. **D)** Mean Session 10 freezing expressed as a percentage of Session 1 freezing. Dots represent individuals. N=8/group (4 females, 4 males). Error bars = S.E.M. *p<0.05 vs. 15s-WS group

Figure 3. Reducing the WS:ITI ratio fails to impair avoidance with a 240s warning signal. A) Total avoidance responses emitted across 10 sessions of training. B) Mean Session 10 freezing expressed as a percentage of Session 1 freezing. N=8/group (4 females, 4 males). Bars represent separate groups. Bar height indicates group mean. Dots represent individuals. Error bars = S.E.M. *p<0.05 for 240s vs. 60s WS groups

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Figure 1

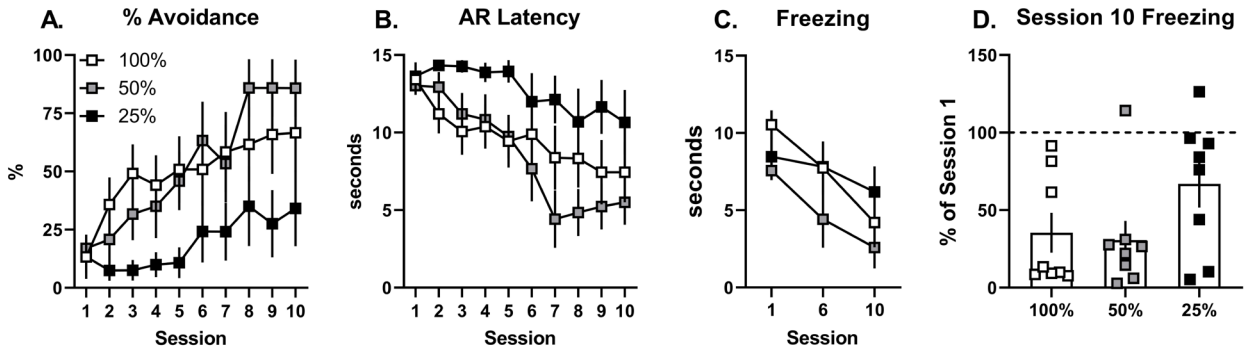

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Figure 2

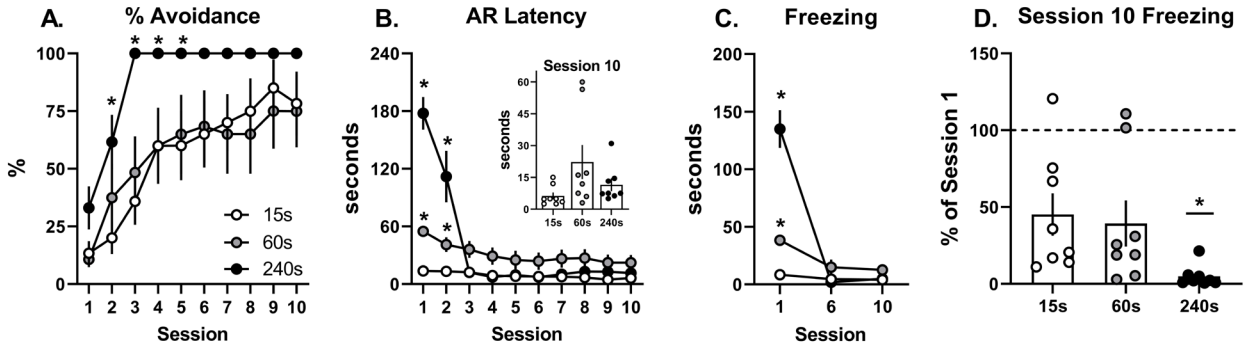

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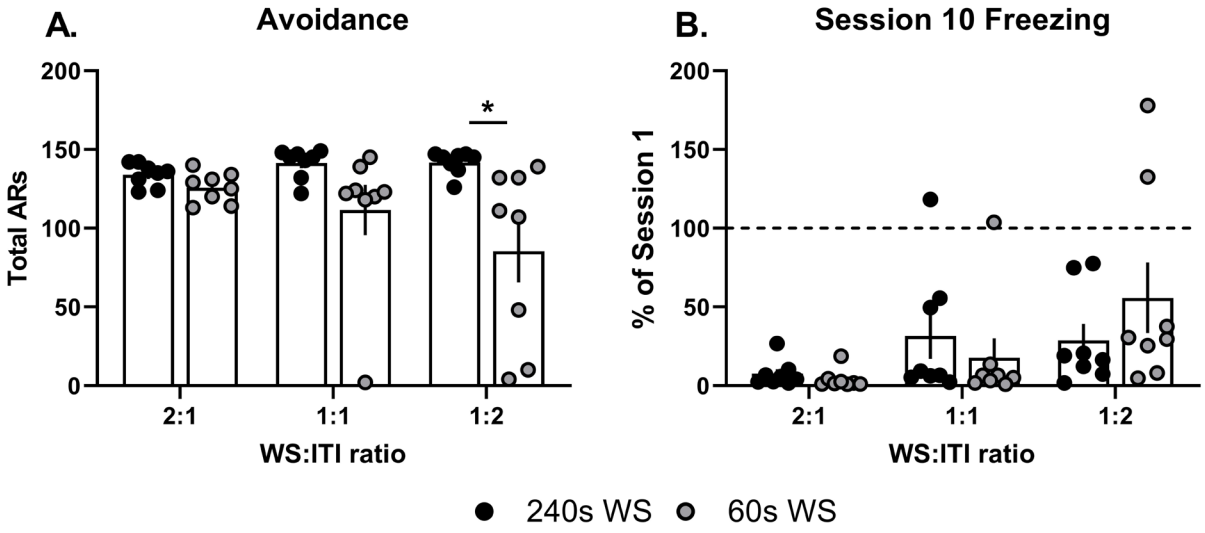
Figure 3



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