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Mild Interoceptive Stressors Affect Learning and Reactivity to Contextual Cues: Toward Understanding the Development of Unexplained Illnesses

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Contextual learning is evident with repeated experiences with agents and treatments that induce frank illness and interoceptive stress. Here, we examined whether acute treatment with mild interoceptive stressors (low doses of pyridostigmine bromide (PB), neostigmine bromide (NB), and interleukin (IL)-1β) may serve as unconditional stimuli supporting contextual learning. Rats were exposed to interoceptive and exteroceptive stressors in contexts distinguished by visual or olfactory cues. Acoustic startle responses (ASRs) were measured the day following exposure and 2 weeks thereafter, without delivery of the unconditional stimuli. The appearance, form, and duration of startle potentiation depended on the distinguishing features of the context and the nature of the interoceptive stressor. Rats given cholinesterase inhibitors (PB and NB), but not IL-1β or exposed to an exteroceptive stressor, exhibited exaggerated ASRs in a novel context distinguished by visual cues. Treatment with either PB or IL-1β led to potentiated ASRs in the presence of odors congruent with those experiences during exposure to the stressor. Startle potentiation by odor was still apparent 2 weeks after treatment. For contexts differentiated by visual stimuli, cholinomimetics transiently alter reactivity within novel contexts. In the case of contexts differentiated by odors, learning is apparent at least 2 weeks after acute treatment of cholinomimetics and IL-1β. Contextual learning and changes in reactivity consequent to mild interoceptive stressors such as PB may play a role in the development of nonspecific symptoms typical of unexplained illnesses, such as Gulf War Illness.
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INTRODUCTION

Veterans returning from the first Gulf War registered a number of nonspecific somatic (eg headaches, rashes, joint pain, fatigue, gastrointestinal distress) and cognitive (lack of concentration, confusion, short-term memory loss) complaints referred to as Gulf War Illness (GWI). GWI is shrouded in controversy stemming from the nonspecific nature of complaints, the lack of consistent biological markers, reporting biases, recollection biases, and issues regarding deployment.

The search for causes has focused attention on factors that could directly induce illness or persistent symptoms. One such factor is pyridostigmine bromide (PB), a carbamate inhibitor of acetylcholinesterase used extensively as a pretreatment under the threat of nerve gas exposure. At the recommended dose, PB is considered relatively safe, because PB does not readily penetrate the blood–brain barrier (Lallement *et al*, 1998; Beck *et al*, 2001, 2003). The safety of PB treatment is less certain in the presence of other chemical exposures (Abou *et al*, 1996; Hoy *et al*, 2000), stress (Friedman *et al*, 1996; Grauer *et al*, 2000; Servatius *et al*, 2000; Sinton *et al*, 2000; Kant *et al*, 2001; Song *et al*, 2002; Beck *et al*, 2003), and in individuals with increased sensitivity to PB (Servatius *et al*, 1998; Beck *et al*, 2001). Safety notwithstanding, a role for PB in GWI would still be in doubt in that the signs and symptoms of GWI are not merely reducible to cholinergic toxicity.

Is a direct biological path necessary? Ferguson and Cassaday suggested that the symptoms of illness arose secondary to Pavlovian conditioning. They noted the resemblance of the symptoms of GWI to the acute-phase responses of proinflammatory cytokines, in particular interleukin (IL)-1β (Ferguson and Cassaday, 1999). In their formulation, GWI is presumed to be the result of

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reactivation of IL-1β through situational reminders of the precipitating event (Ferguson and Cassaday, 2001). If true, proinflammatory activation should be evident, or sensitized in veterans with GWI. Contrary to this prediction, elevated IL-1β in sick veterans is lacking (Soetekouw *et al*, 1999).

Despite the failure of the specific prediction, a conditioning model may still apply to the involvement of both PB and IL-1β. Both IL-1β and PB constitute salient interoceptive stressors. Treatment with of IL-1β results in fever, fatigue, malaise, and anhedonia (Anisman and Merali, 1999). PB produces a number of unpleasant effects: nausea, stomach cramping, fatigue, excessive lacrimation, diarrhea, and urinary urgency (Sharabi *et al*, 1991). Thus, the potential exists for the side effects of PB (Romano and King, 1987) and IL-1β to serve as unconditional stimuli (USs) for Pavlovian conditioning.

Here we focused our attention on the associability of PB and IL-1β as interoceptive stressors with contextual stimuli. Contextual fear or anxiety arising from exposure to stress is typically observed in rodent models as freezing (Fanselow, 1990) or as an exaggerated acoustic startle response (ASR) (Davis, 1990). The advantage of the latter is that an exaggerated ASR is a positive sign of anxiety that is distinct from malaise. To expose contextual learning, a discrimination paradigm was employed. Four otherwise identical chambers were distinguished by either visual or olfactory cues. Exposure to stressors occurred in one of two contexts. Conditioning should be apparent as increased ASRs in the context concordant with stressor exposure. Moreover, such learning should persist, that is, subsequent exposures to contextual cues should elicit potentiated ASRs. The associative strength of contextual features will depend on the nature of the US and the form and saliency of available conditional stimuli (CSs) (Garcia *et al*, 1966, 1968; Garcia and Koelling, 1966). Therefore, contexts were distinguished by visual or olfactory cues.

METHODS

Subjects

Adult male Sprague–Dawley rats (300–450 g) were obtained from Charles River (Willington, MA); rats were allowed 2 weeks to acclimate to laboratory conditions. Rats were individually housed in specially designed chambers (eight rats per chamber) that attenuate sound, and maintain airflow and temperature. Rats were fed *ad lib* Purina Rodent Chow with free access to water. Rats were maintained on a 12 : 12 light:dark cycle with light onset at 0800. All procedures conformed to the Institutional Animal Care and Use Committee of the Department of Veterans Affairs, New Jersey Health Care System.

Materials and Apparatus

The startle apparatus (holders (EO5-15), platforms (E45-11), amplifier, and interface) were obtained from Coulbourn Instruments (Allentown, PA). The software package (Labview) (used for the generation of acoustic stimuli, stimulus delivery, and signal acquisition) and the A/D card (6024E) were obtained from National Instruments (Austin, TX).

Four custom experimental boxes were used for stressor exposure and ASR testing. The boxes are constructed of 2 mm thick PVC, which sandwiches a 3.5 cm foam core. The boxes have smooth inner surfaces with inner dimensions of $26 \times 29 \times 43$ cm. A house light (3 W) is affixed to the top panel as well as a baffled ventilation fan. The front panel is windowed for observation.

Stressors

Interoceptive stress was manipulated by delivering either peripheral cholinesterase inhibitors or proinflammatory cytokines through intraperitoneal (i.p.) injections. PB and neostigmine bromide (NB) (Sigma Chemical, St Louis, MO) were freshly prepared the morning prior to injection and dissolved in physiological saline. Preliminary work established levels of PB and NB that inhibited plasma butyrylcholinesterase activity by 30%. At the levels of PB and NB administered, rats exhibit mild signs of cholinergic overstimulation: barely noticeable muscle fasciculation, increased lacrimation, and loose stool. IL-1β (specific activity $>5 \times 10^8$ U/mg) was obtained from PeproTech (Rocky Hills, NJ). Preliminary work showed that IL-1β (1.0 and 3.0 μg/kg) induced a mild but significant hyperthermia that lasted for 2–4 h in rats. An exteroceptive stressor was devised that would be continuously arousing for 1 h without unduly harming the rat. A clip, part of the system for delivery of tailshock (Ottenweller *et al*, 1992) but without the electrodes, was attached to the rat's tail. Observation of the rat indicated that the clip remained uncomfortable throughout the exposure session, with intermittent gnawing and circling behavior. Neither the clip nor the gnawing by the rat damaged a single rat's tail.

ASR

Rats were placed in holders that sit on force transducers as previously described (Servatius *et al*, 1998). The open cover of the holders is formed by a tubular alignment of rods, spaced 0.3–2 cm apart, allowing the rat to observe their surrounds. Each session consisted of 60, 102-dB white noise bursts (100-ms duration, immediate rise/fall). The interstimulus interval ranged from 25 to 35 s. Response magnitude was calculated on a trial-by-trial basis (Beck *et al*, 2002). 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 six times the standard deviation of that rectified activity. Response amplitudes, the maximum rectified activity within 125 ms after stimulus onset, were only recorded when poststimulus activity exceeded the response threshold. For trials in which activity did not reach this criterion, 'not available' was recorded.

Procedures

Rats were stratified on body weight and randomly assigned to groups. Stressor exposure occurred on DAY 1. For interoceptive stressors, rats received a single i.p. injection. For clip exposure, a clip was attached to the base of the tail. In all cases, rats were placed in the holders used for ASR testing. The experimental chambers were distinguished by

either visual or olfactory cues. To manipulate visual cues, masking tape was affixed to the walls in irregular patterns in two of the boxes; the other two boxes remained unadorned. Thus, wall adornment was the basis for distinguishing chambers in experiments manipulating visually distinguished contexts. To manipulate olfactory cues, segments of commercially available car air fresheners (strawberry or peppermint; Car-Freshener Corporation, Watertown, NY) were affixed to the ceiling of the chambers. Segments of the car air fresheners were of equal area; new segments were used each day of exposure. Order of exposure for this initial period was counterbalanced across conditions. The duration of exposure was 1 h. After this exposure session, rats were returned to their home cages.

The following day (DAY 2), the first ASR test commenced. The rats were placed in the chambers for 5 min prior to the first ASR trial. The SAME designation refers to rats whose ASR testing occurred in a context concordant with initial exposure. The DIFF designation refers to rats whose ASR testing occurred in a chamber not experienced during initial exposure. A second ASR test occurred 2 weeks after stressor exposure (DAY 15). For all rats, the DAY 15 test occurred in a chamber concordant with the DAY 2 test.

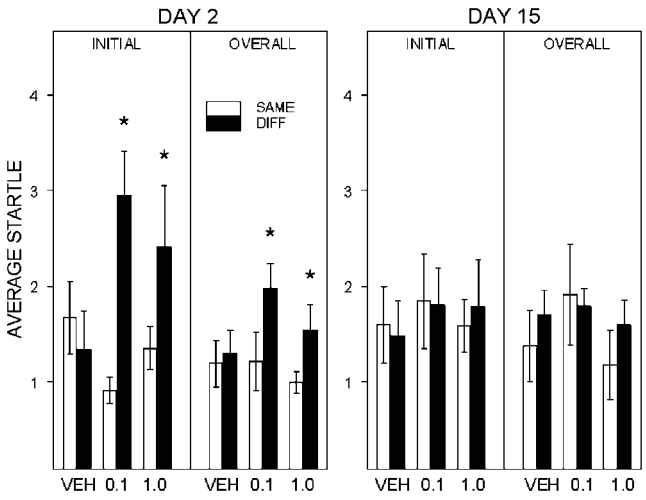

Figure 1 Comparison of ASRs on DAY 2 and DAY 15 in rats given PB (0.1 or 1.0 mg/kg i.p.) or vehicle (VEH) injections in contexts distinguished by visual cues. On DAY 2, both initial ASRs and overall ASRs were exaggerated in DIFF compared to SAME in PB-treated rats, but not VEH rats ($n = 6$ per group). No differences among groups were detected on DAY 15.

Data Analysis

A startle session was reduced to 12 blocks of five responses each. The first block represents initial reactivity; the magnitude of these responses is typically higher with greater variability than the subsequent blocks. In that we were particularly interested in initial reactivity, the initial block of ASRs were separately analyzed. For these data, between-subject (Treatment × Context) analyses of variance (ANOVAs) were performed. For the subsequent blocks, split-plot ANOVAs with repeated measures were performed. With the ASR protocol used herein, typically ASRs decrement substantially from the first block to subsequent blocks indicating habituation. For some, ASRs increment during the subsequent blocks, suggesting the development of sensitization or dishabitutation. Dunnett's and Dunn's tests (tDs) were used to compare SAME and DIFF responses within treatments.

RESULTS

PB with Visual Cuing

We administered to rats PB at 0.1 or 1.0 mg/kg or saline vehicle through intraperitoneal (i.p.) injection. Preliminary data showed that these doses correspond to 15 and 35% inhibition of plasma butyrylcholinesterase activity, respectively. These doses produce inhibition in the range recommended for PB as a pretreatment under the threat of nerve gas.

On DAY 2, PB-treated rats displayed exaggerated ASRs in the DIFF context both initially and over the rest of the test session (see Figure 1). For initial responses, a significant main effect of Context, $F(1,30) = 7.5$, was qualified by the Treatment × Context interaction, $F(2,30) = 4.1$, all p's < 0.05. Overall responses also differed. The main effect of Context, $F(1,300) = 5.6$, and the Treatment × Context × Block interaction, $F(20,300) = 1.7$, was significant, all p's < 0.05. Contrary to expectation, startle reactivity was enhanced in a context that differed from the context in which rats received PB exposure.

Enhanced reactivity was transitory. For DAY 15, initial reactivity did not differ. Although analyses of the overall responses indicated a significant Treatment × Context × Block interaction, $F(20,300) = 2.3$, $p < 0.05$, the significant contrasts were not germane. Thus, the enhanced ASRs of PB-treated rats on DAY 2 dissipated by DAY 15.

PB Compared to NB with Visual Cuing

NB is similar to PB, but with a shorter half-life and greater potency. A direct comparison of PB and NB indicates whether effects attributable to PB are unique to its pharmacokinetic properties or generalize to other cholinomimetics. In addition, the comparison allows for a demonstration of the reproducibility of the phenomena with PB. Inasmuch as the greatest ASRs were observed to 0.1 mg/kg, this dose of PB was compared to an equivalent dose of NB (0.016 mg/kg).

On DAY 2, initial reactivity was exaggerated in both PB- and NB-treated rats in DIFF compared to SAME contexts (see Figure 2). The main effect of Context, $F(1,48) = 9.9$, was qualified by the Treatment × Context interaction, $F(2,48) = 3.6$, all p's < 0.05. For overall responding, only the main effect of Block was significant, $F(10,480) = 2.7$, $p < 0.01$. Specific comparison indicated that PB-treated rats exhibited exaggerated ASRs in DIFF compared to SAME ($tD = 2.25$, $p < 0.05$). Treatment with AChE inhibitors led to exaggerated ASRs in contexts that differed from those of treatment.

Again, enhanced reactivity was transitory. No significant effects were noted in either initial or overall response on DAY 15. Although enhanced reactivity was apparent in rats given cholinomimetics and tested in DIFF, the overall

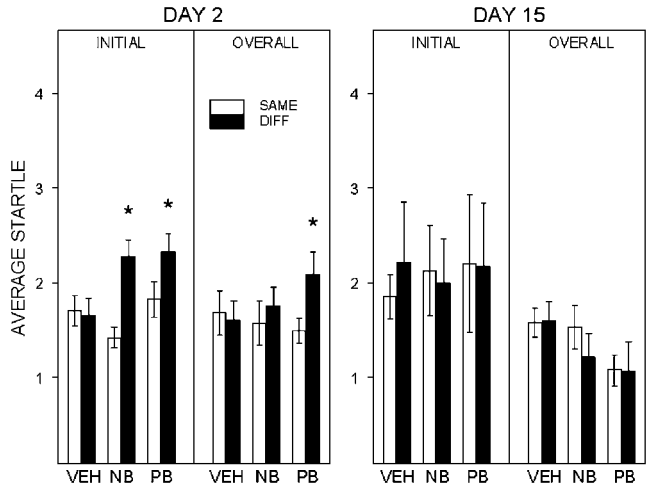

Figure 2 Comparison of ASRs on DAY 2 and DAY 15 in rats given PB (0.1 mg/kg), NB (0.16 mg/kg), or VEH injections in contexts distinguished by visual cues. On DAY 2, initial ASRs were exaggerated in DIFF in NB-treated rats, while overall responses were exaggerated in PB-treated rats in DIFF ($n=9$ per group). No differences among groups were detected on DAY 15.

magnitude of these effects was reduced in comparison to those observed in Experiment 1.

IL-1β with Visual Cuing

Exaggerated ASRs observed after PB and NB treatment in the presence of visual cues are presumed to be attributable to the unwell feelings produced by cholinergic overstimulation. IL-1β is known as an adjunct to sickness behavior, inducing fever, malaise, and anhedonia (Anisman and Merali, 1999). Thus, IL-1β is an interoceptive stressor (Maier and Watkins, 1998). The visual cuing experiment was preformed as above, with the exception that rats were treated with IL-1β (1.0 or 3.0 μg/kg i.p.) or a VEH injection. These doses produce mild hyperthermia, and the higher dose leads to facilitated acquisition of the classically conditioned eyeblink response within 2 h of injection (Servatius and Beck, 2003).

On DAY 2, initial responses did not differ among groups (see Figure 3). Overall responses only differed as a function of Block, $F(10,300)=2.4$, $p<0.01$. Thus, treatment with IL-1β did not affect reactivity in either context. As for DAY 15, no differences were detected in either initial or overall responses, all p's >0.05. Thus, treatment with IL-1β did not induce enhanced reactivity, in SAME or DIFF, regardless of treatment context and day of testing.

Exteroceptive Stressor and Visual Cuing

Contextual learning in the face of exteroceptive stressors is commonly studied. For example, exposure to footshock engenders robust behavioral changes within the exposure context indicative of fear (Iwata and Ledoux, 1988; Fanselow and Tighe, 1988) including the potentiation of the ASR (Davis, 1989; Richardson and Elsayed, 1998). Manipulation of exteroceptive stress with regard to contextual fear typically involves punctate stressors. To manipulate exteroceptive stress in a manner similar to interoceptive stressors, we sought a stressor that would continually engage the rat for an hour, but would not unduly harm or injure the rat. Therefore, a polyurethane clip was attached to the base of the rat's tail. The clip appeared to be annoying to the rat (inducing circling, gnawing, and biting sporadically throughout the 1 h period).

No changes in reactivity were apparent across contextual cues or as a function of stressor exposure on DAY 2, all p's >0.05 (see Figure 4). Similarly, no differences in ASR were noted on DAY 15, all p's >0.05.

PB with Olfactory Cues

Enhanced reactivity in PB-treated rats in the presence of novel visual cues was unexpected. Rats may not readily

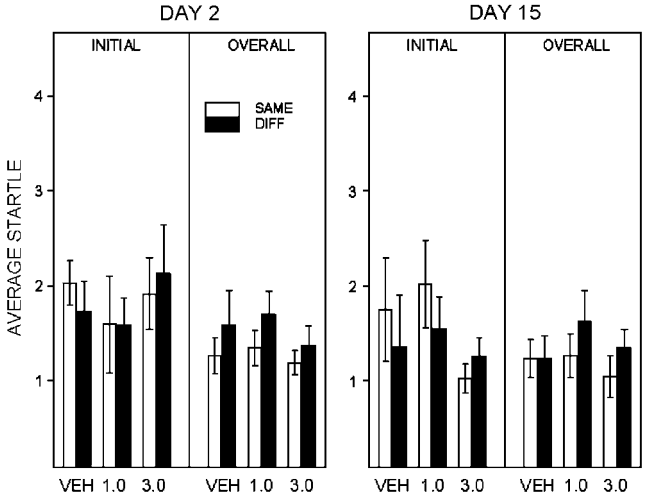

Figure 3 Comparison of ASRs on DAY 2 and DAY 15 in rats given IL-1β (1.0 or 3.0 μg/kg i.p.) or VEH injections in contexts distinguished by visual cues. No differences were detected among groups either DAY 2 or DAY 15 ($n=6$ per group).

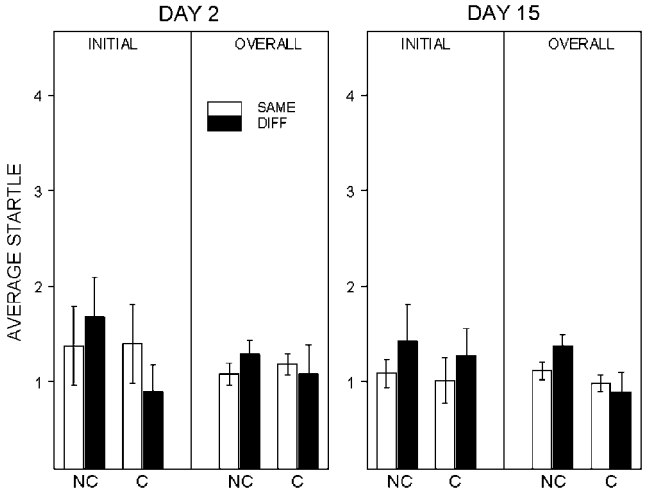

Figure 4 Comparison of ASRs on DAY 2 and DAY 15 of rats with a clip affixed to their tail (C) or no clip (NC) in contexts distinguished by visual cues. No differences were detected among groups either DAY 2 or DAY 15 ($n=6$ per group).

attribute interoceptive stimuli associated with cholinergic overstimulation to the visual features of their surroundings. The classic work of Garcia *et al* (1966) suggests a natural linkage between sickness and olfactory and gustatory sensations. Thus, fear or anxiety attributable to the surrounding in which one became sick (Garcia and Koelling, 1966; Holder and Garcia, 1987) may be more apparent when contexts are differentiated by odors (Herzog and Otto, 1997; Otto *et al*, 2000). Therefore, one would expect that ASR potentiation should be observed in SAME contexts with olfactory cues.

To simplify the experimental design, a single dosage of PB was chosen. In the first two experiments manipulating PB in visually distinguished contexts, PB was administered at 1.0 and 0.1 mg/kg. There was concern that the effects of 0.1 mg/kg may be too subtle, and there was a robust effect in Experiment 1, which was less apparent in Experiment 2. Therefore, we treated the rats with PB at 0.5 mg/kg, approximately midway between the two effective doses of 0.1 and 1.0 mg/kg. Two novel olfactory cues (strawberry and peppermint car air fresheners) were used to distinguish the chambers.

On DAY 2, initial reactivity did not differ. However, exaggerated ASRs were evident in PB-treated rats during the remainder of the test session (see Figure 5). The main effect of Treatment was significant, $F(1,20) = 4.3$, $p < 0.05$. Thus, PB-treated rats exhibited enhanced reactivity in both SAME and DIFF on DAY 2.

On DAY 15, discrimination between SAME and DIFF was evident. For initial responses, the main effects of Treatment, $F(1,20) = 10.5$, and Context, $F(1,20) = 4.9$, were qualified by the Treatment × Context interaction, $F(1,20) = 7.3$, all p's < 0.01. Overall responses yielded a similar pattern, with PB-treated rats exhibiting exaggerated ASRs in SAME. The main effects of Treatment, $F(1,20) = 5.7$, and Block, $F(10, 200) = 2.3$, were significant, all p's < 0.05. Specific comparisons demonstrated that the ASRs of PB-treated rats in SAME were greater than those in DIFF, $tD = 3.1$, $p < 0.01$. PB-treated rats discriminated between SAME and DIFF olfactory contexts 15 days after exposure.

IL-1β with Olfactory Cues

Contextual learning was apparent when odors distinguished contexts in which the rats experienced cholinergic overstimulation. To determine generalizability of contextual learning with interoceptive USs, rats were treated with IL-1β (3.0 μg/kg i.p.) or vehicle. To simplify the design, only the 3.0 μg/kg dose of IL-1β, one that facilitates acquisition of the classically conditioned eyeblink response in rats (Servatius and Beck, 2003), was administered. Again, the chambers were differentiated by peppermint or strawberry scents.

On DAY 2, initial reactivity did not differ (see Figure 6). However, exaggerated ASRs in rats treated with IL-1β developed over time. The main effects of Context, $F(1,20) = 13.2$, and Block, $F(10,200) = 2.3$, were significant. Specific comparison indicated that IL-1β-treated rats in SAME had larger ASRs than those in DIFF, $tD = 2.9$, $p < 0.05$.

Exaggerated reactivity in SAME was still apparent in IL-1β-treated rats on DAY 15. Although the analysis of initial responses did not indicate significance, specific comparison demonstrated that IL-1β-treated rats tested in SAME displayed greater ASRs than those tested in DIFF, $tD = 2.0$, $p < 0.05$. A similar pattern was observed in overall ASRs. The Treatment × Context interaction, $F(1,20) = 4.6$, was significant, $p < 0.05$. Again, the ASRs of IL-1β rats tested in SAME were greater than those tested in DIFF, $tD = 3.3$, $p < 0.01$. Similar to PB treatment, rats treated with IL-1β displayed exaggerated ASRs 2 weeks after acute exposure in contexts congruent with treatment.

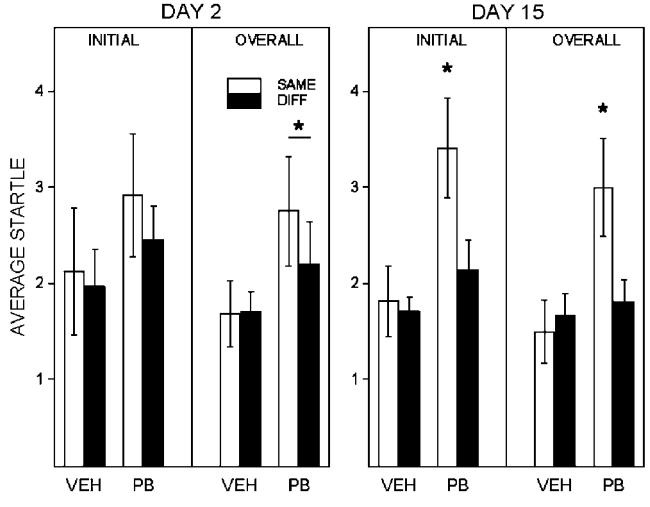

Figure 5 Comparison of ASRs on DAY 2 and DAY 15 in rats given PB (0.5 mg/kg i.p.) or VEH injections in contexts distinguished by odors. On DAY 2, initial responses did not differ, but the overall ASRs of rats given PB were exaggerated regardless of context ($n = 6$ per group). The line below the significance symbol indicates a main effect comparison between PB-treated and VEH-treated rats. On DAY 15, both initial and overall ASRs of rats given PB and tested in SAME were exaggerated compared to those tested in DIFF.

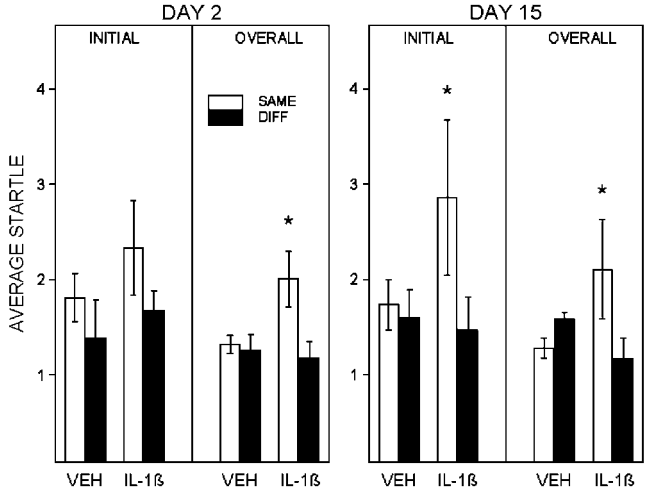

Figure 6 Comparison of ASRs on DAY 2 and DAY 15 in rats given IL-1β (3.0 μg/kg i.p.) or VEH injections in contexts distinguished by odors. On DAY 2, initial ASRs did not differ ($n = 6$ per group). Overall ASRs were exaggerated in SAME relative to DIFF, an effect predominantly derived from rats given IL-1β. On DAY 15, initial ASRs did not differ; however, overall ASRs were exaggerated in SAME relative to DIFF contexts in IL-1β-treated rats.

DISCUSSION

Interoceptive stimuli can be powerful USs for associations with contextual features. Such Pavlovian relations are clearly evident in development of anticipatory nausea in cancer patients or animal models of illness such as treatment with LiCl in rats. In both these cases, the interoceptive stressor is nausea. Do associations form with mild interoceptive stimuli, those that produce sensations less than frank illness? We examined whether visual or olfactory features would form associations with mild interoceptive stimuli accompanying peripheral cholinesterase inhibitors and proinflammatory cytokines.

Visual Cues

Contrary to our initial expectations, rats given peripherally acting cholinomimetics exhibited exaggerated ASRs in novel surroundings. Exaggerated ASRs were evident early in testing and dissipated over the initial test session. Increased reactivity in the presence of changed visual features assumes that rats attended to the visual features during initial exposure. However, those features were not directly associated with physiological disturbances secondary to cholinergic overstimulation. This finding was replicated in rats given PB and reproduced in rats given NB, albeit with a smaller degree of difference in reactivity compared to the first experiment. Changes in reactivity, within either SAME or DIFF environments, were not apparent 2 weeks after initial exposure. We do not know if the apparent increased reactivity to novel visual features persists after the initial test inasmuch as the contexts of the retest were congruent with the initial test session. Thus, exposure to cholinomimetics that do not cross the blood–brain barrier leads to transiently increased reactivity in a novel environment distinguished by visual stimuli.

Exposure to an exteroceptive stressor did not lead to exaggerated ASRs either in SAME or DIFF when environments were distinguished by visual cues. Observation of the rat indicated that focus of the rat's attention was on the tailclip itself with bouts of activity to remove the tailclip. Thus, the US was explicit, causing specific discomfort to the rat. Under these conditions, the place in which stress occurred appears to be irrelevant. Contextual learning during periods of footshock or tailshock exposure is apparent later as freezing (Iwata *et al*, 1986; Fanselow and Tighe, 1988), exaggerated ASRs (Davis, 1989), and facilitated classical conditioning (Shors and Servatius, 1997). A rat is anxious or fearful in the surroundings concordant with shock exposure. The presence of punctate conditional signals attenuates contextual fear (Baldi *et al*, 2004). Thus, knowledge that allows for prediction or control attenuates contextual fear (Jackson and Minor, 1988). In the case of the tailclip, the source of irritation is continuous, known, and observable. Contextual features may be ignored as a potential source of the mild irritation because the source of the irritation is so predictable in this instance.

Potentiated ASRs were not observed in visual contexts after concomitant IL-1β treatment. The doses of IL-1β we used herein produce mild hyperthermia, on the order of 0.3–0.4°C, that lasts for several hours (unpublished observations). Our behavioral data suggest that these levels of IL-1β serve as interoceptive stressors leading to changes in ASRs (Beck and Servatius, 2003) and classical conditioning (Servatius and Beck, 2003), as observed after exposure to inescapable shocks (Servatius and Shors, 1994; Servatius *et al*, 2001). These data suggest that as interoceptive USs, PB and IL-1β can be dissociated with respect to associative learning of contexts. However, a direct test of this possibility would require evidence that the aversive sensations attributable to cholinergic overstimulation and proinflammatory activation are equivalent.

Manipulation along the interoceptive/exteroceptive dimension was not wholly orthogonal or independent. A stressor is considered exteroceptive or interoceptive based on the initial source of stimulation. Exteroceptive stimuli involve the five basic senses of touch, smell, vision, taste, and hearing. Interoceptive stimuli arise from within the body (eg gastric distention, inflammation, fever). Exteroceptive stressors—such as tail pinch, foot shock, and tailshock—will induce a cascade of physiological adjustments secondary to exteroceptive stressors. Indeed the tailclip would be expected to induce activation of the sympathetic nervous system (Antelman and Szechtman, 1975), hypothalamic–pituitary–adrenal axis (HPAA) (Kirby *et al*, 1997), and possibly a proinflammatory response. On the other hand, PB and IL-1β were administered through i.p. injection, thus having an exteroceptive component. While we did not include noninjected controls, the levels of ASR in vehicles were similar to those obtained in other experiments performed at a similar time using noninjected rats. Cessation of afferent traffic during stressor exposure, whether exteroceptive or interoceptive, would be necessary to address directly this point.

Olfactory Cues

Interoceptive sensations attributable to PB and IL-1β treatment were readily associated with odor. Treatment with both PB and IL-1β led to potentiated ASRs when the contexts for testing were concordant with that of exposure. In contrast to the exaggerated responses in visually distinguished novel contexts, exaggerated responses in olfactory contexts developed over the initial test session. These data suggest that the reaction to the odor previously paired with interoceptive stressor required several minutes to develop.

Exaggerated ASRs in SAME were apparent 2 weeks after stressor exposure. Here, both initial ASRs and session-wide ASRs were exaggerated. The increased reactivity specifically in the presence of odors paired with interoceptive stressors is consistent with the induction of fear or anxiety, reinforced through a recapitulation of interoceptive stimuli.

Odors discretely paired with exteroceptive stressors (footshock) (Otto *et al*, 1997; Richardson *et al*, 1999; Paschall and Davis, 2002), but not after pairing with illness from LiCl (Richardson and McNally, 2003), potentiate ASRs. The lack of contextual learning after treatment with LiCl paired with odors could be accounted for by a number of procedural and design differences between the Richardson and McNally studies and the present work. For one, our protocol involved a single pairing of the interoceptive stressor and context; Richardson and McNally used several pairings over a 2-week period prior to the initial startle test.

For another, the discrimination evaluated differed. Our protocol evaluated treated or vehicle rats in one of two odor-distinguished contexts. Richardson and McNally contrasted the ASRs of rats given LiCl in the experimental chamber or home cage, and then tested in the experimental chamber. Also, the procedures varied in the ASR test. Our ASR protocol delivers twice the number of acoustic bursts as the Richardson and McNally protocol. In that the exaggerated ASRs of the initial test were evident later in the test session, this difference in ASR test duration (number) may have played a role. In addition, the discrimination was much more apparent during the second session conducted 2 weeks after the initial; Richardson and McNally evaluated the ASR in a single session conducted the day after the last pairing.

In addition to the learning apparent in contexts differentiated by odors, rats treated with PB exhibited a transient increased reactivity within the novel odor. This increased reactivity was not apparent after IL-1β treatment. Together with the increased reactivity in visually differentiated contexts, it seems that treatment with cholinomimetics alters reactivity to novel features in the environment. Future research needs to examine the stability, generalizability, and robustness of these transient changes in reactivity.

Implications

Dissociation between visual- and odor-differentiated contexts in producing potentiated ASRs is in keeping with the classic work of Garcia and Koelling (1966). Whereas it is likely that the environmental cues used to distinguish contexts vary in saliency (wall adornment and odors), cue saliency would not provide a good explanation for why rats reacted to novelty in visually distinguished contexts and reacted in odor-distinguished contexts congruent with treatment. Here, we held the challenge, ASR, constant across experiments to facilitate comparisons among treatments. Different challenges, such as chemicals, infectious, or biological agents, may reveal distinct patterns of enhanced reactivity relative to contextual features.

A second dissociation was evident between cholinomimetics and IL-1β. Cholinomimetics affect reactivity in both visually and olfactory-distinguished contexts. However, IL-1β only affected reactivity in olfactory-distinguished contexts. While further research would be necessary to validate this impression, it is intriguing that learning related to exteroceptive features could depend on the nature of the interoceptive stressor.

The dissociation between cholinomimetics and IL-1β may reflect their differences in the peripheral organ and tissues affected or the central processing of the respective interoceptive changes. Proinflammatory cytokines comprise acute-phase responses for infections, leading to a cascade of immune (eg IL-1 receptor antagonist, IL-6, and IL-10) and neurohormonal responses (sympathetic and HPAA activation). Systemic administration of IL-1β increases norepinephrine, serotonin, and dopamine turnover in the same regions as many stressors: paraventricular nucleus of the hypothalamus, locus coeruleus, central amygdala, and prefrontal cortex (Brebner *et al*, 2000). Brain stem and amygdala nuclei mediate HPAA responses (Xu *et al*, 1999). On the other hand, PB will affect cholinergic transmission at autonomic ganglia as well as postganglionic parasympathetic terminals, and the neuromuscular junction. Beyond this broad characterization, specific mapping of brain activation following PB treatment has not been performed, likely due to the peripheral nature of PB. Therapeutic levels of PB treatment will induce a mild increase in plasma corticosterone in rats (Servatius *et al*, 2000); however, PB is known to induce growth hormone and inhibit adrenocorticotrophin (ACTH) levels in humans by direct actions on the pituitary (Llorente *et al*, 1996). The elevated corticosterone levels in rats may reflect the emotional components attributable to PB treatment overwhelming the direct suppressive actions on ACTH or species-specific actions. Nonetheless, both IL-1β and PB treatment (and the tailclip for that matter) will induce an HPAA response; however, the behavioral patterns differ between treatments arguing against corticosterone influencing learning in this paradigm. A level of processing sensitive to contextual features, such as the amygdala, hippocampus, entorhinal cortex, and periaquaductal grey, likely mediates the learning and enhanced reactivity in this paradigm.

Unexplained illnesses (chronic fatigue syndrome, fibromyalgia, GWI, multiple chemical sensitivity) have in common the appearance of nonspecific symptoms without an underlying medical explanation. In the case of GWI, there existed the possibility that a single common feature could be identified, given that the lives of troops are more structured than civilians. However, none has been identified. One relatively unexplored possibility is that learning accounts for the generation and maintenance of nonspecific symptoms. Learning processes (eg second order or higher learning and occasion setting) may fill the gap between underlying medical disturbances and the appearance of symptoms. The present data showing first-order conditioning with PB and IL-1β as USs provide initial support for such a hypothesis.

In summary, the aversive properties of even mild interoceptive stressors (those that do not produce frank illness) supported contextual learning, the nature of which depended on the features of the context. Contextual learning, especially in the presence of odors associated with interoceptive stressors, persisted for weeks after a single pairing.

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REFERENCES

Abou D, Wilmarth KR, Jensen KF, Oehme FW, Kurt TL (1996). Neurotoxicity resulting from coexposure to pyridostigmine bromide, deet, and permethrin: implications of Gulf War chemical exposures. *J Toxicol Environ Health* **48**: 35–56.

Anisman H, Merali Z (1999). Anhedonic and anxiogenic effects of cytokine exposure. *Adv Exp Med Biol* **461**: 199–233.

Antelman SM, Szechtman H (1975). Tail pinch induces eating in sated rats which appears to depend on nigrostriatal dopamine. *Science* **189**: 731–733.

Baldi E, Lorenzini CA, Bucherelli C (2004). Footshock intensity and generalization in contextual and auditory-cued fear conditioning in the rat. *Neurobiol Learn Mem* **81**: 162–166.

Beck KD, Brennan FX, Moldow RL, Ottenweller JE, Zhu G, Servatius RJ (2003). Stress interacts with peripheral cholinesterase inhibitors to cause central nervous system effects. *Life Sci* **73**: 41–51.

Beck KD, Brennan FX, Servatius RJ (2002). Effects of stress on nonassociative learning processes in male and female rats. *Integr Physiol Behav Sci* **37**: 128–139.

Beck KD, Servatius RJ (2003). Stress and cytokine effects on learning: what does sex have to do with it? *Integr Physiol Behav Sci* **38**: 179–188.

Beck KD, Zhu G, Beldowicz D, Brennan FX, Ottenweller JE, Moldow RL *et al* (2001). Central nervous system effects from a peripherally acting cholinesterase inhibiting agent: interaction with stress or genetics. *Ann NY Acad Sci* **933**: 310–314.

Brebner K, Hayley S, Zacharko R, Merali Z, Anisman H (2000). Synergistic effects of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha: central monoamine, corticosterone, and behavioral variations. *Neuropsychopharmacology* **22**: 566–580.

Davis M (1990). Animal models of anxiety based on classical conditioning: the conditioned emotional response (CER) and the fear-potentiated startle effect. *Pharmacol Ther* **47**: 147–165.

Davis M (1989). Sensitization of the acoustic startle reflex by footshock. *Behav Neurosci* **103**: 495–503.

Fanselow MS, Tighe TJ (1988). Contextual conditioning with massed *versus* distributed unconditional stimuli in the absence of explicit conditional stimuli. *J Exp Psychol Anim Behav Process* **14**: 187–199.

Fanselow MS (1990). Factors governing one-trial contextual conditioning. *Anim Learn Behav* **18**: 264–270.

Ferguson E, Cassaday HJ (1999). The Gulf War and illness by association. *Br J Psychol* **90**(Part 4): 459–475.

Ferguson E, Cassaday HJ (2001). Theoretical accounts of Gulf War Syndrome: from environmental toxins to psychoneuroimmunology and neurodegeneration. *Behav Neurol* **13**: 133–147.

Friedman A, Kaufer D, Shemer J, Hendler I, Soreq H, Tur-Kaspa I (1996). Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. *Nat Med* **2**: 1382–1385.

Garcia J, Ervin FR, Koelling RA (1966). Learning with prolonged delay of reinforcement. *Psychonomic Sci* **5**: 121–122.

Garcia J, Koelling RA (1966). Relation of cue to consequence in avoidance learning. *Psychonomic Sci* **4**: 123–124.

Garcia J, McGowan BK, Ervin FR, Koelling RA (1968). Cues: their relative effectiveness as a function of the reinforcer. *Science* **160**: 794–795.

Grauer E, Alkalai D, Kapon J, Cohen G, Raveh L (2000). Stress does not enable pyridostigmine to inhibit brain cholinesterase after parenteral administration. *Toxicol Appl Pharmacol* **164**: 301–304.

Herzog C, Otto T (1997). Odor-guided fear conditioning in rats: 2. Lesions of the anterior perirhinal cortex disrupt fear conditioned to the explicit conditioned stimulus but not to the training context. *Behav Neurosci* **111**: 1265–1272.

Holder MD, Garcia J (1987). Role of temporal order and odor intensity in taste-potentiated odor aversions. *Behav Neurosci* **101**: 158–163.

Hoy JB, Cornell JA, Karlix JL, Tebbett IR, van Haaren F (2000). Repeated coadministrations of pyridostigmine bromide, DEET, and permethrin alter locomotor behavior of rats. *Vet Hum Toxicol* **42**: 72–76.

Iwata J, Ledoux JE, Reis DJ. (1986). Destruction of intrinsic neurons in the lateral hypothalamus disrupts the classical conditioning of autonomic but not behavioral emotional responses in the rat. *Brain Res* **368**: 161–166.

Iwata J, Ledoux JE. (1988). Dissociation of associative and nonassociative concomitants of classical fear conditioning in the freely behaving rat. *Behav Neurosci* **102**: 66–76.

Jackson RL, Minor TR (1988). Effects of signaling inescapable shock on subsequent escape learning: implications for theories of coping and 'learned helplessness'. *J Exp Psychol Anim Behav Process* **14**: 390–400.

Kant GJ, Bauman RA, Feaster SR, Anderson SM, Saviolakis GA, Garcia GE (2001). The combined effects of pyridostigmine and chronic stress on brain cortical and blood acetylcholinesterase, corticosterone, prolactin and alternation performance in rats. *Pharmacol Biochem Behav* **70**: 209–218.

Kirby LG, Chou-Green JM, Davis K, Lucki I (1997). The effects of different stressors on extracellular 5-hydroxytryptamine and 5-hydroxyindoleacetic acid. *Brain Res* **760**: 218–230.

Lallement G, Foquin A, Baubichon D, Burckhart MF, Carpentier P, Canini F (1998). Heat stress, even extreme, does not induce penetration of pyridostigmine into the brain of guinea pigs. *Neurotoxicology* **19**: 759–766.

Llorente I, Lizcano F, Alvarez R, Diez N, Sopena M, Gil MJ *et al* (1996). Cholinergic modulation of spontaneous hypothalamic–pituitary–adrenal activity and its circadian variation in man. *J Clin Endocrinol Metab* **81**: 2902–2907.

Maier SF, Watkins LR (1998). Cytokines for psychologists: implications of bidirectional immune-to-brain communication for understanding behavior, mood, and cognition. *Psychol Rev* **105**: 83–107.

Ottenweller JE, Servatius RJ, Tapp WN, Drastal SD, Bergen MT, Natelson BH (1992). A chronic stress state in rats: effects of repeated stress on basal corticosterone and behavior. *Physiol Behav* **51**: 689–698.

Otto T, Cousens G, Herzog C (2000). Behavioral and neuropsychological foundations of olfactory fear conditioning. *Behav Brain Res* **110**: 119–128.

Otto T, Cousens G, Rajewski K (1997). Odor-guided fear conditioning in rats: 1. Acquisition, retention, and latent inhibition. *Behav Neurosci* **111**: 1257–1264.

Paschall GY, Davis M (2002). Second-order olfactory-mediated fear-potentiated startle. *Learn Mem* **9**: 395–401.

Richardson R, Elsayed H (1998). Shock sensitization of startle in rats: the role of contextual conditioning. *Behav Neurosci* **112**: 1136–1141.

Richardson R, McNally GP (2003). Effects of an odor paired with illness on startle, freezing, and analgesia in rats. *Physiol Behav* **78**: 213–219.

Richardson R, Vishney A, Lee J (1999). Conditioned odor potentiation of startle in rats. *Behav Neurosci* **113**: 787–794.

Romano JA, King JM (1987). Conditioned taste aversion and cholinergic drugs: pharmacological antagonism. *Pharmacol Biochem Behav* **27**: 81–85.

Servatius RJ, Beck KD (2003). Facilitated acquisition of the classically conditioned eyeblink response in male rats after systemic IL-1beta. *Integr Physiol Behav Sci* **38**: 169–178.

Servatius RJ, Brennan FX, Beck KD, Beldowicz D, Coyle-DiNorcia K (2001). Stress facilitates acquisition of the classically conditioned eyeblink response at both long and short interstimulus intervals. *Learn Motiv* **32**: 178–192.

Servatius RJ, Ottenweller JE, Beldowicz D, Guo W, Zhu G, Natelson BH (1998). Persistently exaggerated startle responses in rats treated with pyridostigmine bromide. *J Pharmacol Exp Ther* **287**: 1020–1028.

Servatius RJ, Ottenweller JE, Guo W, Beldowicz D, Zhu G, Natelson BH (2000). Effects of inescapable stress and treatment with pyridostigmine bromide on plasma butyrylcholinesterase

and the acoustic startle response in rats. *Physiol Behav* **69**: 239–246.

Servatius RJ, Shors TJ (1994). Exposure to inescapable stress persistently facilitates associative and nonassociative learning in rats. *Behav Neurosci* **108**: 1101–1106.

Sharabi Y, Danon YL, Berkenstadt H, Almog S, Mimouni-Bloch A, Zisman A *et al* (1991). Survey of symptoms following intake of pyridostigmine during the Persian Gulf war. *Isr J Med Sci* **27**: 656–658.

Shors TJ, Servatius RJ (1997). The contribution of stressor intensity, duration, and context to the stress-induced facilitation of associative learning. *Neurobiol Learn Mem* **68**: 92–96.

Sinton CM, Fitch TE, Petty F, Haley RW (2000). Stressful manipulations that elevate corticosterone reduce blood–brain barrier permeability to pyridostigmine in the rat. *Toxicol Appl Pharmacol* **165**: 99–105.

Soetekouw PM, de Vries M, Preijers FW, Van Crevel R, Bleijenberg G, van der Meer JW (1999). Persistent symptoms in former UNTAC soldiers are not associated with shifted cytokine balance. *Eur J Clin Invest* **29**: 960–963.

Song X, Tian H, Bressler J, Pruett S, Pope C (2002). Acute and repeated restraint stress have little effect on pyridostigmine toxicity or brain regional cholinesterase inhibition in rats. *Toxicol Sci* **69**: 157–164.

Xu Y, Day TA, Buller KM (1999). The central amygdala modulates hypothalamic–pituitary–adrenal axis responses to systemic interleukin-1beta administration. *Neuroscience* **94**: 175–183.