The effects of acute stress on Pavlovian-instrumental transfer in rats

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Abstract Pavlovian stimuli invigorate ongoing instrumental action, a phenomenon termed the *Pavlovian-instrumental transfer* (PIT) effect. Acute stressors can markedly enhance the release of corticotropin-releasing factor (CRF), and CRF injection into the nucleus accumbens increases the PIT effect. However, it is unknown whether acute stressors by themselves would amplify the PIT effect. Here, we examined the effects of acute stressors on PIT. Rats first received Pavlovian and instrumental training, and then the impact of the Pavlovian stimuli on instrumental responding was analyzed in the subsequent PIT test. Acute stressors were applied prior to the PIT test. Because the effects of acute stressors critically depend on stressor type and time of day, we used two acute stressors that involved one or several distinct stressors (denoted here as “single” vs. “multiple” stressors) applied either in the light or the dark period of the light:dark cycle. The results revealed that single and multiple stressors applied in the light period did not alter the PIT effect—that is, the ability of an appetitive Pavlovian stimulus to enhance leverpressing—or the basal leverpress rate. When applied in the dark period, single and multiple stressors also did not alter the PIT effect, but they did markedly reduce the basal leverpress rate. Diazepam pretreatment did not counteract the declines in basal instrumental responding in the PIT test that were induced by either a single or multiple stressors. Our findings suggest that acute stressors were unable to amplify the incentive salience of reward-predictive Pavlovian stimuli to activate instrumental responding, but, depending on the time of day of stressor exposure, they did reduce basal instrumental responding.

Keywords Acute stress · Incentive motivation · Anxiety · Pavlovian-instrumental transfer · Rat

Pavlovian stimuli predictive of natural rewards such as food can increase leverpressing for food, a phenomenon termed the *Pavlovian-instrumental transfer effect* (PIT effect) (Estes, 1948; Lovibond, 1983). One important mechanism by which Pavlovian stimuli influence the vigor of an action is to create a general appetitive arousal that elevates instrumental responding (Rescorla & Solomon, 1967). The central nucleus of the amygdala (Corbit & Balleine, 2005; Holland & Gallagher, 2003), the nucleus accumbens core (Corbit & Balleine, 2011; Hall, Parkinson, Connor, Dickinson, & Everitt, 2001), the ventral tegmental area (Corbit, Janak, & Balleine, 2007; Murschall & Hauber, 2006), and the meso-accumbens dopamine system (Hall, et al., 2001; Lex & Hauber, 2008; Wyvell & Berridge, 2000) are major components of a neural circuitry mediating the PIT effect. Notably, Pecina, Schulkin, and Berridge (2006) demonstrated that the PIT effect was markedly enhanced not only by intra-accumbens amphetamine infusion but also by intra-accumbens infusion of corticotropin-releasing factor (CRF), and they concluded that activation of CRF subsystems in the nucleus accumbens—and, thus, acute stress—could amplify positive motivation for signaled rewards. Importantly, such paradoxical positive incentive effects could explain why acute stress can produce stimulus-triggered bursts of binge eating, drug relapse, or other excessive pursuits of reward (Pecina et al., 2006).

It is well known that acute stress is associated with increased brain CRF and dopamine release (Bale & Vale, 2004; Imperato, Puglisi-Allegra, Casolini, Zocchi, & Angelucci, 1989). However, although CRF plays a crucial role (Bale & Vale, 2004), it is important to note that acute stress responses involve the release of multiple stress mediators, including a number of other neuropeptides, such as

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vasopressin or dynorphin, as well as monoamine neurotransmitters and steroids (Joels & Baram, 2009) and entail complex interacting circuits, in particular in the limbic forebrain, the hypothalamus, and the brainstem (Ulrich-Lai & Herman, 2009). Because intra-accumbens CRF infusion may mimic the multifaceted neurochemical responses induced by acute stressors only to a minor extent, it is questionable whether acute stress would be able to amplify the PIT effect in a manner similar to intra-accumbens infusions of CRF. Therefore, in the present study we sought to explore the as-yet-unknown effects of acute stressors on PIT. Rats first received Pavlovian and instrumental training, and thereafter, the impact of the Pavlovian stimuli on instrumental responding was analyzed in a subsequent PIT test. Acute stressors were applied immediately prior to the PIT test. As the impact of acute stress critically depends on the stressor type and the time of day (Koolhaas et al., 2011), we included two distinct stressors that involved either one or several stressors (denoted here as “single” vs. “multiple” stressors) that were applied during the light or the dark period of the light:dark cycle. To assess hypothalamus–pituitary–adrenocortical (HPA) axis activity in response to these stressors, we also measured plasma corticosterone levels. Furthermore, we examined whether the standard anxiolytic drug diazepam would be able to reverse the impact of acute stressors on PIT.

Materials and method

All animal experiments were in compliance with the German Law of Animal Protection and were approved by the proper authorities.

Subjects and apparatus

Experimentally naive male Lister hooded rats (Harlan Winkelmann, Borcheln, Germany) served as the subjects. The rats were housed in groups of four in transparent plastic cages (55 × 39 × 27 cm; Ferplast, Nürnberg, Germany) in a temperature- and humidity-controlled room (20 ± 2 °C, 50 %–60 %) on a 12:12-h light:dark cycle (lights on at 7.00 a.m.; Exp. 1) or on an inverted 12:12-h light:dark cycle (lights off at 8.00 a.m.; Exps. 2 and 3). The rats had ad libitum access to water. Standard laboratory maintenance chow (Altromin, Lage, Germany) was given ad libitum for 5 days after arrival. Thereafter, food was restricted to 15 g per animal per day to maintain them at ~85 % of their free-feeding weight.

Training and testing took place in identical operant chambers (24 × 21 × 30 cm; Med Associates, St. Albans, VT) housed within sound-attenuating cubicles. Each operant chamber was equipped with a pellet dispenser that delivered a 45-mg pellet into a receptacle that was positioned in the

center of one wall. In addition, each chamber contained a retractable lever located on the left side of the receptacle. A 24-V/3-W houselight mounted at the top center of the opposite wall illuminated the chambers. The speaker that delivered the auditory conditioned stimuli was mounted on the wall opposite to the levers and the receptacle. Two auditory stimuli (white noise and a tone) served as conditioned stimuli. A computer system (MedPC Software; Med Associates) controlled the equipment and recorded the data.

Pavlovian–instrumental transfer

The testing for PIT consisted of three separate parts: instrumental training and Pavlovian training, followed by a transfer test in which the invigorating influences of the Pavlovian stimuli on instrumental behavior were determined in extinction. Each session started with the illumination of the houselight and the insertion of the appropriate lever, and each ended with the retraction of the lever and the turning off of the houselight.

Magazine training First, all subjects received one session of magazine training in order to habituate the animals to the operant chamber. During the magazine training, food pellets (45-mg pellets; Bio-Serv, Frenchtown, NJ) were delivered on a random-time (RT) 30-s schedule with no lever available.

Instrumental and Pavlovian training Thereafter, eight training days were given with two sessions per day, an instrumental training session in the morning and a Pavlovian training session in the afternoon. In the instrumental training sessions, the lever was inserted, and responding on the lever was reinforced on a random-interval (RI) schedule, starting with RI 2 s during the first session. For the next two sessions, the schedule was increased to RI 15 s and RI 30 s, and for the remaining five sessions, to RI 60 s; a session ended after 30 min. The rats received Pavlovian training sessions with the lever retracted. An 80-dB sound pressure level (SPL) auditory stimulus (3-kHz tone or white noise) served as the CS+ (conditioned stimulus, rewarded) in a counterbalanced fashion. Each session contained six 2-min presentations of the CS+, followed by an interstimulus interval (ISI) of 2–4 min. Food reward pellets were delivered only during the presentation of the CS+, on an RT-30-s schedule. Subsequent to this combined instrumental–Pavlovian training, the animals received a single 30-min instrumental extinction session, with the lever available but not reinforced. In addition, one Pavlovian reminder was given. This session was similar to the Pavlovian training sessions, except that two additional, nonrewarded 2-min presentations of an 80-dB SPL auditory (3-kHz tone or white noise) CSo stimulus (neutral stimulus, nonrewarded),

as well as an additional ISI, were given preceding the fifth and following the sixth presentation of the CS+. If the tone was the CS+, the white noise was the CSo, and vice versa.

Transfer test The PIT effect was tested in extinction, while the lever was inserted into the operant chamber. Each stimulus (CS+ and CSo) was 2 min in duration and was preceded by a 2-min ISI period. The session always started with the presentation of the white noise, thus counterbalancing the order of the presentations of CS+ and CSo within each group, and the session ended after four presentations of each stimulus type (CS+ and CSo).

Experimental design

Three experiments were performed.

Experiments 1 and 2: We examined whether two distinct types of stressors, involving one or several prototypical types of acute stressors (“single” vs. “multiple” stressors; see below) and applied in either the light (Exp. 1) or the dark (Exp. 2) period of the light:dark cycle could alter the PIT effect measured in the transfer test. Instrumental and Pavlovian training and the transfer test were performed during either the light or the dark period; single or multiple stressors were applied to separate groups of rats immediately prior to the transfer test. Nonstressed rats served as the controls.

Experiment 3: Experiments 1 and 2 had revealed stressor effects on PIT when the stressors were applied during the dark, but not during the light, period. Therefore, in Experiment 3 we examined whether the observed dark-period-related single- or multiple-stressor effects on the PIT test could be reduced by pretreatment with the standard anxiolytic drug diazepam. Nonstressed rats subjected to vehicle injections served as the controls.

Types of acute stressors

Single stressor This stressor consisted of acute restraint for 60 min in a brightly lit novel room (see, e.g., Reis et al., 2011; Wong et al., 2007): Rats were placed in air-accessible Plexiglas cylinders of 7-cm diameter and variable length, in order to accommodate different-sized animals.

Multiple stressor This stressor consisted of a combination of acute stressors, applied for 60 min in total in a brightly lit, novel room with music from a local radio station (~70 dB SPL), similar to the conditions used in previous studies (Chen et al., 2010). First, the rats were exposed to restraint for 20 min by placing them in Plexiglas cylinders. Thereafter, the rats were placed on an elevated platform

(12 × 12 cm, 1 m tall) for 20 min (Wong et al., 2007). Eventually, the rats were reexposed to restraint for 20 min by placing them back in the Plexiglas cylinders. During this restraint, five tail shocks (1 mA, 1 s) were applied (e.g., Waddell, Mallimo, & Shors, 2010).

Rats were exposed to single or multiple stressors during the light or the dark phase of the light:dark cycle. Note that in those rats examined during the dark phase, stressor exposure in the bright room represented a light pulse that might act, per se, as an additional stressor (Song & Rusak, 2000; Zubidat, Ben-Shlomo, & Haim, 2007).

Drug treatment

Diazepam (1 mg/kg, i.p.; Sigma Aldrich, Steinheim, Germany) or vehicle (1 ml/kg, i.p.) was given 90 min prior to the onset of PIT testing (i.e., 30 min before stressor exposure). Previous studies and our own pilot experiments had revealed that diazepam at 1 mg/kg i.p. produced anxiolytic-like effects devoid of marked locomotor inhibition (Boissier, Simon, & Aron, 1968; Treit & Fundytus, 1988) or suppression of instrumental responding (George, Hutson, & Stephens, 2009). Due to sedative effects of this drug, higher doses reduced basal rates of leverpressing on their own (see also George et al., 2009). Diazepam was dissolved in a vehicle of 50 % saline (0.9 % NaCl), 10 % pure ethanol, and 40 % polyethylene glycol.

Blood sampling and corticosterone analysis

To assess the stressor effects in Experiments 1 and 2 on glucocorticoid secretion, we measured plasma corticosterone levels in separate groups of experimentally naive rats exposed to no, single, or multiple stressors in the light ($N = 3$, respectively) or in the dark ($N = 3$, respectively) period. In addition, to assess the stressor and drug effects in Experiment 3 on glucocorticoid secretion, plasma corticosterone levels were measured in experimentally naive rats subjected to vehicle administration and no stress or to diazepam administration and single or multiple stressors ($N = 3$, respectively). Upon completion of the stressor exposure, the rats were anesthetized with an overdose of isoflurane (cp-pharma, Burgdorf, Germany) and decapitated. Short-term treatment with isoflurane per se, as used here, does not alter corticosterone levels (Zardooz, Rostamkhani, Zaringhalam, & Faraji Shahriar, 2010). Trunk blood was collected in a falcon tube (50 ml) for 15 min and centrifuged (4,000 rpm for 10 min; 8 °C). Subsequently, the blood plasma was centrifuged (4,000 rpm for 4 min, RT) and stored at −20 °C. Plasma corticosterone levels (ng/ml) were measured using the corticosterone ELISA kit RE52211 (IBL International GmbH, Hamburg, Germany).

Data analysis

The data from the instrumental and Pavlovian training were subjected to an analysis of variance (ANOVA) for repeated measures, with Groups Exposed to Stressors as a between-subjects factor and Training Day as a within-subjects factor. The PIT data were also subjected to an ANOVA for repeated measures, with Stressor Type as a between-subjects factor and Interval (ISI, CS₀, or CS₊) as a within-subjects factor. Post hoc analyses were performed using the least significant difference test. Plasma corticosterone values were also subjected to an ANOVA, with Stressor Type and Time of Day as between-subjects factors. The level of statistical significance was $p \leq .05$. All statistical computations were carried out with STATISTICA (Version 7.1; StatSoft, Inc., Tulsa, OK).

Results

Instrumental training

Experiment 1 Separate groups of rats to be exposed to no stressor ($n = 22$), a single stressor ($n = 23$), or multiple stressors ($n = 20$) were used; the rats were randomly assigned to their respective groups. Leverpress rates during instrumental training were similar across groups (Fig. 1a). An ANOVA revealed a significant effect of day [$F(7, 427) = 190.61, p < .01$] but not of group [$F(2, 61) = 1.73, p = .19$], and no Day $\times$ Group interaction [$F(14, 427) = 1.60, p = .08$].

Experiment 2 Separate groups of rats to be exposed to no stressor ($n = 15$), a single stressor ($n = 12$), or multiple stressors ($n = 11$) were used; the rats were randomly assigned to the respective groups. We found a significant effect of day [$F(7, 245) = 110.85, p < .01$], a significant effect of group [$F(2, 35) = 10.60, p < .01$], and a trend toward a significant Day $\times$ Group interaction [$F(14, 245) = 1.62, p = .07$] (Fig. 1b).

Experiment 3 Separate groups to be exposed to no stressor ($n = 14$), a single stressor ($n = 12$), or multiple stressors ($n = 12$) were used; the rats were randomly assigned to the respective groups. An ANOVA revealed a significant effect of day [$F(7, 245) = 97.042, p < .01$] and a significant Day $\times$ Group interaction [$F(14, 245) = 2.13, p = .01$], but no effect of group [$F(2, 35) = 0.37, p = .69$] (Fig. 1c).

The results from Experiments 2 and 3 indicated that the leverpress performance over days differed somewhat between groups during instrumental training. The reasons for the differences between groups are unknown, but they may not influence the PIT effect measured in the subsequent PIT test, for several reasons: (1) the approach ratios during Pavlovian training were similar across groups (see below); (2) the instrumental response rates during training did not

predict the instrumental response rates in the PIT test performed in extinction; and (3) the PIT effect size does not critically depend on the instrumental response rate in the PIT test (Lex & Hauber, 2008).

Pavlovian training

Stimulus-directed behavior during Pavlovian training was measured by calculating the approach ratio—that is, the percentages of time spent in the food receptacle during the CS₊ versus during the ISI—as follows: approach ratio = (%CS₊) / (%CS₊ + %ISI) (Hall et al., 2001).

Experiment 1 The approach ratios were similar in all groups (Fig. 2a). An ANOVA demonstrated a significant effects of day [$F(7, 392) = 6.48, p < .01$], but no effect of group [$F(2, 56) = 0.79, p = .46$] and no Day $\times$ Group interaction [$F(14, 392) = 0.54, p = .91$].

Experiment 2 Likewise, in Experiment 2, the approach ratios of all groups were similar (Fig. 2b). An ANOVA showed a significant effect of day [$F(7, 245) = 3.65, p < .01$], but no effect of group [$F(2, 35) = 1.14, p = .33$] and no Day $\times$ Group interaction [$F(14, 245) = 1.19, p = .28$].

Experiment 3 Also, in Experiment 3 (Fig. 2c) the approach ratios increased to a similar extent in all groups, and an ANOVA revealed a significant effect of day [$F(7, 238) = 2.81, p < .01$], but no significant group effect [$F(2, 34) = 0.20, p = .82$] and no Day $\times$ Group interaction [$F(14, 238) = 1.24, p = .25$].

Transfer test

Experiment 1 Presentation of the CS₊ enhanced leverpressing relative to the ISI in both nonstressed control rats and rats exposed to single and to multiple acute stressors immediately before the transfer test. Furthermore, a minor decrease in overall response rates across all intervals was observed in rats subjected to multiple stressors, relative to control rats (Fig. 3a). An ANOVA demonstrated no effect of stressor type (no, single, or multiple stressors) [$F(2, 61) = 1.85, p = .17$], a significant effect of interval (CS₊, CS₀, or ISI) [$F(2, 122) = 55.99, p < .01$], and no Interval $\times$ Stressor Type interaction [$F(4, 122) = 1.94, p = .11$]. Post hoc comparisons further indicated higher responding in control rats during the CS₊ relative to the ISI ($p < .05$), but not during the CS₀ relative to the ISI ($p = .53$). Likewise, in rats exposed to single and multiple stressors, leverpressing during the CS₊ was higher relative to the ISI (single stressor, $p < .01$; multiple stressors, $p < .01$), but not during the CS₀ relative to the ISI (single stressor, $p < .23$; multiple stressors, $p = .69$).

Fig. 1 Instrumental training: Mean leverpresses per minute ($\pm$ SEMs) during instrumental training sessions in Experiments 1–3 (a–c) in control rats and in rats to be exposed to single or multiple stressors. Training was given during the light (Exp. 1) or during the dark (Exps. 2 and 3) period

Fig. 2 Pavlovian training: Mean approach ratios ($\pm$ SEMs) during Pavlovian training in Experiments 1–3 (**a–c**) in control rats and in rats to be exposed to single or multiple stressors. Training was given during the light (Exp. 1) or during the dark (Exps. 2 and 3) period

Fig. 3 Pavlovian–instrumental transfer (PIT): Mean lever-presses per minute ($\pm$ SEMs) in control rats and in rats exposed to single and multiple stressors immediately prior to the PIT test. In Experiment 1 (**a**), transfer testing was performed in the light period, and in Experiments 2 and 3 (**b** and **c**), in the dark period. In Experiment 3, controls rats were pretreated with vehicle, and rats exposed to single or multiple stressors were pretreated with diazepam (DZ; 1 mg/kg, i.p.). * $p < .05$ for comparisons of performance during the interstimulus interval (ISI), during the unconditioned, neutral stimulus (CS₀), or during the conditioned stimulus (CS⁺)

Experiment 2 This experiment demonstrated a PIT effect in both control rats and rats exposed to single and multiple stressors. However, baseline responding was lower in the rats exposed to single and multiple stressors, relative to control rats (Fig. 3b). Consistent with this notion, an ANOVA demonstrated significant effects of stressor type [$F(2, 35) = 7.05, p < .01$] and interval [$F(2, 70) = 14.09, p < .01$], but no Interval $\times$ Stressor Type interaction [$F(4, 70) = 0.62, p = .65$]. Post hoc comparisons further indicated higher responding during the CS+ relative to the ISI in control rats and in rats exposed to single and multiple stressors (controls, $p = .02$; single stressor, $p < .01$; multiple stressors, $p = .03$), but not during the CS0 relative to the ISI (controls, $p = .65$; single stressor, $p = .63$; multiple stressor, $p = .87$). For a more detailed analysis of the treatment effect on overall leverpress rates, we performed separate pair-wise ANOVAs. The results revealed that the overall leverpress rates across all intervals (ISI, CS+, and CS0) were lower in the single-stressor group than in the control group [$F(1, 25) = 5.97, p = .02$] and also lower in the multiple-stressor group than in the control group [$F(1, 24) = 13.47, p = .01$], but not between the single-stressor and multiple-stressor groups [$F(1, 21) = 1.98, p = .17$].

Experiment 3 A PIT effect was observed in the vehicle-treated control rats exposed to no stressor, as well as in the diazepam-pretreated rats exposed to a single stressor and to multiple stressors (Fig. 3c). An ANOVA revealed significant effects of stressor type [$F(2, 35) = 3.26, p = .05$] and interval [$F(2, 70) = 22.68, p < .01$], but no Interval $\times$ Stressor Type interaction [$F(4, 70) = 0.61, p = .66$]. Post hoc comparisons further indicated higher responding in all treatment groups during the CS+ relative to the ISI (control, $p = .01$; single stressor, $p < .01$; multiple stressor, $p < .01$), but not during the CS0 relative to the ISI (control, $p = .52$; single stressor, $p = .55$; multiple stressor, $p = .88$). For a more detailed analysis, we analyzed the treatment effect on overall leverpress rates using separate pair-wise ANOVAs. The results revealed that the overall leverpress rates across intervals (ISI, CS+, and CS0) did not differ between the single-stressor and control groups [$F(1, 24) = 0.01, p = .92$], but that they were lower in the multiple-stressor group than in the control group [$F(1, 24) = 4.37, p < .05$], as well as in the single-stressor group [$F(1, 22) = 7.70, p = .01$].

Collectively, the data from Experiments 2 and 3 point to the possibility that diazepam moderately reduced the stressor-induced decline in overall leverpress rates, in particular in rats exposed to the single stressor. To characterize the diazepam effects in more detail, we performed an ANOVA on the PIT effects measured in Experiments 2 and 3, with Experiment and Stressor Type as between-subjects factors and Interval as a within-subjects factor. The results revealed significant effects of experiment [$F(1,$

$70) = 11.06, p < .01$], stressor type [$F(2, 70) = 8.80, p < .01$], and interval [$F(2, 140) = 36.31, p < .01$], but, importantly, no Experiment $\times$ Stressor Type interaction [$F(2, 70) = 1.14, p = .32$], suggesting that diazepam had only marginal effects that failed to reach significance.

Plasma corticosterone levels

Corticosterone levels were higher in the untreated rats exposed to single and multiple stressors than in controls, and the effects of time of day seemed to be moderate (Table 1). In line with this notion, an ANOVA with Time of Day (light or dark period) and Stressor Type (no, single, or multiple stressors) as factors showed significant effects of stressor type [$F(2, 12) = 35.6, p < .01$] and time of day [$F(1, 12) = 5.3, p < .05$], but no interaction between these factors. Post hoc comparisons indicated that in both the light and dark periods, corticosterone levels were increased in stressor-exposed rats relative to control rats (Table 1). By contrast, corticosterone levels in the rats treated with vehicle or diazepam did not differ. An ANOVA with Stressor Type (no, single, or multiple stressors) as a factor revealed no significant treatment effects [$F(2, 5) = 1.81, p > .05$]. The corticosterone value of one diazepam-treated animal exposed to the single stressor was excluded, as it deviated from the group mean by more than 2 SDs.

Discussion

Here, we examined the impact of two types of acute stressors when they were applied in the light or the dark period of the light:dark cycle immediately prior to the PIT test. The main finding of our study was that both types of stressors applied in the light did not alter the PIT effect—that is, the ability of an appetitive Pavlovian stimulus to enhance

Table 1 Time of day, stressor, and drug effects on mean plasma corticosterone levels

Time of Day	Stressor	Drug	Corticosterone (ng/ml)	n
Light period	Control	–	195.4 $\pm$ 72	3
Light period	Single	–	738.6 $\pm$ 36*	3
Light period	Multiple	–	1,053.3 $\pm$ 99* #	3
Dark period	Control	–	503.7 $\pm$ 103	3
Dark period	Single	–	902.8 $\pm$ 101*	3
Dark period	Multiple	–	1,060.2 $\pm$ 81*	3
Dark period	Control	Vehicle	661.9 $\pm$ 233	3
Dark period	Single	Diazepam	1,259.8 $\pm$ 358	2
Dark period	Multiple	Diazepam	896.8 $\pm$ 36	3

* $p < .01$ vs. the respective control; # $p < .05$ single vs. multiple stressors

leverpressing—and also left basal leverpress rates unchanged. By contrast, when applied in the dark period, both types of stressors did not amplify the PIT effect but did markedly reduce basal leverpress rates.

Acute stressor effects: Influence of time of day

In the light period, the acute single stressor stimulated the HPA axis, resulting in increased plasma corticosterone, as was shown by earlier studies (e.g., Keim & Sigg, 1976; Porterfield et al., 2011; Ulrich-Lai et al., 2010), but it did not alter the PIT effect or basal instrumental response rates. Our pilot studies using other prototypical acute stressors applied in the light period—for instance, elevated platform stress (Howland & Cazakoff, 2010) or extended restraint stress (Yuen et al., 2009)—gave similar results. Therefore, we employed a novel multiple stressor involving a combination of typical acute stressors used in previous studies (e.g., Howland & Cazakoff, 2010; Keim & Sigg, 1976; Musazzi et al., 2010; Yuen et al., 2009). Notably, exposure to the multiple stressor during the light period increased plasma corticosterone to a larger extent than did the single stressor applied during the light period, but it did not alter the PIT effect and instrumental response rates.

By contrast, in the dark period, single and multiple stressors elevated plasma corticosterone to about the same extent and significantly reduced instrumental response rates, but they left the PIT effect per se unaffected. This finding suggests that our acute stressors, which involved solely or predominately restraint, were, in behavioral terms, most effective in the dark period. This view is consistent with an earlier notion that, for rats, stress procedures involving restraint elicit more pronounced physiological and behavioral effects during the dark (activity) period than during the light (inactivity) period (Koolhaas et al., 2011). As was pointed out by the authors of this previous study, from an ecological point of view this observation is not surprising, as, for instance, during the light (inactivity) period feral Norway rats hide in narrow burrow systems.

Consistent with earlier reports (e.g., Atkinson & Waddell, 1997; Chacon et al., 2005; Gallo & Weinberg, 1981), plasma corticosterone levels in our rats were generally higher during the dark period than during the light period. Moreover, plasma corticosterone elevations after single or multiple stressor correspond to those reported in previous studies using similar or other acute stressors (e.g., Kavushansky, Klein, & Ben-Shachar, 2010; Kavushansky & Richter-Levin, 2006; Ulrich-Lai & Herman, 2009; Woodson, Macintosh, Fleshner, & Diamond, 2003). The plasma corticosterone elevations reported in previous studies were not or were only partially related to stressor type or intensity, the timing of the stress exposure, or the magnitude of the stress-induced behavioral effects. For instance, male

rats exposed to a predator such as a cat or to a female receptive rat showed equivalent increases in plasma corticosterone levels, but only cat-exposed rats exhibited working memory deficits (Woodson et al., 2003). Furthermore, the effects of acute restraint stress on cost/benefit decision making in rats were not mimicked by systemic administration of corticosterone, suggesting that increased corticosterone levels may be not sufficient to impair this form of behavioral choice (Shafiei, Gray, Viau, & Floresco, 2012). Overall, these findings suggest that plasma corticosterone levels may not always be a sensitive index of stress intensity (e.g., Natelson et al., 1987). Correspondingly, our present findings provide strong support for the notion that, in rats, the behavioral effects of acute stressors involving restraint critically depend on the time of day, but depend less on stressor intensity.

Acute stress effects on PIT

A major mechanism by which Pavlovian stimuli might influence the vigor of an action is to create a general appetitive arousal that elevates instrumental responding (e.g., Rescorla & Solomon, 1967). Notably, this PIT effect has been markedly enhanced not only by intra-accumbens amphetamine infusion (Wyvell & Berridge, 2000) but also by intra-accumbens CRF infusion, suggesting that the activation of CRF subsystems in the nucleus accumbens and, by implication, acute stress could amplify positive motivation for signaled rewards (Pecina et al., 2006). Importantly, such paradoxical positive incentive effects could explain why acute stress sometimes produces stimulus-triggered bursts of binge eating, drug relapse, or other excessive pursuits of reward (Dallman, Pecoraro, Houshyar, & Griffith, 2005; Marinelli & Piazza, 2002). Contrasting with this view, our results indicate that, unlike intra-accumbens CRF, acute stressors applied prior the PIT test did not enhance the ability of an appetitive CS+ to invigorate instrumental responding, but rather impaired basal instrumental responding for an expected reward. As only high but not low doses of intra-accumbens CRF have been effective (Pecina et al., 2006), the failure to observe an amplified PIT effect could be due simply to subthreshold accumbens CRF levels induced by the acute stressors used here. To our knowledge, the effects of the stressors used here on intra-accumbens CRF levels are unknown. Microdialysis studies have revealed that restraint stress for 20 min produced a threefold increase of CRF levels in limbic brain areas such as the amygdala (Merlo Pich et al., 1995), and possibly in the nucleus accumbens as well; however, direct evidence for the latter claim is missing. Furthermore, restraint stress for 90 min markedly increased intra-accumbens dopamine release (Imperato et al., 1989), indicating that the stressors used in the present study can produce marked neurochemical effects.

Furthermore, our observation that both single and multiple stressors applied in the dark period reduced basal leverpressing suggests that the stressors were behaviorally effective.

Importantly, acute stress responses involve the release of multiple stress mediators, including a number of other neuropeptides, such as vasopressin or dynorphin, as well as monoamine neurotransmitters and steroids (Joels & Baram, 2009). Furthermore, acute stress responses are mediated by complex neural circuits in the limbic forebrain, the hypothalamus, and the brainstem (Ulrich-Lai & Herman, 2009). Thus, an intra-accumbens CRF infusion may mimic the multifaceted neurochemical responses in many brain areas induced by acute stressors only to a minor extent. For instance, it is conceivable that acute stressors activate the CRF subsystems that mediate aversive states (Schulkin, Morgan, & Rosen, 2005), possibly counteracting the expression of an amplified PIT effect governed by accumbens CRF subsystems. Overall, our finding that acute stressors were unable to amplify the incentive salience of a reward-predictive CS+ in order to activate reward-directed responding puts in question a general role of acute stressors in precipitating paradoxical incentive effects—for instance, stimulus-triggered bursts of binge eating or drug relapse (Pecina et al., 2006). Yet, it is important to note that acute stressor effects depend on multiple variables; thus, other types of expected rewards, such as drugs, could enhance the incentive motivation triggered by an appetitive CS+, in particular in rats chronically pretreated with addictive drugs. Furthermore, the fact that the PIT effect was insensitive to acute stressors implies that the retrieval of well-learned memories for CS–US associations, unlike other forms of memory (de Kloet, Oitzl, & Joels, 1999), and the behavioral impact mediated by the appetitive CS+ seem to be relatively resistant to acute stress. To some extent, the resistance of the PIT effect to acute stressors may be explained by Pavlovian training over not less than 8 days, as it has been implemented in most PIT protocols (Holmes, Marchand, & Coutureau, 2010). In other words, it is possible that the PIT effect could become sensitive to acute stressors if a PIT protocol with more limited Pavlovian training would be used.

Importantly, our results further show that acute stressors significantly reduced basal instrumental responding for an expected reward during the PIT test. As acute stressors can compromise performance of skilled paw movements (Metz, Jadavji, & Smith, 2005), the reduced leverpressing seen here could simply reflect impaired motor function per se. However, such a chronic, state-like motor impairment should have applied for the entire transfer test, regardless of whether or not the CS+ stimulus was present. By contrast, we observed that presentation of an appetitive CS+ was still able to invigorate leverpressing, an observation that seems to rule out basic motor impairments as an explanation for reduced instrumental responding. Considerable evidence

suggests that, rather than reducing reward-seeking behavior, exposure to an acute stressor can have the opposite effect, stimulating palatable food intake or reinstating drug-taking behavior (Cador et al., 1992; Kalivas, Duffy, & Latimer, 1987; Stewart, 2003). In turn, pleasurable behaviors such as palatable food intake were able to reduce neuroendocrine, cardiovascular, and behavioral responses to acute stress (Ulrich-Lai & Herman, 2009). By contrast, a large number of studies have suggested that both acute stress and intracerebroventricular CRF inhibited food intake and behaviors associated with food intake. For instance, CRF decreased food-maintained instrumental responding under various reinforcement schedules, including differential reinforcements of low rate or combined fixed-interval and fixed-ratio schedules (see Glowa, Barrett, Russell, & Gold, 1992, for a review). Remarkably, depending on a number of variables, such as stress intensity or context, acute stress can either reinstate or inhibit drug seeking (Lu, Shepard, Hall, & Shaham, 2003; Shaham, Erb, & Stewart, 2000). Furthermore, it is well documented that acute stress can produce fear and anxiety (Davis, 2006; Schulkin et al., 2005). For instance, by producing anxiety, a footshock-predictive stimulus can markedly suppress instrumental responding (Blanchard & Blanchard, 1969; Hunt & Brady, 1951). These findings point to the possibility that stress-induced anxiety could have contributed to the reduced instrumental responding observed here. However, our data do not provide support to this notion, as the standard anxiolytic drug diazepam at a dose of 1 mg/kg only marginally reduced leverpressing after stressor exposition. Notably, in acute-stress paradigms, low doses of diazepam (<3 mg/kg) can produce anxiolytic effects on a variety of behavioral measures (Grahn, Kalman, Brennan, Watkins, & Maier, 1995; Kalman, Kim, Cole, Chi, & Spencer, 1997) without decreasing stress-induced rises in plasma corticosterone (Keim & Sigg, 1976). Nevertheless, we cannot exclude the possibility that higher doses of diazepam could have antagonized stress-induced behavioral effects. However, our pilot studies indicated that higher doses reduced basal rates of leverpressing on their own. Likewise, a study by George et al. (2009) demonstrated that, due to sedative effects, diazepam at doses of 3 and 10 mg/kg reduced basal rates of leverpresses in the PIT test, an effect not seen with a dose of 1 mg/kg, as used here. Thus, anxiolytic or antidepressant drugs with a lower incidence of sedative effects could be an option for future studies. Taken together, our findings clearly show that acute stressors impaired basal instrumental responding; however, the underlying behavioral mechanisms remain to be determined.

Conclusions

Our study demonstrated for the first time that acute stressors did not amplify the PIT effect, but rather reduced basal

instrumental responding during the PIT test, an effect that critically depended on the time of day of stressor exposure. Importantly, these findings suggest that, unlike intra-accumbens CRF (Pecina et al., 2006), acute stress was unable to enhance the incentive salience of a CS+ that was predictive of natural reward so as to invigorate reward-directed responding, but instead impaired basal instrumental responding.

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