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Cholinergic overstimulation supports conditioned-facilitated startle but not conditioned hyperalgesia

Kevin D. Beck [a,b,c,*], Michele Hsu [d], Xilu Jiao [b,c], Richard J. Servatius [a,b,c]

[a] Neurobehavioral Research Laboratory (129), Department of Veterans Affairs New Jersey Health Care System, East Orange, NJ, USA
[b] Stress and Motivated Behavior Institute, New Jersey Medical School, University of Medicine and Dentistry of New Jersey, Newark, NJ, USA
[c] Department of Neuroscience, New Jersey Medical School, University of Medicine and Dentistry of New Jersey, Newark, NJ, USA
[d] Department of Biology, Honors College, Rutgers University, Newark, NJ, USA

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Abstract

Pyridostigmine bromide (PB), a peripheral cholinesterase inhibitor, has been shown to support odor-potentiated startle responding in rats. Here we conducted 2 sets of experiments that further characterize aspects of this learned association. First we conducted experiments designed to further characterize the learning parameters of the odor–PB association that leads to startle facilitation weeks later. We found that an acute injection of PB causes an increase in startle reactivity that lasts less than 2 h. This is evidence for PB's direct action on the startle response as an enhancing agent. We also delineated the duration of the conditioned enhancement to less than 4 weeks. Second, we conducted similar studies but substituted a nociceptive paw-lick response (thermal pain reflex) for the startle reflex. PB did not have an unconditional action upon the latency to paw-lick to a 48.5 °C heated plate nor did any subsequent changes in paw-lick occur in the presence of the previously paired odor. These results suggest that the actions of PB, as an unconditional stimulus, are limited to specific behaviors. Future work examining this compound as a source of conditioned symptoms (as in the case of Gulf War Illness) should focus on those symptoms that are directly influenced by peripheral cholinergic activity. Published by Elsevier Inc.

Keywords: Pyridostigmine bromide; Contextual learning; Startle; Pain; Paw-lick; Odor

Unexplained symptom clusters following coalition troop deployment in the first Gulf War (Operation Desert Storm) led to years of speculation concerning the cause of diverse symptoms in thousands of returning troops. In the last couple years, a new approach to the etiology of these symptoms has arisen. The human research suggests that veterans experience more and/or more severe symptoms following exposure to certain salient odors (such as diesel fumes) (Ferguson et al., 2004). Animal research subsequently showed that either mild cholinergic overstimulation, caused by the antinerve gas medication pyridostigmine bromide (PB), or initiation of an immune response, elicited by an injection of interleukin-1β (IL-1β) could be associated with a proximal odor, leading to later exaggerated startle responses when the same conditional odor was presented again (Servatius and Beck, 2005). This behavioral response to the odor was shown to be evident 2 weeks following the initial pairing of the odor with the drug injections. These odor-elicited exaggerated startle responses appear to mimic conditioned responses (CRs) associated with fear, similar to the conditioned freezing and analgesia found when odors are paired with lithium chloride (Richardson and McNally, 2003). Further characterization of the learned responses to these conditioned odors could provide additional information as to the extent to which this type of conditioning could potentially cause conditioned symptoms. These conditioned symptoms could, in part, be a cause of certain unexplained symptoms in Operation Desert Storm veterans.

The data described in Ferguson and colleagues' diary study suggests that the veterans experienced a worsening of some physical symptoms following exposure to distinct odors (Ferguson et al., 2004). The most common odors reported in this study were:

* Corresponding author. Neurobehavioral Research Laboratory (129), Department of Veterans Affairs New Jersey Health Care System, East Orange, NJ 07018, USA. Tel.: +1 973 676 1000x3682; fax: +1 973 395 7114.
E-mail address: beck@njneuromed.org (K.D. Beck).

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car exhaust, diesel, petrol, sweat, burning, perfume, and vomit. Because their patients reported increased symptom prevalence following exposure to these types of stimuli but not distinct sounds, the authors concluded that these odors were serving as conditional stimuli (CSs) for conditional responses that are perceived as health ailments. Coinciding with that research, Van den Bergh and colleagues have shown individuals will condition to specific odorous CSs when they are paired with CO_2 exposure (the unconditional stimulus, US) under controlled conditions (Van den Bergh et al., 1997, 1998; Devriese et al., 2000).

The physiological mechanisms for such a conditioned-illness process have not been delineated outside of the realm of contextual conditioning in drug dependence. Still, the hypothesis is a provocative one, especially given that the drug dependence literature suggests nociception is an area where changes in sensitivity can be elicited by conditional stimuli (Siegel, 1975, 1976; Siegel et al., 1978). The epidemiological research conducted on Gulf War veterans has consistently revealed a symptom cluster of unexplainable pain (Haley et al., 1997; Fukuda et al., 1998; Unwin et al., 1999; Doebbeling et al., 2000). We hypothesized that agents used in the Persian Gulf War may have served as USs causing later CRs to odors that may have been present during deployment. This hypothesis was supported by our initial work showing the Gulf War-issued nerve-gas prophylactic treatment PB could serve as a US that would associate with an odorous CS, as would a proinflammatory cytokine US (which could represent an immune response to various vaccinations) (Servatius and Beck, 2005). In this regard, we are using this apparent conditioned fear response as a tool for exploring this learning process.

In addition to expanding our knowledge of the characteristics pertaining to exaggerated startle responses in the presence of the odor CS, we also explored whether PB could serve as a US for a conditional shift in pain sensitivity. The logic for this assumes the mechanisms by which PB acts as an interoceptive stressor. PB can cause mild but significant gastrointestinal distress in humans (Sharabi et al., 1991). Gastrointestinal distress could cause a release of oxytocin (Verbalis et al., 1986). Oxytocin can potentiate central opiate signaling pathways (Robinson et al., 2002; Gao and Yu, 2004), thus, leading to a possible acute analgesic effect. In this case, PB would be causing an analgesic unconditional response (UR). If an odor is present during this exposure (as a CS), then, we need to ponder what the resulting CRs could be when the odor is re-experienced. CRs involving opiate stimulation are commonly found to be compensatory (Siegel, 1988). Given the possible unconditional analgesic effect of PB, a compensatory CR would be hyperalgesia. This hypothesis was tested here in the context of pairing PB with specific odors.

The following experimentation had three goals: 1) document that an UR from PB exposure is an exaggerated startle response; 2) further characterize the duration of the conditioned exaggerated startle response elicited by an odor CS; 3) document that an UR from PB is a decrease in pain sensitivity; and 4) test the hypothesis that the odor CS could also elicit a second CR involving hyperalgesia. In this paradigm, PB serves as an US, causing peripheral cholinergic over-stimulation, and a distinct strawberry or peppermint odor serves as a conditional stimulus.

1. Methods

1.1. Subjects

Ninety male Sprague–Dawley rats (350–500 g, Charles River, Kingston NY) were used in the 4 experiments. All subjects were allowed 2–3 weeks to acclimate to our facility before experimentation began. Animals were housed individually in shoebox type cages (12:12/L:D cycle, lights on 0700), and allowed free access to rodent chow and water throughout the study except for the odor-exposure periods and behavioral testing.

1.2. Apparatus/materials

These experiments involve 2 testing apparatus. The startle apparatus included sound-attenuating chambers (Med Associates, Georgia, VT), rat holders and weight displacement platforms (Coulbourne Instruments, Allentown, PA), audio amplifiers (Optimus, Radio Shack), and a custom designed software program to control the stimulus generation and data sampling (Labview, National Instruments). The sampling rate from the weight displacement platforms occurred at 1000 Hz. Startle testing involved 60 presentation trials of a 100-ms 102 dB white noise stimulus (immediate rise/fall). The interstimulus interval was 15–25 s.

Pain sensitivity was assessed using an II TC Model 39D Hot Plate Analgesia Meter (Life Science USA) set at 48.5 °C. Differences in thermal pain sensitivity were assessed via the timing of the rats to lick their paws in response to being on the heated plate. Latency measures were assessed manually by having the experimenter watch for a clearly conducted paw-lick and stopping the timer on the apparatus. Pilot testing was conducted to determine a temperature setting that was sensitive to changes reflecting either hyperalgesia or hypoalgesia.

1.3. Procedure

Across all experiments, PB administration and odor manipulations followed consistent procedures. For the injections of PB, each rat was injected (i.p.) with either saline vehicle or 0.5 or 1.0 mg/kg PB (Sigma). The timing of the injections was held constant, in that, across studies it always occurred during the light phase between 0900 and 1200. For the experiments involving the use of odor as a CS, the odor manipulation was conducted through the placement of 1 in. square pieces of commercial car air-fresheners in the startle boxes. Two scents were used, strawberry and peppermint. Only one odor was ever used in a box to keep the odor in the environment constant.

Experiment 1 involved an acute assessment of PB affects on the startle response (defining the characteristics of the UR). We tested the rats in either a lighted chamber or in a darkened chamber. Others have suggested that startle reactivity shifts based on the lighting conditions of the chambers and the time of testing (Ison et al., 1991; Walker and Davis, 1997); thus, we tested startle reactivity under both lighted and darkened conditions to discern if this factor influenced the expression of the UR in this learning paradigm. Consequently, following a 2 (Drug)×2 (Visual

Context) factorial design ($N=16$), subjects were injected with either saline or PB (0.5 mg/kg) and subsequently placed in either a lighted or darkened startle chamber where startle testing occurred 10 min, 120 min, and 24 h following the injections.

In Experiment 2 involved testing rats that had been previously used for a similar experiment 1 month earlier. Care was taken to assure that a different odor was paired with each of the animals. A 2-group repeated measures design ($N=11$) comparing vehicle versus PB (1.0 mg/kg) administration was used to compare startle responses measured 1, 7, 14, 21, and 28 days after the PB-odor paired exposure. A larger dose was used, compared to our previous work (Servatius and Beck, 2005), because we planned to increase the number of CS probe trials to track the duration of the effect. We hypothesized that a higher dose could have a stronger association with the CS odor thereby increasing the number of test trials that would elicit a response before extinction processes reduced the response.

Experiment 3 was principally conducted to document the acute effects of PB administration on nociception. Rats were pretested and matched on their latencies to paw-lick to a 48.5 °C hot-plate stimulus. One week later, the rats were injected either with saline ($n=5$), 0.5 mg/kg PB ($n=5$), 1.0 mg/kg PB ($n=5$) and placed in the conditioning boxes with one of the odors. After 10 min, the rats were subsequently placed on the hot-plate for assessing the unconditional effects of PB on pain sensitivity. Subsequent testing in the presence of the odor CS occurred over the following 3 days. During the pain tests, the same odor stimulus as experienced on the initial treatment day was present (exposed on the underside of the plastic container placed over the apparatus during the test trial).

Experiment 4 served to test if the same odor–PB session pairing that is sufficient to cause a conditioned exaggerated startle response in the later presence of the odor CS can also cause a conditional shift in pain sensitivity. The saline and PB groups were split where the CS would either remain the same throughout testing (CS+) or change after initial CS–US pairing (CS−). Subjects received i.p. injections of saline ($n=12$), 0.5 mg/kg PB ($n=6$), or 1.0 mg/kg PB ($n=6$). Paw-lick latencies were measured 1, 7, and 14 days after CS–US exposure by a single experimenter for both tests.

All the described procedures occurred with the approval of the institutional animal care and use committee of the VA NJ Health Care System. The VANJHCS adheres to all the federal regulations for the care and use of rodents and by the standards set forth by AAALAC.

1.3.1. Data analysis and statistics

Prior to startle computation, the whole body response for a given rat was divided by the rat's body weight on the day of startle measurement. For each stimulus presentation, a response threshold for whole body response was computed as the average rectified activity 200 ms prior to stimulus onset plus 6 times the standard deviation of that rectified activity (Servatius et al., 1998). Response amplitudes, the maximum rectified activity within 125 ms after stimulus onset, were only recorded when post-stimulus activity exceeded the response threshold. For trials in which activity did not reach this criterion "not available" was recorded. Startle latencies were computed based on the initiation of the startle response. This was calculated by determining the point where rectified activity first exceeded the response threshold.

2. Results

2.1. Experiment 1

Startle testing occurred 10 and 120 min after PB or saline injections to determine the acute, unconditional effects of PB on startle reactivity. A third startle session was conducted 24 h later to show that any unconditional effect of PB on startle reactivity is confined to the day of drug administration. As shown in Fig. 1, startle magnitudes were generally higher in PB-treated rats. A 2 (Drug) × 2 (Light Context) × 3 (Session) × 10 (Block) repeated measures ANOVA was used to determine group and testsession differences. The resulting analysis revealed a main effects of Session, F (2, 343) = 70.3, $p<.001$ and Block, F (9, 343) = 5.7, $p<.001$. An additional significant Drug x Session interaction, F (2, 343) = 35.0, $p<.001$, and Drug × Light Context × Session interaction, F (2, 343) = 6.4, $p<.005$ were also found. Post-hoc analyses showed that PB increased startle reactivity during the first startle test but not thereafter.

2.2. Experiment 2

As shown in Fig. 2, the pairing of a single injection with a subsequent 1 h exposure to a distinct odor yielded significant effects on startle magnitude when tested in the presence of the same odor for weeks thereafter. Using a 2 (Group) × 5 (Session) × 10 (Block) mixed ANOVA, we found significant main effects of Session, F (4, 441) = 7.0, $p<.001$, and Block, F (9, 441) = 11.9, $p<.001$. In general, the main effect of Session reflects that startle magnitudes on post-pairing days 1, 7, and 28 were less than those on post-pairing day 14 (regardless of

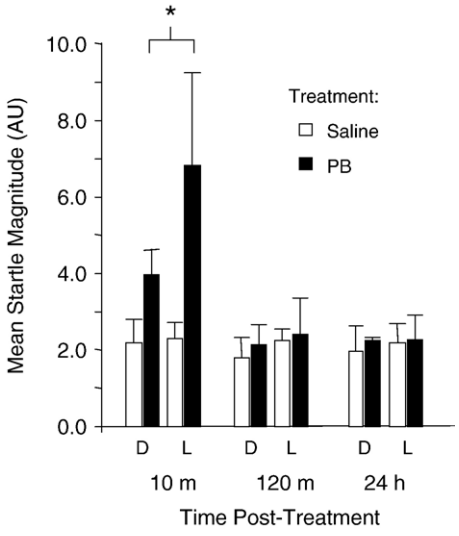

Fig. 1. Periodic startle magnitudes (unconditional responses) in male rats following a single dosing of pyridostigmine bromide (PB). Testing occurred under either dark (D) or lighted (L) conditions in the conditioning chamber. An asterisk (*) represents a significant difference between the 2 PB-treated groups compared to the 2 saline-treated groups. There are no significant differences due to lighting condition.

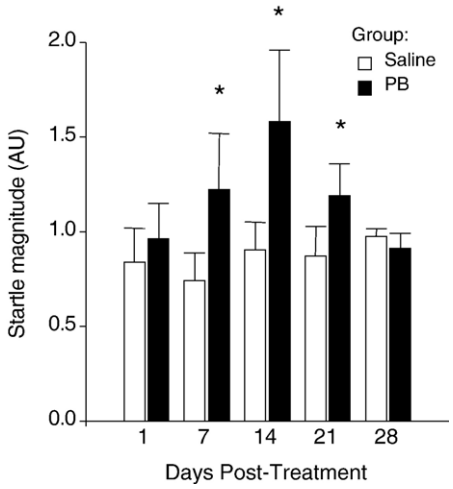

Fig. 2. Startle magnitudes of saline and pyridostigmine (PB)-treated rats tested once a week in the presence of a specific order following an initial pairing of the drug treatment with that same odor. An asterisk (*) represents a significant difference between the PB-treated group and the saline-treated group on that specific test day.

treatment condition). In addition, both treatment groups showed a robust decrease in responding from the first trial bock on each test day (data not shown). More importantly, an additional Group × Session interaction was also found, F (4, 441) = 4.2, $p < .005$. Post-hoc analyses determined that the PB-treated group had greater startle magnitudes than their vehicle-control counterparts 7, 14, and 21 days following the drug–odor pairing.

2.3. Experiment 3

Fig. 3 shows the responses to thermal pain stimulation shortly after the administration of PB, as well as the 3 days thereafter. We utilized a 2 (Group) × 5 (Session) mixed ANOVA

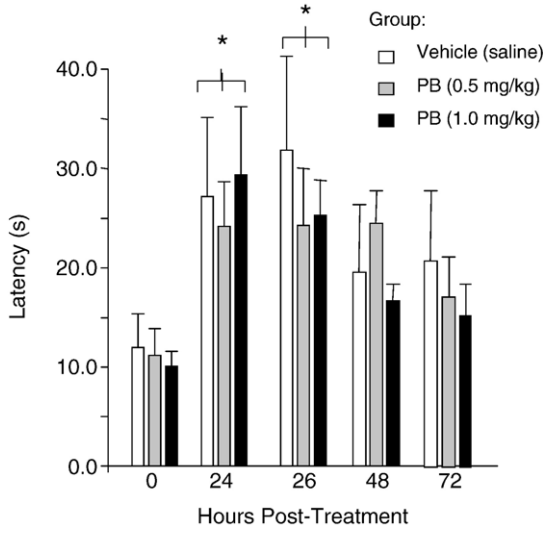

Fig. 3. Paw-lick latencies for rats treated with saline or pyridostigmine bromide (PB) in the presence of a specific odor. Testing occurred 0 (10 min), 24, 26, 48, and 72 h after the treatment. The rats were in the presence of the odor during all of the subsequent tests (for the duration of their time on the hot-plate). An asterisk (*) represents a significant difference between the initial test session (regardless of treatment group).

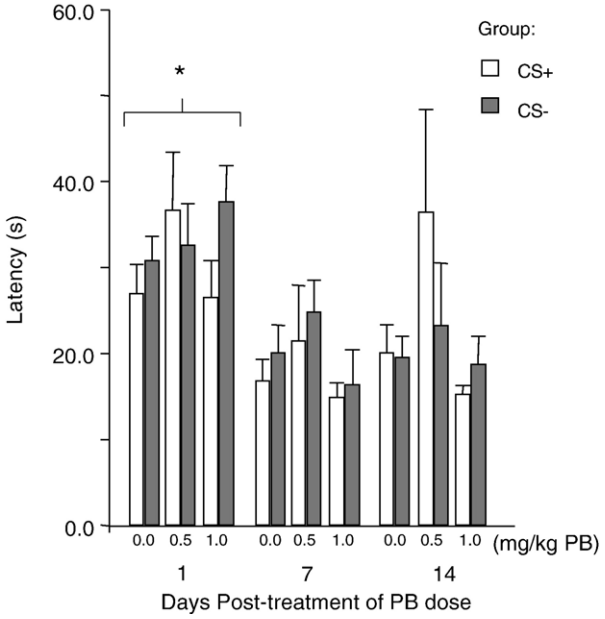

Fig. 4. Paw-lick latencies for rats treated with saline vehicle (0.0), 0.5 mg/kg dose of PB, or a 1.0 mg/kg dose of PB in the presence of a specific CS+ or CS− odor 1, 7 and 14 days after the initial CS+ odor–PB pairing. A significant main effect of Session was found with Day 1 startles being significantly higher than subsequent sessions (as denoted by *).

to test for significant differences in paw-lick latency. As evidenced by the plotted values, there were no group differences; rats dosed with saline, 0.5 mg/kg, or 1.0 mg/kg PB exhibited similar latencies to paw lick. Only a significant effect of Session was evident, F (4, 48) = 9.3, $p < .001$. The initial hot-plate test elicited quicker paw lick responses than the subsequent tests, regardless of treatment condition.

2.4. Experiment 4

Rats were pretested and matched on paw-lick latencies to conditions where they were administered PB (0.5 or 1.0 mg/kg) either in the presence of 1 of 2 possible odors. Subsequent testing for pain sensitivity occurred in the presence of the same odor as during PB administration (CS+) or the other odor (CS−) 1, 7, and 14 days after the initial odor–PB pairing. Thus, we subjected the paw-lick latencies from each of these cohorts to a 3 (Group) × 2 (Test Odor) × 3 (Session) mixed ANOVA. Although differences in pain sensitivity were not evident in Experiment 3, the added factor of odor discrimination could provide additional sensitivity in detecting a shift in sensitivity due to conditioning. As shown in Fig. 4, only a main effect of Session, F (2, 83) = 22.8, $p < .001$ was significant. The first test session latencies to paw-lick were longer than all subsequent sessions. These findings suggest that the repeated testing of pain sensitivity the under the same contextual cues facilitates the initiation of the paw-lick response, without any discernable effect of PB.

3. Discussion

The results from these experiments clearly show that PB is sufficient for supporting behavioral CRs. Importantly, we show that

there is specificity in the associability of PB-induced interoceptive stress to certain behavioral responses. Despite a clear unconditional and conditional effect of PB exposure on startle responsivity, there was no significant unconditional or conditional effect on thermal pain responsivity. With particular regard to startle reactivity, we also show that the duration of the conditioned exaggerated startle response approximates several weeks. Extinction of the conditional exaggerated startle response occurs after several exposure sessions. Overall, these data further delineate the characteristics of PB as a potential US and adds to our understanding of the extent to which secondary, long-lasting behavioral effects can occur following PB usage through associative learning.

The issue of PB as a potential cause of any number of unexplained symptoms has been discussed and debated for over a decade. The difficulty in finding parallels between the acute effects of PB and the chronic symptoms reported by Gulf War veterans has been the temporal discrepancy between the ingestion of the drug and the onset of the symptoms days to weeks later. Many studies have tried to expose a purely pharmacological mechanism by which PB could cause persistent symptoms. These have involved single PB-dosing effects (Hoy et al., 1999, 2000a; van Haaren et al., 1999), multi-day PB-dosing patterns (Servatius et al., 1998, 2000; van Haaren et al., 1999), and combining PB with other possible toxins (Hoy et al., 2000a,b). Persistent effects (beyond 1 week) were observed in chronically dosed Wistar–Kyoto (WKY) rats in the past (Servatius et al., 1998). After a week of PB ingestion by WKY rats (but not Sprague Dawley rats), exaggerated startle responses were reported. Although that study was not particularly designed to assess the role of contextual learning, the repeated dosing across days coupled with repeated testing over several weeks may have supported the development of an exaggerated startle CR to a CS that was not identified. This is speculative, but, the WKY rat may have a lower threshold for perceiving certain stimuli (e.g. odors) that were present during the experiment. On the other hand, the WKY was found to have a different level of circulating cholinesterase (Servatius et al., 1998), which could translate into a different level of coping to cholinesterase inhibition and a qualitatively different amount of interoceptive stress. In order to make such a conclusion, in the future we will have to directly compare the two strains. If strain differences in olfactory stimulus saliency and PB responsiveness are apparent, we would examine whether such characteristics affect the processes of associative learning in a manner than would explain whether certain individual differences increase the expression or duration of such CRs.

Using PB as a US gives our model a rather specific context of interest (i.e. Operation Desert Storm). Because of the linkage to Gulf War Illness, there is an expectation that the CRs elicited should match the symptoms reported by the veterans. In this study, we did not observe any changes in pain sensitivity that may have been expected due to the immediate effects of PB or as a function of a CR to the odor CS. There are two possible explanations for this. First, PB does not elicit an unconditional change in pain sensitivity and, therefore, will not support a CR that involves a shift in pain sensitivity. Servatius et al. tested pain sensitivity following a chronic dosing regimen of PB and did not observe any differences from the saline-treated controls (Servatius et al., 1998). With the different dose regimens conducted in this study, it appears that PB may not reliably affect thermal nociception (at least at levels considered in the normal physiological range). A second explanation is that there may be a mismatch between our CS olfactory stimuli and the UR pain sensitivity change. Garcia showed that there are optimum stimulus conditions for persistent conditioned sickness responses (such as avoidance) (Garcia and Koelling, 1966). In the context of conditioned changes in nociception, Siegel found that contextual visual and auditory stimuli could support conditioned hyperalgesia after those stimuli are paired with subsequent morphine injections (Siegel et al., 1978). On the other hand, when van den Bergh and colleagues examined the effectiveness of different odors as CSs in humans (using CO_2 as the symptom-causing US), they found particularly noxious (i.e. negatively perceived) odors were more effective (Van den Bergh et al., 1997). Therefore, there could also be a mismatch between the particular CSs used here (because they were sweet odors) and the noxious thermal stimulus. Possibly an ammonia or acidic quality odor could be a more effective CS for conditioning pain responses. Of course, there is also the possibility that olfactory stimuli serve as a reliable CS for causing shifts in startle reactivity, but olfactory stimuli, in general, may not associate as readily to thermal nociceptive URs.

An additional difficulty in this assessment was the obvious shifting of pain sensitivity across all groups with repeated testing. We saw in both pain experiments that there is a general increase in the paw-lick latencies the day following either a pain test (regardless of treatment) or treatment with PB. The former can be viewed as an exteroceptive stressor and the later an interoceptive stress (with the obvious combined group that was both treated with PB and tested shortly thereafter). Others have reported analgesic effects with repeated hot-plate testing (Hawranko et al., 1994), and in both experiments, those treated with PB exhibited longer latencies to respond with a paw-lick the day following treatment (with reductions occurring over subsequent tests). Moreover, when we examined the post-hoc power analyses for the pain studies, most main effects and interactions pertaining to PB administration or conditional cues had low power calculations ($p < .5$). In the end, this type of pain testing may require a substantial greater number of rats in order to be sensitive to this type of conditioning, and thus, it may not be useful for testing a PB-induced change in pain sensitivity. Moreover, a lack of a clear UR to the thermal stimulus suggests that a different pain model system needs to be utilized (e.g. weight-bearing measures). Consequently, this type of conditioning may have to involve pairing a specific inflammatory agent with a salient CS. We have already shown that IL-1β can serve as a US for a conditioned enhancement of startle reactivity (Servatius and Beck, 2005), but further exploration in the use of proinflammatory cytokines as USs for eliciting conditional changes in pain sensitivity still have to be conducted.

As we learn more about the types of stimuli that can serve as salient USs in classical conditioning, we need to explore the possible range of potential CRs those stimuli can produce. In our research concerning possible USs applicable to supporting the unexplained symptoms of Gulf War Illness, we have examined PB and IL-1β. To this point, it appears that PB, as an exogenous substance that elicits an interoceptive stressor response, supports an association of fear to the paired odor. Similar conditioned fear

to the paired odor occurs when IL-1β is used as the US (Servatius and Beck, 2005). This pattern suggests that there is a common fear response elicited to a novel CS that occurs in the presence of internal distress. The conditioned fear elicited in this paradigm could either be viewed simply as a probe for the occurrence of interoceptive stressor conditioning, or it may reflect other processes occurring in the brain that could affect symptom severity. For instance, anxiety has been reported to have a higher prevalence in the deployed personnel (Black et al., 2004), and some have suggested that a heightened level of anxiety prior to deployment may have been a key factor in the development of the unexplained symptoms (Blanchard et al., 2006). Could prior abnormal activity in the brain areas associated with fear and anxiety influence this conditioning process and the development of conditioned symptoms? Future research directed toward identifying factors that make individuals more or less able to make these learned associations is needed, and those studies may provide conditions where individual attributes are more or less predictive of conditioned illness susceptibility.

In summary, these results clarify the extent to which PB can serve as an US for eliciting persistent hypervigilance in the presence of an odor CS. The exaggerated startle CR is evident for several weeks after a single CS–US pairing of 1 h. The exaggerated CR mimics the UR elicited by PB alone (startle exaggeration). Odor–PB associations do not appear to support any change in sensitivity to a thermal pain stimuli in the presence of the CS odor. This lack of conditioning is likely due to a lack of an analgesic UR in response to PB or it could reflect a mismatch in CS–US associability pertaining to the type of pain tested here. Thus, we have learned that interoceptive stressor associability, stemming from exposure to PB, occurs more readily to behavioral responses that are directly regulated by cholinergic input.

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