Enhanced fear acquisition in individuals with evening chronotype. A virtual reality fear conditioning/extinction study

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Highlights

• Evening chronotype has been associated with enhanced risk to develop anxiety and post-traumatic stress disorder.

• We examined the role of chronotype to predict fear conditioning and extinction.

• We found higher fear acquisition in evening compared to intermediate chronotype.

Abstract

Circadian rhythms have received increasing attention within the context of mental disorders. Evening chronotype has been associated with enhanced risk to develop anxiety and post-traumatic stress disorder (PTSD). The classical fear conditioning paradigm is a powerful tool to reveal key mechanisms of anxiety and PTSD. We used this paradigm to study the neurocognitive basis of the association between chronotype and fear responses in healthy humans.

20 participants with evening chronotype and 20 controls (i.e., intermediate chronotype) completed a 2-day Pavlovian fear learning and extinction virtual reality task. Participants received fear conditioning, and extinction learning on day 1. Extinction memory recall was tested on day 2. To address interactions between chronotype and time of day of the fear conditioning, and extinction performance, half of the participants were tested in the morning, and the other half in the evening. Skin conductance response (SCR) and subjective fear ratings were measured as primary outcomes. Chronotype was established via the morningness–eveningness questionnaire (MEQ).

We found an overall higher SCR for fear acquisition in participants with the evening chronotype profile, compared to controls. Moreover, the higher the MEQ scores –indicative of less eveningness – the lower the SCR was. No effects of chronotype were found for extinction and extinction recall. The higher vulnerability of the evening chronotype for anxiety and related disorders may thus be explained by enhanced fear acquisition of this group.

Keywords:

Chronotype; Fear acquisition; Affective learning; Eveningness; Trait anxiety related disorders; Virtual reality; Skin conductance.

1. Introduction

Circadian rhythms are involved in the regulation of numerous aspects of physiology and behaviour, including locomotor functions, sleep/wake cycles, hormonal rhythms, metabolism, and cognitive functions (Foster and Kreitzman, 2014), such as attention (Valdez, 2019) and memory (Gerstner and Yin, 2010). For instance, a time-of-day effect on overall response inhibition in the Sustained Attention to Response Task was reported, where accurate inhibition of responses in no-go trials was lower during early morning and the night as compared to the afternoon and evening times (Manly et al., 2002).

In this context, chronotype causes interindividual differences regarding both, the preferred time of day to perform activities and sleep timing (Lara et al., 2014). Three main circadian typologies or chronotypes have been identified: morning-type ('larks'), intermediate-type ('neutral'), and evening-type ('owls') (Adan et al., 2012; Di Milia et al., 2013). According to this general classification, differences in task performance as a function of both, chronotype and time-of-day, have been described. Lara et al. (2014) demonstrated that executive task performance reaches its optimal level when individuals are tested at the optimal time of day according to specific circadian profiles (i.e., the morning-type group tested in the morning, and evening-types tested in the evening). Similar results have been provided by two recent studies exploring school performance (Goldin et al., 2020), and the interplay between cognitive performance (i.e., motor learning, working memory and attention) and brain physiology (Salehinejad et al., 2021).

Overall, the literature in the field provides evidence on how cognitive performance is influenced by time-of-day/chronotype interplay. Nevertheless, knowledge about the role of this interplay on affective processes is limited. Evidence links eveningness to anxiety symptoms in healthy samples (Alvaro et al., 2014; Park et al., 2015) and anxiety disorders in clinical populations (Lemoine et al., 2013). However, a large cohort study (Antypa et al., 2016) focusing on anxiety disorders (social phobia, panic disorder with/without agoraphobia, generalized anxiety disorder) did not detect a relation with eveningness (for a review see also Kivelä et al., 2018). On the other hand, more consistent findings have been reported with regard to depression (e.g., Coleman and Cain, 2019; Gaspar-Barba et al., 2009; Kivelä et al., 2018) and PTSD, with military veterans (Hasler et al., 2013, Hasler et al., 2013) firefighters (Yun et al., 2015), showing increased PTSD symptoms in evening as compared to other chronotypes (for a review see Kivelä et al., 2018).

The classical fear conditioning paradigm offers a clinically relevant model for studying acquisition, treatment, and relapse of fear (Borgomaneri et al., 2020; Lonsdorf and Merz, 2017). This protocol, which is widely used in research on anxiety and related disorders (Marković et al., 2021), allows to study the influence of environmental and intrinsic factors on affective learning processes, namely fear acquisition and fear extinction. To the best of our knowledge, only one study so far however explored the role of chronotype on fear acquisition and extinction in healthy humans. In that study, Pace-Schott et al. (2015) showed that extinction and extinction recall in the morning (7 am–10 am) was better in early, as compared to late chronotypes. Moreover, eveningness predicted anxiety levels (Pace-Schott et al., 2015). The study however focused on two extreme groups and did not test intermediate chronotypes, which makes it difficult to establish whether the reported results reflect improved learning by the morning type or impaired learning by the evening type when learning took place in the morning. Intermediate chronotype individuals are the ideal control group for several reasons. They are most frequent in the population (Partonen, 2015). Moreover, they have a "in between" profile, compared to the morning and evening chronotype regarding their preferred times for waking up and falling asleep (Terman and Terman, 2005), respective physiological parameters such as, for example, body temperature (Horne and Ostberg, 1976), and risk for mental disorders (Hittle and Gillespie, 2018). Building on prior reports of a relationship between eveningness and anxiety/depressive traits (Alvaro

et al., 2014; Park et al., 2015; Lemoine et al., 2013; Coleman and Cain, 2019; Hasler et al., 2013, Hasler et al., 2013) and the study of Pace-Schott et al. (2015), here, we focused on evening chronotypes and addressed prior limitations by testing fear acquisition and extinction in a group of individuals with evening chronotype and in a "control" group of individuals with intermediate chronotype. We focused on eveningness as this chronotype is closely related to poor mental health and we are interested to provide new insights into its relationship with anxiety and related mental disorders such as PTSD (Antypa et al., 2016), for which the association with eveningness is more consistent in the literature (Kivelä et al., 2018). The goal is exploring whether abnormal fear acquisition and/or fear extinction learning is a relevant mechanism for explaining the higher risk for anxiety and/or PTSD in evening chronotype individuals.

According to the results of the study conducted by Pace-Schott and co-workers, we predicted reduced extinction learning in eveningness, as compared to intermediate chronotypes. We also expected to detect higher fear acquisition in evening-type individuals in line with evidence for altered fear acquisition (and extinction) responses in clinical populations associated with eveningness, such as PTSD (Orr et al., 2000; Peri et al., 2000; Blechert et al., 2007). Moreover, we expected that these differences would be larger when late chronotypes would be explored at non-preferred times of the day. A virtual reality Pavlovian fear conditioning-extinction paradigm was used, to provide a realistic, ecologically valid, and immersive scenario (Lucifora et al., 2020; Lucifora et al., 2021a; Lucifora et al., 2021b; Daher et al., 2021; Grasso et al., 2020; Grasso et al., 2019). Lastly, in view of the relationship between evening chronotype and anxiety-related disorders (Alvaro et al., 2014; Park et al., 2015; Lemoine et al., 2013) and the influence of these disorders on classical fear conditioning (Duits et al., 2015), we sought to clarify whether any difference in fear conditioning between the two groups reflected the unique contribution of chronotype or alternatively, could be accounted by interindividual differences in trait anxiety.

2. Methods

2.1. Participants

This study involved a total of 76 participants recruited among students at the University of Messina. The preselection was based according to the Morningness–eveningness questionnaire (MEQ) scores which allows to identify individual chronotypes (see below). The MEQ was administered online via Google form. 26 of the prospective participants were identified as "morning type" and excluded; 10 participants ("intermediate type") were included in a pilot investigation to determine if the conditioning protocol was working as expected; 40 participants (10 males and 30 females, average age of 21.9, SD 3.23) completed the test. The sample was divided into 2 sub-groups: evening chronotype (N = 20, 3 males and 17 females, mean age 22.4, SD 3.08) and intermediate chronotype (N = 20, 7 males and 13 females, mean age 21.5, SD 3.35). 10 participants of each sub-group were tested in the morning (i.e., from 8 am to 10 am), and the other 10 participants in the evening time (i.e., from 8 pm to 10 pm). Informed consent was obtained from all participants before inclusion, and the protocol was approved by the local ethics committee of the Department of Cognitive Science, n. COSPECS_4_2021.

2.2. Instruments

2.2.1. Morningness–eveningness questionnaire (MEQ)

To identify chronotypes, we used the MEQ (Horne and Ostberg, 1976; Mecacci and Zani, 1983), which consists of 19 questions that ask individuals to determine their "feeling best" rhythms, indicate preferred

clock time blocks rather than the actual real time for sleep and engagement in other daily/weekly activities (e.g., physical exercise, tests, work), and assess morning alertness, morning appetite, and evening tiredness. Each question has a score (from 1 – not at all - to 4 - a lot), and the sum score ranges from 16 to 86, with scores below 42 indicating evening chronotype, and scores higher than 58 indicating morning chronotype (Horne and Ostberg, 1976; Tonetti and Natale, 2019). Scores between these values (42–58) correspond to intermediate chronotype. Our sample comprised 20 intermediate and 20 evening chronotype individuals (see Table 1). Because of a technical failure in SCR recording (see below), the final sample comprised 20 intermediate and 19 evening chronotype individuals.

	Intermediate chronotype	**Evening chronotype**	**Statistical comparison**
Age	M = 22.4 y, SD = 3.08	M = 21.5 y, SE = 3.35	p = 0.60
Gender	17 females, 3 males	13 females, 7 males	p = 0.27
MEQ	M = 49.3, SD = 5.23	M = 34.15, SD = 5.36	p < 0.001
STAI-Y2	M = 47.15, SD = 4.08	M = 48.5, SD = 3.59	p = 0.27
STAI-Y2 based anxiety groups	14 intermediate, 6 high scores	12 intermediate, 7 high scores	p = 0.74

Table 1. Demographic characteristics, chronotype (MEQ) and anxiety levels (STAI-Y2) of the intermediate and evening chronotype groups. Statistical comparisons were conducted either with two-tailed *t*-tests or Fisher's exact tests.

2.2.2. State-Trait Anxiety Disorder Inventory

We used the trait anxiety (STAI-Y2) subscale of State-Trait Anxiety Disorder Inventory STAI (Spielberger et al., 1970) to investigate relationships between trait anxiety and fear acquisition and extinction. The scale includes 20 statements for which the volunteers indicate the intensity at present and the frequency with which these occur by a scale ranging from1 to 4. The total score of each scale ranges from 20 to 80 points. The results of the scores were classified according to Mayer et al. (2016) in three types of anxiety levels: low anxiety (<33 points), moderate anxiety (33 to 49 points), and high anxiety (>49 points).

Our final sample included 26 moderate anxiety and 13 high anxiety individuals, similarly distributed across the intermediate and evening chronotypes (see Table 1).

2.2.3. Virtual reality Pavlovian fear conditioning/extinction task

To study fear conditioning and extinction, we created a 3D environment in virtual reality by using the Oculus Rift device. Our experimental setup includes a helmet for virtual reality presentation, the Oculus Rift, equipped with two Pentile OLED displays, 1080 × 1200 resolution per eye, 90 Hz refreshing rate and a 110° field of view. The device includes also features for rotation, position tracking and integrated headphones that provide a 3D sound effect (Grasso et al., 2019). A graphics workstation, equipped with NVIDIA Titan X graphics card, was used to run the simulation, ensuring a

uniform high-resolution rendering of the virtual environment, which was projected to the VR headset. Our VR paradigm consisted of four sessions: habituation, acquisition, and extinction on day 1, and the recall session on day 2. Stimuli of the protocol consisted of two doors of different colors. During the habituation session (duration about 7 min), the participant watched the two different doors (blue and red) for a total of 8 trials (i.e., 4 times the blue door, 4 times the red door) presented in randomized order. During presentation, doors were kept closed for 3 s and opened for 9 s. The inter-trial interval was between 6 and 20 s.

After a short break of 60 s, participants were prompted to start the fear acquisition stage (duration about 10 min) when ready. In the acquisition session, the blue door served as conditioning stimulus (CS+) as it was paired (in 80% of cases, in 8 trials) with a threatening stimulus (i.e., a monster - serving as the Unconditioned stimulus – US) jumping in the direction of the participant and screaming (80 dB) to induce a fear response. Both the duration and timing of appearance of the US were 3 s. The red color door was not paired with the US (CS−: the safety signal). The acquisition phase involved presentation of 10 CS+ trials and 10 CS− trials. The inter-trial interval was between 6 and 20 s. See Fig. 1 for details about a typical sequence for CS– and CS+.

The conditioned fear response was extinguished in the extinction stage, which took place 5 min after the acquisition phase. During this break, participants stayed out of virtual reality. In this session, the blue door (CS+) was no longer accompanied by the jumping and screaming monster. This session consisted of two blocks, early and late extinction (duration about 10 min), involving presentation of 20 trials (10 times the red door and 10 times the blue door), presented in randomized order.

In the recall session, which was administered 24 h after the extinction session, the presentation of the blue door (CS+) was again not followed by the jumping and screaming monster, as in the extinction session. Duration and number of trials were identical to the extinction session.

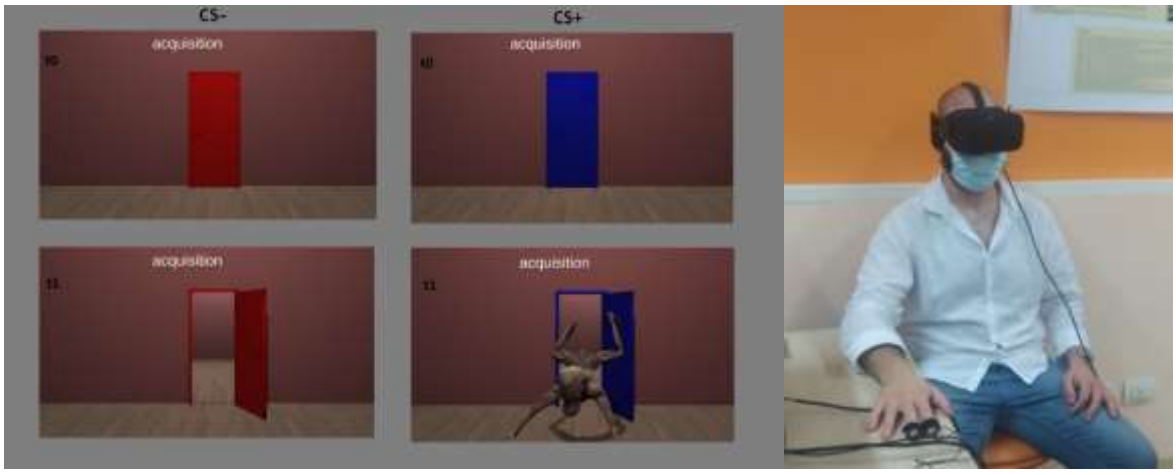

Fig. 1. Left panel. Typical sequences related to CS– (Safe condition) and CS+ (fear condition) in the acquisition session. T0 and t1 indicate the event sequence for CS– and CS+ conditions. Right panel: a typical experimental session recording SCR via the e-Sense device during the virtual reality fear conditioning/extinction task.

2.3. Measurements

2.3.1. Skin conductance

Skin conductance level (SCL) was measured via eSense (Mindfield Biosystems, Inc., Berlin, Germany) on a Xiaomi Redmi note 8 t, with electrodes attached to the middle and index finger with Velcro straps

(see Fig. 1). The electrodes were connected to the Xiaomi Redmi note 8 t using the audio connection input. eSense acquired data at a sampling rate of 5 Hz and the data were exported via email using csv files. The skin conductance response (SCR) to the CS+ and CS− was calculated in microSiemens (μS) by subtracting the mean SCL during the 2 s prior to stimulus onset from the maximum SCL during the 12 s stimulus presentation duration.

2.3.2. Fear stimulus rating

At the end of each session the participants were asked to rate "how scary were the presented stimuli (i.e., red and blue door)". Ratings were provided by using a 10 points Likert scale, where 1 indicates not scary at all and 10 indicated extremely scary.

2.4. Procedure

2.4.1. Timeline of the experimental procedure

The experiment was conducted over two consecutive days, using the same experimental context (time of day, room). Participants were allocated to one of two different times of day with respect to task performance (morning, evening). After the participants had given informed consent, they completed the STAI-Y2 questionnaires. Next, participants were connected to the GSR Amp, (eSense), and two ring-shaped skin conductance electrodes were placed over the middle and index fingers of the right hand. Then, the virtual reality helmet (Oculus Rift) was placed on the head, and fear conditioning/extinction task was conducted. On day two, all participants underwent a repetition of the extinction stage to monitor fear extinction recall. Finally, participants were debriefed.

2.4.2. Data analysis

Statistical comparisons of age, gender, STAI-Y2 and MEQ scores were conducted via two-tailed t-tests or Fisher's exact tests. The SCR amplitude was determined off-line by subtracting the baseline of 2 s prior to CS presentation from the highest skin conductance level during each CS presentation. This was done for each individual value of each participant. For SCR data analysis, a square root transformation was applied to the SCR data to reduce variability, in accordance with previous studies (e.g., van't Wout et al., 2016; Vicario et al., 2020; Ney et al., 2021).

We tested the effects of the factors chronotype, time-of-day of performance on the two key dependent variables, i.e., the SCR amplitude and subjective fear stimulus rating scores, by separate 2 (Chronotype: evening, intermediate) × 2 (Time-of-day: morning, evening) × 4 (Session: habituation, acquisition, extinction, recall) × 2 (Stimulus: CS+, CS–) mixed factors ANOVAs. Partial-eta squared (ηp2) and Cohen's d were calculated as effect sizes. Post-hoc analyses using the Tukey test to correct for multiple comparisons were conducted in case of significant results of the ANOVAs. In a series of control analyses we additionally tested the influence of chronotype and trait anxiety level on SCR differences (CS+ minus CS–). In this case, Bonferroni-corrected independent t-tests were applied. Moreover, we tested the influence of anxiety by treating STAI-Y2 scores as a dichotomous variable with two levels (intermediate and high anxiety) and entered this variable as a between-subject factor in an Anxiety x Chronotype x Time-of-day x Session x Stimulus ANOVA. Lastly, to further check the influence of anxiety on our results, we treated STAI-Y2 scores as a continuous variable and entered it in Pearson correlation and partial correlation analyses conducted with SCR differences (CS+ minus CS–) as second variable. and testing the influence of MEQ and STAI-Y2 scores. A critical alpha level of $\alpha = 0.05$ served

as significance threshold for all tests. Statistical analysis was performed using STATISTICA (StatSoft. Inc., Tulsa, OK, USA) version 12.0.

3. Results

3.1. Demographics

Comparisons between the intermediate and evening chronotype groups revealed only the expected difference of the MEQ scores ($p < 0.001$; Table 1), but no differences for age, gender, or trait anxiety levels (all $p \geq 0.27$; see Table 1).

3.2. Fear stimulus ratings

The Chronotype × Time-of-day × Session × Stimulus ANOVA conducted on fear stimulus ratings showed significant main effects of Session ($F_{3,105} = 18.67$, $p < 0.001$, $\eta_p^2 = 0.35$) and Stimulus ($F_{1,35} = 25.09$, $p < 0.001$, $\eta_p^2 = 0.42$), which were qualified by a significant Session x Stimulus interaction ($F_{3,105} = 56.81$, $p < 0.001$, $\eta_p^2 = 0.62$). Post-hoc analyses showed that ratings of CS+ (mean rating values ± standard deviation: 7.3 ± 2.2) were higher than ratings of CS– (3.2 ± 2.6; $p < 0.001$, Cohen's d = 1.46) only during acquisition, whereas no significant differences were found in the remaining sessions (all $p \geq 0.13$, all Cohen's $d \leq 0.45$; Fig. 2).

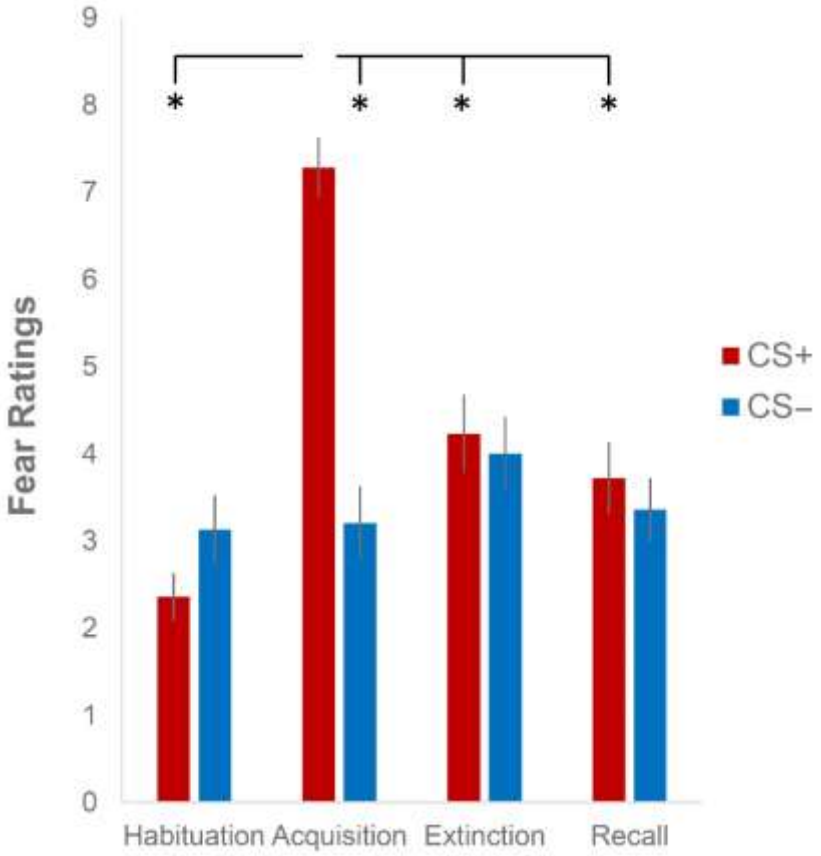

Fig. 2. Mean fear ratings of CS+ and CS− in the four sessions. The figure shows the Session x Stimulus interaction. (*) Indicates significant post-hoc differences between CS+ and CS− stimuli in the acquisition session and between CS+ stimulus across the four sessions. See main text for further details. Vertical bars denote ± standard error of means.

Focusing on CS+ ratings, we observed higher values in the acquisition (7.3 ± 2.2) than in the other conditions (range: 2.4–4.2; all $p < 0.001$, all Cohen's $d \geq 1.20$); SCR in the extinction session (4.2 ± 2.8) showed higher values than in the habituation (2.4 ± 1.7; $p < 0.001$, Cohen's $d = 0.71$), but not in the recall session (3.7 ± 2.6; $p = 0.62$, Cohen's $d = 0.28$). Furthermore, we observed higher ratings of CS+ during recall than during habituation ($p < 0.001$, Cohen's $d = 0.49$). In contrast, no consistent differences in the ratings of CS– were observed across sessions (range: 3.1–4.0; all $p \geq 0.054$, all Cohen's $d \leq 0.41$).

No effect of the factors Chronotype or Day-of-time were detected in the ANOVA (all $F \leq 1.60$, all $p \geq 0.16$, all $\eta_p^2 \leq 0.05$), thus suggesting that greater fear for CS+ than for CS– during acquisition was similarly induced in the morning and at evening sessions, and across chronotype groups.

3.3. Skin conductance response

Table 2 shows the results of the Chronotype × Time-of-day × Stimulus × Session ANOVA on SCR. The results show a significant main effect of Chronotype ($F_{1,35} = 10.99$, $p = 0.002$, $\eta_p^2 = 0.24$), with higher SCR in the evening group (mean ± standard deviation: 0.22 µS ± 0.10), as compared to the intermediate group (0.13 µS ± 0.08); a significant main effect of Stimulus ($F1,35 = 8.89$, $p = 0.005$, $\eta_p^2 = 0.20$), with higher SCR for CS+ (0.19 µS ± 0.11), as compared to CS– (0.15 µS ± 0.10), and a significant main effect of the factor Session ($F_{3,105} = 47.72$, $p < 0.001$, $\eta_p^2 = 0.58$), which was qualified by the significant two way interaction Session x Time of day ($F_{3,105} = 5.83$, $p = 0.001$, $\eta_p^2 = 0.14$), showing higher SCR during acquisition in both, morning (0.39 µS ± 0.10) and evening (0.27 µS ± 0.17) sessions relative to the other sessions (mean range: 0.7–0.16 µS: all $p \leq 0.022$, all Cohen's $d \geq 0.86$), and moreover, higher SCR during acquisition in the morning than at evening ($p = 0.043$, Cohen's $d = 0.61$). This higher SCR for acquisition in the morning did not differ between the two chronotypes and across stimulus types, as no higher order interactions involving these factors were detected (all $F \leq 1.33$, all $p \geq 0.27$, all $\eta_p^2 \leq 0.04$).

The two-way interactions of the factor Session with the factors Chronotype and Stimulus were significant (all $F_{3,105} \geq 4.74$, all $p \leq 0.004$, all $\eta_p^2 \geq 0.12$), and, importantly, these interactions were qualified by the significant higher-order Session x Stimulus x Chronotype interaction ($F_{3,105} = 7.43$, $p < 0.001$, $\eta_p^2 = 0.18$; Fig. 3). Post-hoc analyses showed larger SCR to CS+ than to CS– in the acquisition session of both, intermediate (CS+: 0.30 µS ± 0.21; CS–: 0.16 µS ± 0.13; $p = 0.001$; Cohen's $d = 1.04$) and evening chronotypes (CS+: 0.60 µS ± 0.29; CS–: 0.26 µS ± 0.17; $p < 0.001$; Cohen's $d = 1.43$), suggesting that the VR protocol was successful with respect to fear conditioning in both groups of participants. For both groups, SCR to CS+ in the acquisition session was larger than in the other sessions (all $p < 0.001$, all Cohen's $d \geq 0.97$). Remarkably, SCR to CS+ was larger in evening than in intermediate chronotypes during acquisition ($p < 0.001$; Cohen's $d = 1.18$). No other between group differences or differences between CS+ and CS– were detected across sessions (all $p \geq 0.71$).

No other effects were observed in the ANOVA (all $F \leq 2.77$, all $p \geq 0.10$, all $\eta_p^2 \leq 0.07$; see Table 2), including the 4-way interaction ($F_{3,105} = 1.33$, $p = 0.27$, $\eta_p^2 = 0.04$), suggesting that the increased response to CS+ observed in the evening as compared to intermediate chronotypes was similar in morning and evening sessions

Effect	F statistics	P-level	Effect Size
Time of day	$F_{1,35} = 0.13$	$p = 0.718$	$\eta_p^2 < 0.01$
Chronotype	$F_{1,35} = 10.99$	$*p = 0.002$	$\eta_p^2 = 0.24$
Time of day × chronotype	$F_{1,35} = 2.77$	$p = 0.105$	$\eta_p^2 = 0.07$
Session	$F_{3,105} = 47.72$	$*p < 0.001$	$\eta_p^2 = 0.58$
Session × time of day	$F_{3,105} = 5.83$	$*p = 0.001$	$\eta_p^2 = 0.14$
Session × chronotype	$F_{3,105} = 4.74$	$*p = 0.004$	$\eta_p^2 = 0.12$
Session × time of day × chronotype	$F_{3,105} = 1.31$	$p = 0.276$	$\eta_p^2 = 0.04$
Stimulus	$F_{1,35} = 8.89$	$*p = 0.005$	$\eta_p^2 = 0.20$
Stimulus × time of day	$F_{1,35} = 0.96$	$p = 0.334$	$\eta_p^2 = 0.03$
Stimulus × chronotype	$F_{1,35} = 1.41$	$p = 0.242$	$\eta_p^2 = 0.04$
Stimulus × time of day × chronotype	$F_{1,35} = 1.60$	$p = 0.214$	$\eta_p^2 = 0.04$
Session × stimulus	$F_{3,105} = 39.42$	$*p < 0.001$	$\eta_p^2 = 0.53$
Session × stimulus × time of day	$F_{3,105} = 1.10$	$p = 0.352$	$\eta_p^2 = 0.03$
Session × stimulus × chronotype	$F_{3,105} = 7.43$	$*p < 0.001$	$\eta_p^2 = 0.18$
Session × stimulus × time of day × chronotype	$F_{3,105} = 1.33$	$p = 0.267$	$\eta_p^2 = 0.04$

Table 2. Statistical effects observed in the Chronotype × Time-of-day × Session × Stimulus ANOVA on SCR. (*) Indicates significant effects.

3.4. Control analyses

The most relevant finding of the former analyses was that chronotype affects the physiological expression of fear conditioning during the acquisition phase, with larger SCRs to CS+ in the evening relative to intermediate chronotypes (Fig. 3).

These results were further investigated in three analyses. First, we carried out a series of Bonferroni-corrected independent t-tests computed on SCR differences (CS+ minus CS–) which showed that in the acquisition session there were larger SCR differences in evening chronotypes (0.34 µS ± 0.24) relative to intermediate chronotypes (0.14 µS ± 0.13; $p = 0.009$; Cohen's $d = 1.05$), whereas there were no differences between groups in the other sessions (all $p \geq 0.27$), thus further confirming the results of the main analysis. Second, as expected (e.g., Silva et al., 2020), MEQ and STAI-Y2 scores were weakly but significantly related ($r = -0.38$, $p = 0.016$). Therefore, in the further analyses we aimed to ensure that the influence of chronotype on fear acquisition was not merely mediated by trait anxiety. Based on STAI-Y2 scores, we thus assigned participants to intermediate and high anxiety groups (see Table 1) and introduced this additional between-subject factor in the ANOVA. In this second analysis, all statistical effects observed in the main analysis were confirmed, including the critical Chronotype × Session × Stimulus interaction ($F_{3,93} = 6.07$, $p < 0.001$, $\eta_p^2 = 0.16$), showing that during acquisition there was a larger SCR to CS+ in the evening relative to the intermediate chronotypes ($p < 0.001$), but no other significant between group differences emerged (all $p \geq 0.84$). The main effect of Anxiety, and most interactions including anxiety were not significant ($F \leq 2.25$, all $p \geq 0.14$, $\eta_p^2 \leq 0.07$), except for the interaction Anxiety × Session × Stimulus ($F_{3,93} = 3.85$, $p = 0.012$, $\eta_p^2 = 0.11$). Post-hoc analyses showed that during acquisition SCR differences between CS+ and CS– were present in both anxiety

groups (all $p \leq 0.022$, Cohen's $d \geq 0.92$), and did not differ between groups (all $p \geq 0.42$). This was further confirmed by Bonferroni-corrected independent t-tests on SCR differences (CS+ minus CS–) showing no difference between intermediate and high anxiety groups in any session (all $p \geq 0.15$). Thus, although anxiety exerted some influence on SCR, this influence was weaker than, and independent from, the influence exerted by chronotype.

Third, we investigated the pattern of correlations between MEQ, STAI-Y2, and SCR differences in the acquisition session. Further confirming the main analyses, we found that MEQ scores were associated with the magnitude of SCR differences ($r = -0.32$, $p = 0.025$); in contrast, STAI-Y2 scores were not significantly associated with SCR differences ($r = -0.21$, $p = 0.20$). Moreover, the relationship between MEQ and SCR differences remained significant while controlling for STAI-Y2 as shown by partial correlation ($r = -0.44$, $p = 0.005$). All in all, these findings provide evidence that chronotype has a clear impact on fear acquisition and this effect is not accounted for by anxiety levels.

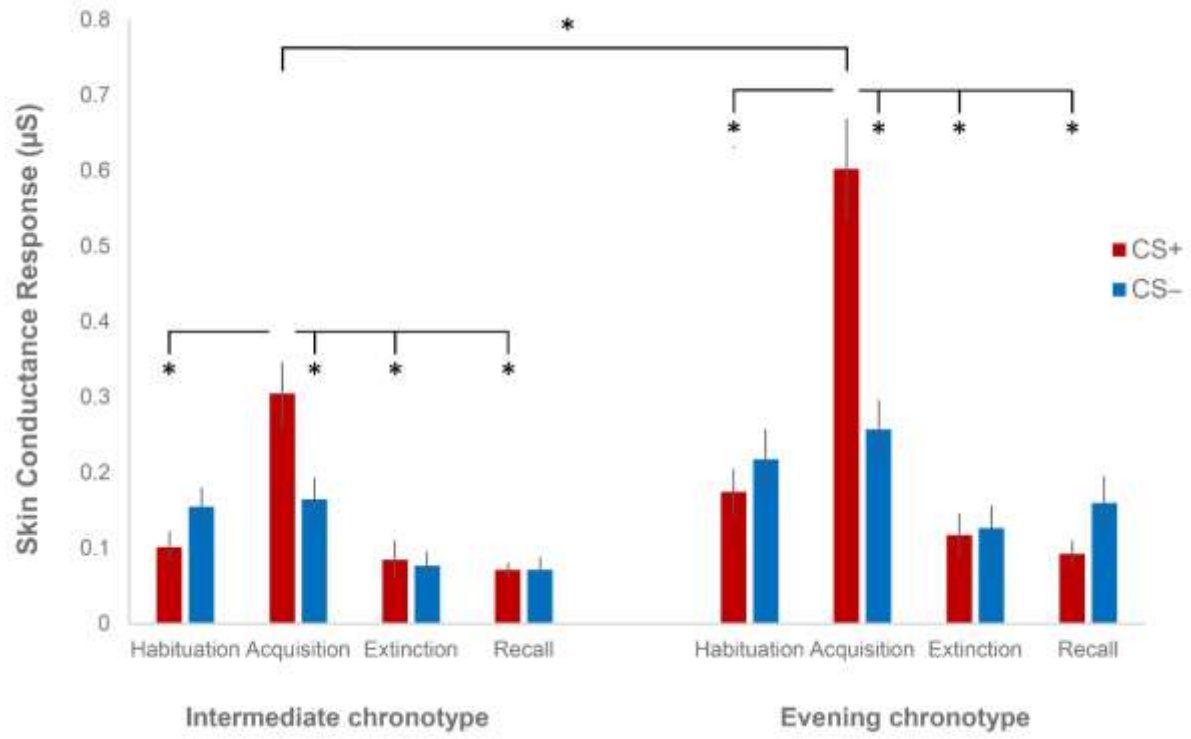

Figure 3. Mean SCR to CS+ and CS– across sessions in the intermediate and evening chronotype groups. The figure shows higher SCR to CS+ compared to CS– in the acquisition session of both groups. Moreover, it shows that in the acquisition session there was larger SCR to CS+ in evening chronotype as compared to intermediate chronotype individuals. (*) indicates significant post-hoc differences, see main text for further details. Vertical bars denote ± standard error of means.

4. Discussion

We tested the influence of time-of-day and chronotype on fear acquisition and extinction using a virtual reality affective learning paradigm. We wanted to clarify the specific role of evening chronotype, which is considered a risk factor for several mental disorders, including PTSD (Kivelä et al., 2018). Hence,

we tested behavioural and physiological response in a Pavlovian fear conditioning-extinction paradigm, which is a valid and widely used translational model for the experimental investigation of mechanisms underlying pathological fear and anxiety (Borgomaneri et al., 2021; Milad and Quirk, 2012; Vervliet and Raes, 2013). In this way, we tested whether abnormal fear acquisition and/or fear extinction learning characterize individuals with evening chronotype, thus highlighting a possible mechanism explaining the higher risk for anxiety and/or PTSD associated with evening chronotype.

Overall, the results show the expected fear conditioning-extinction response pattern in intermediate-chronotype and evening-chronotype groups of participants – with higher SCR for CS+ trials, compared to CS− trials, in the acquisition session, and no difference in habituation, extinction learning and recall sessions. Importantly, this effect was independent from chronotype or time-of-day interplay during fear acquisition. Fear ratings during acquisition were higher for CS+, compared to CS−, in both intermediate and evening chronotype individuals. These results confirm the validity of the proposed VR fear conditioning/extinction protocol as tool to study fear learning and extinction in combination with monitoring of the neurovegetative response of participants. Our results support prior evidence linking the evening chronotype with a higher risk for anxiety disorders (Alvaro et al., 2014; Park et al., 2015), and PTSD (e.g., Hasler et al., 2013, Hasler et al., 2013; Yun et al., 2015). As predicted, the between group analysis shows a higher fear acquisition response in evening chronotype individuals, compared to intermediate chronotype participants. Furthermore, the higher the MEQ score (indicative for less eveningness), the lower was the neurovegetative response (SCR signal) to the conditioned stimulus (CS+) in the acquisition session. Importantly, this pattern of response, as well as the general learning process across the different sessions, was not modulated by time-of-day of performance, nor by the time-of-day/chronotype interplay. Notably, chronotype correlated with anxiety, suggesting that trait anxiety is larger in eveningness. However, control analyses show that the influence of anxiety does not account for the effect of chronotypes on fear conditioning. Scientific and clinical implications of these results are discussed in the following paragraphs.

4.1. Evening chronotype enhances the neurovegetative response in fear conditioning

In line with our hypothesis, evening chronotype was associated with a higher neurovegetative response (SCR) to the conditioned stimulus in the fear acquisition session, as compared to the intermediate chronotype. This result is novel since it was not reported in the only previous investigation in the field (Pace-Schott et al., 2015). On the other hand, no between group difference was revealed for the explicit fear measure, the fear rating task. This suggests that circadian rhythm may primarily modulate the neurovegetative component of the fear acquisition response.

Pace-Schott et al. (2015) reported that extinction and recall were more effective in morning compared to evening chronotypes. However, these data do not clarify the question if morning chronotypes perform better, or evening chronotypes worse compared to intermediate chronotype controls, because in that study such a control group was not included. Our study helps to clarify this point, as the absence of differences between our samples in the extinction and recall sessions suggests that fear extinction and recall may be selectively improved by morningness (Pace-Schott et al., 2015). One reason for the performance difference observed in the present study, namely fear acquisition, might be that eveningness mediates learning of emotionally negative stimuli. This is in line with evidence for negative emotional biases in late chronotype individuals (e.g., Horne and Norbury, 2018a). Accordingly, one may speculate that eveningness may facilitate learning of negative outcomes and morningness the learning of positive outcomes, although future work is needed to test this hypothesis.

Time-of-day/chronotype interplay does not modulate the neurovegetative response of affective learning or subjective ratings of fear.

Affective learning, as explored via Pavlovian fear conditioning and extinction processes (Lipp and Purkis, 2005), was not modulated by the time-of-day in our study, as well as by the time-of-day/chronotype interplay. We observed that during acquisition, SCR signals recorded in morning sessions were larger than those obtained in evening sessions, but this general effect did not depend on chronotype, or the specific stimulus being presented. Indeed, we found a stronger neurovegetative (SCR) response during fear acquisition in the evening-chronotype independently from time-of-day. However, a respective chronotype/time-of-day interplay might take place in morning-type individuals, as described by Pace-Schott et al. (2015).

The absence of a time-of-day/chronotype interplay in the affective learning task used in the present study contrasts with recent results on motor learning (Salehinejad et al., 2021), where performance was best when individuals were tested during the optimal time of day according to their specific circadian profiles. Substantial difference between these types of learning might explain the different results. Specifically, fear learning might show little or no sensitivity to time-of-day for evolutionary reasons, because of its central relevance for the survival of individuals (i.e., being responsive to danger at any time of the day can be essential for survival).

Moreover, emotional, and affective processing are central in fear learning which was among those variables that showed converging outperformance in late chronotypes (Salehinejad et al., 2021). It is thus possible that cognitive and emotional systems respond differently to chronotype-related time of day.

4.2. Evening chronotype and enhanced fear acquisition: implication for anxiety and PTSD

In line with a recent work (Silva et al., 2020), we found a negative correlation between STAI-Y2 and the MEQ score. This may indicate a higher risk for anxiety and related syndromes in the evening chronotype, in line with previous investigations (for a review see Kivelä et al., 2018). For instance, patients with a diagnosis of anxiety disorder exhibit greater evening preference than those without such a diagnosis (e.g., Fares et al., 2015). Similar results were found in patients affected by PTSD (Yun et al., 2015; Hasler et al., 2013, Hasler et al., 2013).

According to earlier reports documenting a stronger CS+ response in PTSD (Grillon, 2002) and in individuals with high trait anxiety (Indovina et al., 2011), the stronger fear acquisition response in the evening chronotype individuals may be a key factor to explain the higher vulnerability to anxiety and related syndromes in eveningness (Kivelä et al., 2018). On the other hand, the absence of a direct association between the STAI-Y2 score, and fear acquisition (as measured via SCR) aligns with the results of some earlier studies (e.g., Torrents-Rodas et al., 2013; Pace-Schott et al., 2015). Together this might mean that abnormal fear acquisition in anxiety may be mediated, at least in part, by chronotype.

The missing difference between intermediate and evening chronotypes in extinction and recall sessions is a further aspect of potential clinical relevance to be discussed. This suggests that eveningness may not be a relevant issue for successful treatment of anxiety and related syndromes via exposure therapy, which aims to promote recovery by boosting extinction.

In summary, the evening chronotype may be a risk factor for anxiety and related disorders by strengthening fear acquisition, a learning process considered to be relevant for these mental disorders, although not all investigations support this view (for a review see Pittig et al., 2018).

4.3. How does eveningness affect fear acquisition? Insights from neurobiology

This study did not investigate the brain physiology of participants during Pavlovian fear conditioning and extinction task performance. However, neurobiological studies provide insights on how eveningness could affect fear acquisition.

Stress hormone levels may contribute to explain the current results, although the literature offers mixed results. For example, a recent study (D'Elia et al., 2021) provides evidence for a dysbalanced hypothalamic-pituitary-adrenal (HPA) axis in PTSD, with higher adrenocorticotropic hormone (ACTH) levels in PTSD compared to healthy control, with furthermore a positive correlation between PTSD severity and ACTH (see also D'Elia et al., 2021). Even more interestingly, a similar HPA alteration pattern was reported by Lucassen et al. (2013) in evening chronotype individuals. However, there also studies providing evidence for lower levels of stress hormones in PTSD (e.g., Yehuda et al., 1996), or no differences between patients and controls (e.g., see Meewisse et al., 2007, for a systematic review and meta-analysis). Moreover, evidence regarding the effects of acute stress on fear acquisition is also mixed (Simon-Kutscher et al., 2019; Shors, 2001; see Raio and Phelps, 2014 for a review). Therefore, the potential relevance (if any) of stress hormones to explain the higher fear acquisition in evening chronotype individuals, as reported in our study, is probably linked to a long-term effect of stress on neuroplasticity of brain structures involved in fear acquisition (see Marković et al., 2021 for a review).

For brain areas involved in this effect, it is well known that the amygdala, anterior cingulate cortex (ACC) and hippocampus are involved in fear acquisition (Myers and Davis, 2007; see also Vicario et al., 2019; Marković et al., 2021, Vicario et al., 2022 for discussion). A positive correlation between SCR and amygdala activation was shown during fear acquisition (MacNamara et al., 2015; Furmark et al., 1997). Moreover, SCR responses to the CS+ were positively correlated with thickness of the dorsal anterior cingulate cortex (Milad et al., 2007). Furthermore, amygdala overactivation is considered a neural hallmark of anxiety and PTSD (e.g., Rauch et al., 2000; Etkin and Wager, 2007). Critically, a recent work (Horne and Norbury, 2018b) reported enhanced amygdala activity, and reduced amygdala-ACC connectivity to fearful faces in the evening chronotype. In summary, the literature discussed in this paragraph provides insights to explain enhanced fear acquisition in evening chronotype individuals and that should be directly tested in future work.

4.4. Limitations

Some limitations of this study should be mentioned. First, sample size was small. However, the large effect size of the most relevant results (i.e., the between group difference in physiological response to CS+ in the acquisition session) suggest a robust outcome, with a low risk of a type I error (i.e., the risk of a false positive). On the other hand, we found no evidence that such effect was influenced by time of day. While we do not rule out that more powered studies could detect such influence, our data suggests this influence to be small at the best.

A second limitation refers to the gender disbalance of the study, with a higher number of female participants. However, there were no gender differences between the evening and intermediate chronotype groups. Therefore, gender cannot account for the different physiological reactivity of the two groups.

Third, we did not include morning type participants. To test our hypotheses, we specifically focused on evening chronotype and intermediate chronotype. However, future studies could include morning types in order to provide a more complete picture of the impact of different chronotype on fear conditioning.

Finally, adding brain physiological data would have helped to clarify underlying mechanisms, and should be the topic of future research.

5. Conclusion and perspectives

This study provides new insights about the influence of circadian rhythms on cognitive and affective processes by showing higher fear acquisition in evening chronotype individuals. This result has clinical implications as it suggests that the higher vulnerability of the evening chronotype to anxiety and related disorders may be mediated by altered fear acquisition. Moreover, the chronotype-dependency of fear might help to explain inter-individual variability of fear acquisition in the Pavlovian fear conditioning paradigm (see Marin et al., 2020, for an overview).

Prospectively, it would be interesting to investigate the role of evening chronotype for learning of other emotionally negative associations - e.g., associations of unconditioned stimuli with disgusting and sad emotions. This may help to explain the relevance of the evening chronotype as a risk factor for further mental disorders such as obsessive-compulsive disorder and depression (Cox et al., 2018; Gaspar-Barba et al., 2009; Kivelä et al., 2018). Disgust and sadness are considered as core symptoms of these clinical conditions (Arnone et al., 2012; Montag et al., 2017; Vicario et al., 2017; Mouchet-Mages and Baylé, 2008) and in further support of this hypothesis, preliminary evidence suggests difficulties of inhibiting acquired disgust in OCD (Armstrong and Olatunji, 2017), and increased recognition of sad facial expressions in evening chronotype individuals (Horne et al., 2017).

CRediT authorship contribution statement

Study concept and design: Chiara Lucifora, Carmelo Mario Vicario, Giorgio Mario Grasso, Alessandra Falzone, Alessio Avenanti; Mohammad Ali Salehinejad; Acquisition of data: Chiara Lucifora, Giovanni D'Italia, Mauro Sortino; Analysis and interpretation of data: Alessio Avenanti, Carmelo Mario Vicario, Michael A. Nitsche. Drafting of the manuscript Chiara Lucifora, Alessio Avenanti, Carmelo Mario Vicario, Michael A. Nitsche, Mohammad Ali Salehinejad; Alessandra Falzone. Critical revision of the manuscript for important intellectual content: Alessio Avenanti, Carmelo Mario Vicario, Michael A. Nitsche. All authors read and approved the final manuscript.

Role of the funding source

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (protocol number 316803389 - SFB 1280) awarded to M.A.N.; grants from the Ministero Istruzione, Università e Ricerca, Italy (protocol number 2017N7WCLP) and Bial Foundation (protocol number 347/18) awarded to A.A.; and a grant from the Bial Foundation (protocol number 160/18) awarded to C.M.V.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

We thank Abed Hadipour Lakmehsari for technical support with data extraction.

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