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Parametric exploration of the fear-inhibited light reflex

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Abstract

The effect of various parameters on the mediation of the fear-inhibited light reflex was examined. The light reflexes of 16 healthy men were measured across four light probe intensities, either in the presence of white noise alone or when the white noise was associated with the threat of either an electric shock or an acoustic sound blast. The white noise alone did not affect the light reflex amplitude. Both types of threat were subjectively anxiogenic and inhibited the light reflex across all light probe intensities, the threat of shock being more potent than the threat of sound blast. Importantly, the effect of either type of threat on the light reflex amplitude was found to increase with increasing light probe intensity, suggesting that brighter light probes may become more relevant motivationally in the threat condition, thus attracting greater allocation of attentional/cognitive resources.

Descriptors: Anxiety, Conditioned fear, Anxiety models, Light reflex, Pupil, Healthy volunteers

The startle reflex is a fast defensive response with the likely purpose of facilitating the flight reaction and protecting the organism from a sudden threat. In both animals (Davis, 1992) and humans (Grillon & Davis, 1997; Hamm, Greenwald, Bradley, & Lang, 1993; Lipp, Sheridan, & Siddle, 1994) the startle reflexes elicited during an aversive conditioned stimulus (CS) are potentiated relative to those elicited during a nonreinforced CS. Thus, the startle reflex has become an important measure of conditioned fear responses in humans, with the particular advantage that, as opposed to the traditional conditioned skin conductance response, it measures aversive learning rather than nonspecific arousal (Hamm & Vaitl, 1996; Lipp et al., 1994). Other startle studies in humans focus more on the expression rather than the acquisition of fear and find consistent startle potentiation during anxious anticipation following verbal threat (Grillon, Ameli, Woods, Mericangas, & Davis, 1991; Patrick & Berthot, 1995); this paradigm has been fruitfully exploited in the study of human anxiety (Grillon & Baas, 2003). The startle reflex has also become a unique measure in the study of emotion (Lang, Bradley, & Cuthbert, 1990) as it importantly distinguishes between emotions with negative and positive valence with startle facilitation and inhibition, respectively (for a review, see Bradley, Cuthbert, & Lang, 1999); the latter is a less robust phenomenon, however (Jackson, Malmstadt, Larson, & Davidson, 2000).

The dynamic light reflex possesses several features that make it another potentially useful tool in the investigation of normal and abnormal emotional and attentional processes, as a paradigm alternative or complementary to that of the startle reflex. The dynamic light reflex is a homeostatic parasympathetic reflex and consists of a brisk and transient contraction of the smooth iris sphincter muscle in response to rapid increments in light flux to the retina, thus reflecting the amount of light captured by the eye. Similar to the startle reflex, the light reflex is a primitive, cross-species automatic/reflexive response, not primarily influenced by intentional control, and is mediated by a subcortical mesencephalon-based, simple neural circuit. Important synapses include the retinal ganglion cells, the olivary pretectal nucleus, the parasympathetic Edinger-Westphal nucleus, the ciliary ganglion, and the sphincter iris muscle (Gamlin, Zhang, & Clarke, 1997; Kardon, 1998). The light reflex can be elicited and recorded accurately in the laboratory using light probe stimuli, by means of computerized infrared television pupillometry (Loewenfeld, 1999).

Using a verbal threat paradigm, we have shown that the amplitude of the pupillary light reflex is reduced when the light probe is presented after a warning cue (e.g., a tone) that has been previously verbally associated with an aversive electric shock. These changes in pupillary activity are accompanied by increases in subjective alertness and anxiety, and, importantly, light reflex amplitude was shown to correlate negatively with subjective anxiety (Bitsios, Szabadi, & Bradshaw, 1996; Bitsios, Szabadi, & Bradshaw, 2002). This phenomenon was termed "fear-inhibited light reflex" and the threat-induced decrease in light reflex amplitude was proposed as a potential laboratory model for human anxiety (Bitsios et al., 1996).

Within a broad theory of emotional responses, the startle reflex is conceptualized as a protective/defensive reflex, primed with a matching aversive ongoing emotional state (Lang et al., 1990). By the same token, light reflex inhibition may be mediated by the mismatch between an aversive ongoing emotional state (e.g., anxious anticipation in the threat condition) and the homeostatic nature of the light reflex. Threat-induced light reflex

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inhibition can be conceptualized as an adaptive defense response by which the organism manages to achieve maximal visual fields in order to locate the source of threat, at the cost of the fine regulation of retinal illumination, a homeostatic function that under threat conditions becomes less important. Interestingly, during simultaneous recording of the startle and light reflexes, shock anticipation modified both reflexes in the predicted direction (Bitsios, Philpott, Langley, Bradshaw, & Szabadi, 1999). Moreover, the fear-inhibited light reflex, in common with the fear-potentiated startle reflex, was found to be sensitive to the anxiolytic drug diazepam in a dose-dependent manner (Bitsios et al., 1999; Bitsios, Szabadi, & Bradshaw, 1998a), suggesting that a common mechanism may mediate the effect of threat in both cases (Bitsios et al., 1999).

Based on the abundant evidence on the amygdala being the critical structure mediating fear responses (LeDoux, Iwata, Cicchetti, & Reis, 1988), including the fear-potentiated startle reflex in rodents (for reviews, see Davis, Falls, Campeau, & Kim, 1993; Koch & Schnitzler, 1997; Walker, Toufexis, & Davis, 2003) and in humans during verbally instructed threat cues (Phelps et al., 2001), we have argued that this structure, through its connections to the locus coeruleus (Cederbaum & Aghajanian, 1978), drives established direct locus coeruleus (Breen, Burde, & Loewy, 1983; Koss, Cherezghiler, & Nomura, 1984; Loewy, Aranjo, & Kerr, 1973) and/or direct hypothalamic (Koss & Wang, 1972; Saper, Loewy, Swanson, & Cowan, 1976; Szabadi & Bradshaw, 1996) inhibitory projections to the Edinger-Westphal nucleus (the motor center of the reflex), thus inhibiting the light reflex during threat of shock (Bitsios et al., 1996, 1998a, 1999; Bitsios, Szabadi, & Bradshaw, 1998b).

An interesting feature of the light reflex paradigm is that, during the shock anticipation condition, there is also an increase in initial pupil diameter, a sympathetically mediated response (Giakoumaki, Hourdaki, Grinakis, Theou, & Bitsios, 2005) preceding reflex inhibition (Bitsios et al., 1996). Despite the close temporal proximity of the two pupillary changes, there is mounting evidence that the two effects of shock anticipation on the pupil may reflect different central processes. This is likely to be due to the different central autonomic mechanisms underlying the two pupillary responses to threat (Giakoumaki et al., 2005).

In previous fear-inhibited light reflex studies, a low-intensity 500-ms warning tone, which on its own had no effect on the amplitude of the light reflex (Bitsios, Bradshaw, & Szabadi, 2004), was presented 3 s before the light probe and was used as the CS in order to signal the possibility of shock administration. This procedure slightly departs from the procedures used in the startle-potentiation paradigm, however, where startle probes are presented during a constant visual CS (usually a red light) signaling the threat condition (Grillon et al., 1991). In the present study, an attempt to minimize procedural differences between the two paradigms was made by presenting the light probes while low-intensity constant white noise signaling the threat condition was being heard. The pupil is, however, known to dilate in response to sensory stimuli (Loewenfeld, 1958, 1999); therefore, the results may become contaminated by the pupillary reactions to the physical properties of the constant white noise. The first aim of this study was to investigate whether the constant white noise may itself have an effect on initial pupil diameter or on the size of the light reflex amplitude. For this reason, the effect of the presence versus the absence of the white noise alone on the light reflex was examined without any association with fear.

Our previous studies with the fear-inhibited light reflex have uniformly employed a standardized light intensity as the reflex-eliciting probe, while they have employed the (verbal) association of the CS to a potentially painful tactile electric shock. The second aim of this study was to investigate whether the threat-induced pupillary changes were sensitive to manipulations of the light probe intensity or to the sensory modality of the fear-inducing stimulus. The effects of the anticipation of an aversive electrical stimulus (threat of shock) versus those to an adverse acoustic stimulus (threat of sound blast) on the light reflex elicited by four graded probe intensities, were, therefore, examined.

Methods

Participants

The study protocol was approved by the University of Crete Medical School Ethics Committee. Sixteen healthy male volunteers aged 22–31 years (mean $\pm$ *SD*; 24.6 $\pm$ 2.8) were tested between 9:00 and 13:00 hours, following written informed consent. Each received the nominal fee of € 25 for study participation.

Tests and Apparatus

Pupillometry. Recordings took place in a dark, sound-attenuated room. A binocular infrared television pupillometer (PROCYON, P2000D) was used to elicit and record the light reflex in the dark, in previously dark-adapted eyes. The sampling rate of the pupillometer was 25 Hz, spatial resolution >0.05 mm, and accuracy $> \pm 3\%$. The light probes were 200-ms-duration flashes delivered through a light-emitting diode, and they were presented to the subject's right eye as a white uniformly illuminated field of 8° diameter at an apparent distance of 10 m from the subjects' cornea, providing thus "full-field" light stimulation (at four levels of stimulus luminance: 0.35, 5, 50, and 140 cd m^{-2}). The left eye was fixating to a target dot projected at a distance of approximately 10 m. Stimulus presentation was computer controlled, and pupillary measures were digitized and stored for off-line analysis. The parameters studied were initial pupil diameter (i.e., the mean pupil diameter recorded over 500 ms prior to the onset of the light probe) and light reflex amplitude (i.e., the difference between initial pupil diameter and the diameter reached at the trough of the pupillary response to the light probe).

Subjective ratings. The subjects' mood and feelings were self-rated on visual analogue scales (Aitken, 1969; Norris, 1971) on several occasions throughout the session (for details see Procedures). For each subject, the raw values (in millimeters) for each item were weighted by multiplication with their respective factor loading, and the weighted values for each item were then allocated to "alertness" and "anxiety" factors, based upon a principal component analysis (Bond & Lader, 1974). Each factor's average weighed value was entered in the statistical analysis.

Procedures

The experiment consisted of a training session and an experimental session.

Training session. Upon arrival at the laboratory, participants received a detailed description of all procedures including a demonstration of the pupillometer and the shock and white noise generators (see below). Subsequently, in order to familiarize

subjects with pupillometry, they underwent a brief training session (application of a few light flashes in the dark to evoke the pupillary light reflex). Subjects were then exposed to a mild electric stimulus (constant current square pulse 1.5 mA, 50 ms) delivered through disposable silver surface electrodes by a Grass stimulator (SD 9) to the skin over the left wrist median nerve. This stimulus is known to cause minimal or negligible discomfort (Bitsios et al., 1996). Subjects were also exposed to a loud acoustic stimulus (115 dB, 50 ms white noise) delivered via headphones (model TDH-39-P, Maico, Minneapolis, MN) over a 70-dB background white noise generated by a white noise generator (EMG SR-LAB, San Diego Instruments, San Diego, CA). At this point, participants were told that the shock and the sound blast would be 50 times stronger during the experimental session, and, therefore, the discomfort would be greater than the one just experienced. No further demonstration of electrical or acoustic stimuli took place during the training session.

Experimental session. Experimental sessions took place 1 or 2 days following the training sessions, and started with the subjects adapting to dim red illumination using red goggles for 15 min. The experimental recording consisted of three phases. Each phase comprised six identical blocks of four 200-ms light probes (one of each of the four light intensities used) in a pseudorandom order. Therefore, there were 24 light probes per phase and 72 light probes in total. All subjects started with Phase 1, where each block was associated either with the presence or the absence of the white noise (constant, 70 dB, delivered via headphones) in an alternating and counterbalanced fashion. The white noise started 3 s prior to the onset of the block and lasted throughout the duration (20 s) of this block. The light probes were, thus, presented while the white noise was heard. At the end of Phase 1, after a 3-min rest, which served for preparing the skin on the subjects' left wrists and for the application of the electrodes, the headphones were placed again on the subjects' ears. Phases 2 and 3 were then started. The headphones and the electrodes remained fixed throughout the rest of the session.

Phases 2 and 3 were identical in structure to Phase 1; however, white noise presentation was now associated with the possibility of an electric shock (Phase 2) or with the possibility of a sound blast (Phase 3). Phases 2 and 3 comprised, therefore, three threat blocks alternating with three safe blocks each. Half of the subjects started with Phase 2 and half with Phase 3. Subjects who started with a safe block in Phase 2 also started with a safe block in Phase 3, and similarly, subjects who started with a threat block in Phase 2 also started with a threat block in Phase 3. Finally, although the pseudorandom order of presentation of the four light intensities was different between subjects, it was kept identical within subjects across the threat blocks and their respective safe blocks in Phases 2 and 3. Thus, although Phases 2 and 3 and the threat and safe blocks were all counterbalanced and the light flashes were delivered in a pseudorandom order, subjects were examined under the same conditions for both the electric and the acoustic stimuli. The subjects were informed 30 s prior to the onset of each block about the nature of the condition (safe or threat) with which the block was associated.

To reduce the overall length of the session and the ensuing subject fatigue, the interstimulus interval within a block was relatively short and it was kept constant at 5 s. Previous studies have shown that the 75% recovery time (the time required for the pupil to reach 75% of its original size after stimulus offset), a sympathetically mediated response (Smith, 1992), would not exceed 3.5 s and 2.6 s in the safe and the threat conditions, respectively, even following sympathetic outflow reduction by systemic administration of the sympatholytic clonidine (Bitsios et al., 1998b). Each block ended 5 s after delivery of the fourth light flash; thus, the duration of each block was 20 s. To investigate changes in mood and feelings from safe to the next threat condition, the subjects were asked to rate themselves retrospectively, immediately after each safe and threat block, with a mood/feelings battery of Visual Analogue Scales. The interblock interval was 90–120 s, allowing sufficient time for the completion of the ratings. Thus, the experimental session lasted for 40 min (15 for dark adaptation +2 for Phase 1+3 for preparation of skin +10 for Phase 2 +10 for Phase 3).

Instructions to subjects. Following application of the headphones and the electrodes, an electric or an acoustic stimulus was delivered, as in the training session described above, depending on the Phase (2 or 3) of the recording session to which the subject was assigned. It was then emphasized again, as in the training session, that the shock and the sound blast would be 50 times stronger. To convince the subjects, a pseudo-switch on the shock box and the white noise generator were switched to a 50-fold higher shock and sound blast intensity, respectively.

In the safe condition the subjects were instructed to relax and were told that no electric shocks (in Phase 2) or acoustic sound blasts (in Phase 3) would be administered. In the threat blocks of Phase 2, the subjects were instructed to anticipate a total of one to three electric shocks, delivered to their left wrists during the 20-s duration of the block, while the white noise was being heard. In the threat blocks of Phase 3, the subjects were similarly instructed to anticipate a total of one to three acoustic sound blasts delivered by the headphones, during the 20 s of the block that the white noise was being heard. The subjects were blind to the exact number of electric shocks or acoustic sound blasts and to the exact threat block(s) in Phases 2 and 3 that electric shock(s) or sound blast(s) respectively would occur.

Only one mild shock (1.5 mA, 50 ms) and only one acoustic sound blast (115 dB, 50 ms white noise) were delivered at the end of the last threat block in Phases 2 and 3, respectively. The shocks were described by the experimenter as painful stimuli inducing a short-lived localized unpleasant sensation on the wrist. The acoustic sound blasts were described as high intensity, and difficult to tolerate, yet posing no risk for their eardrums. All subjects were, thus, successfully conditioned to become apprehensive in the presence of the white noise (presented in the threat blocks of Phases 2 and 3).

Data Reduction and Analysis

Pupillary data at each of the four graded luminance levels were collapsed across blocks for the four conditions (white noise on/no threat, white noise off/no threat in Phase 1, white noise on/threat of shock in Phase 2 and white noise on/threat of sound blast in Phase 3). Separate repeated measures ANOVAs with Condition (4 levels) $\times$ Luminance (4 levels) as the within-subject factors were used to analyze the pupillary measures (initial pupil diameter and light reflex amplitude). The relationship between initial pupil diameter and light reflex amplitude was examined with an analysis of covariance (ANCOVA). Significant main effects or interactions were followed up using separate ANOVAs with the same factorial design as above, in order to compare (1) the pupillary data in Phase 1 (white noise on/no threat vs. white noise off/no threat) and (2) the pupillary data in

Phases 2 and 3 (white noise on/threat of shock and white noise on/threat of sound blast, respectively) to the baseline conditions in Phase 1 when subjects did not anticipate any aversive stimulation at all and (3) the pupillary data in Phase 2 to the pupillary data in Phase 3. Analyses of the light reflex amplitude data were always followed by ANCOVAs with initial pupil diameter as the covariate in order to examine the relationship between light reflex amplitude and initial pupil diameter.

The safe blocks (white noise off/no threat) in Phases 2 and 3 were used as a second control condition in order to define the individual's response to threat of shock and threat of sound blast, respectively; for each pupillary measure, the within-subject (threat/safe) differences obtained at each one of the four luminance levels in Phase 2 (electric fear stimulus), as well as in Phase 3 (acoustic fear stimulus) were calculated. These within-subject differences were defined as the individual's response to threat of shock and threat of sound blast, respectively. Separate two-way (Fear-Stimulus Type $\times$ Luminance) ANOVAs with repeated measures were used to analyze these data. Finally, the safe blocks in Phases 2 and 3 were also used as a second control condition in order to control for threat effects in the subjective ratings.

These subjective ratings were obtained as described above (see Subjective ratings), and the average weighted values for "alertness" and "anxiety" were entered in the statistical analysis. Data for each rating were collapsed across blocks for the two conditions (threat, safe) and the two stimulus types (electric shock, acoustic sound blast). Two-way repeated-measures analyses of variance with fear-stimulus type (two levels) and condition (two levels) as within-subject factors were used to analyze these data. In the case of a significant interaction, the two stimulus types were compared under each condition with the least significant difference test (criterion, $p < .05$).

All repeated measures with more than two levels (or one degree of freedom) employed the Greenhouse–Geisser epsilon correction. Uncorrected degrees of freedom are reported in this case, with the corrected p values and the epsilon value. Effect sizes (η^2) are also reported.

Results

Initial Pupil Diameter

The initial pupil diameter data in Phase 1 (white noise on/no threat vs. white noise off/no threat) and Phases 2 (white noise on/threat of shock) and 3 (white noise on/threat of sound blast) are shown in Figure 1 (top left panel). An overall repeated-measures ANOVA with condition (4 levels) and luminance (4 levels) as the within-subject factors showed a significant main effect of condition, $F(3,45) = 6.2$, $p = .001$, $\varepsilon = .68$, $\eta^2 = .293$, but no significant main effect of luminance or interaction, Fs < 1.

In Phase 1 (no threat), initial pupil diameter was greater in the presence of white noise but a follow-up ANOVA with condition (white noise on, white noise off) and luminance (4 levels) as the within-subject factors did not reveal a significant white noise main effect, $F(1,15) = 1.8$, $p > .1$. Follow-up two-way ANOVAs comparing the initial pupil diameter data in Phase 2 (threat of shock) to the baseline condition (white noise on/no threat) of Phase 1 showed that the threat of shock increased significantly the initial pupil diameter compared to the baseline, $F(1,15) = 8.98$, $p = .009$, $\eta^2 = .375$. Identical comparisons between the initial pupil diameter data in Phase 3 (threat of sound blast) and the baseline in Phase 1 showed that the threat of blast did not increase the initial pupil diameter, $F < 1$.

ANOVAs with the same factorial design, comparing the initial pupil diameter data in Phases 2 and 3 to the white noise off/no threat baseline condition of Phase 1, yielded identical results. Finally, follow up ANOVAs comparing the initial pupil diameter data in Phase 2 and Phase 3 revealed that the initial pupil diameter was significantly greater with the threat of shock compared to the threat of blast, $F(1,15) = 16.34$, $p = .001$, $\eta^2 = .521$.

Light Reflex Amplitude

Figure 1 (top right panel) shows the group means of the light reflex amplitude, obtained at the four graded luminance levels (each one of them averaged across the three blocks) for the four conditions (white noise on/no threat and white noise off/no threat in Phase 1, white noise on/threat of shock in Phase 2 and white noise on/threat of blast in Phase 3). An overall repeated-measures ANOVA with condition (4 levels) and luminance (4 levels) as the within-subject factors showed significant main effects of condition, $F(3,45) = 47.17$, $p = .001$, $\varepsilon = .71$, $\eta^2 = .759$, and luminance, $F(3,45) = 245.03$, $p = .001$, $\varepsilon = .72$, $\eta^2 = 0.942$, but no significant interaction, $F(9,135) = 1.26$, $p > 0.1$. ANCOVA of the light reflex amplitude data with initial pupil diameter as the covariate revealed a significant effect of the regression in the case of Condition $\times$ Luminance interaction, $F(1,134) = 16.81$, $p < .001$, $\eta^2 = .111$, following which the interaction remained nonsignificant. There was no effect of the regression in the case of condition, $F < 1$, or luminance, $F(1,44) = 3.31$, $p = .075$, $\eta^2 = .070$, and both main effects remain significant at the level of $p < .001$.

Figure 1 (top right panel) shows that light reflex amplitude was not affected by the presence of white noise in Phase 1. Indeed, follow-up ANOVA of the amplitude data in Phase 1 with condition (white noise on, white noise off) and luminance (4 levels) as the within-subject factors revealed an expected light intensity main effect, $F(3,45) = 166.7$, $p < .001$, $\varepsilon = .79$, $\eta^2 = .917$, but not a white noise main effect or interaction, Fs < 1.

Inspection of the amplitude data in Phases 2 and 3 (Figure 1, top right panel) shows clear reductions in light reflex amplitude with threat of shock and threat of blast across all luminance levels. Indeed, follow-up ANOVA comparing the amplitude data in Phase 2 (threat of shock) to the baseline condition (white noise on/no threat) of Phase 1 showed that the threat of shock reduced significantly the amplitude across all luminance levels, $F_{\text{condition}}(1,15) = 67.19$, $p = .001$, $\eta^2 = .817$; $F_{\text{luminance}}(3,45) = 151.62$, $p = .001$, $\varepsilon = .73$, $\eta^2 = .910$; $F_{\text{interaction}}(3,45) = 1.28$, $p > .1$, and the ANCOVA with the initial pupil diameter data as the covariate revealed no relationship between the initial pupil diameter and the amplitude data (all regressions $F < 1.5$). Identical comparisons between the amplitude data in Phase 3 (threat of sound blast) and the baseline condition (white noise on/no threat) in Phase 1 showed that the threat of blast significantly reduced the light reflex amplitude across all luminance levels, $F_{\text{condition}}(1,15) = 47.14$, $p = .001$, $\eta^2 = .759$; $F_{\text{luminance}}(3,45) = 196.32$, $p = .001$, $\varepsilon = .66$, $\eta^2 = .929$; $F_{\text{interaction}}(3,45) = 2.09$, $p > .1$. An ANCOVA with the initial pupil diameter data as the covariate revealed a significant effect of the regression in the case of luminance, $F(1,44) = 4.85$, $p = .033$, $\eta^2 = .099$, but not in the case of condition or Condition $\times$ Luminance interaction, Fs < 1. Following this ANCOVA, the main effect of luminance remained significant at the level of $p < .001$.

All the above comparisons were also carried out using the white noise off/no threat condition of Phase 1 as the baseline, but they are not reported here, as they yielded identical results.

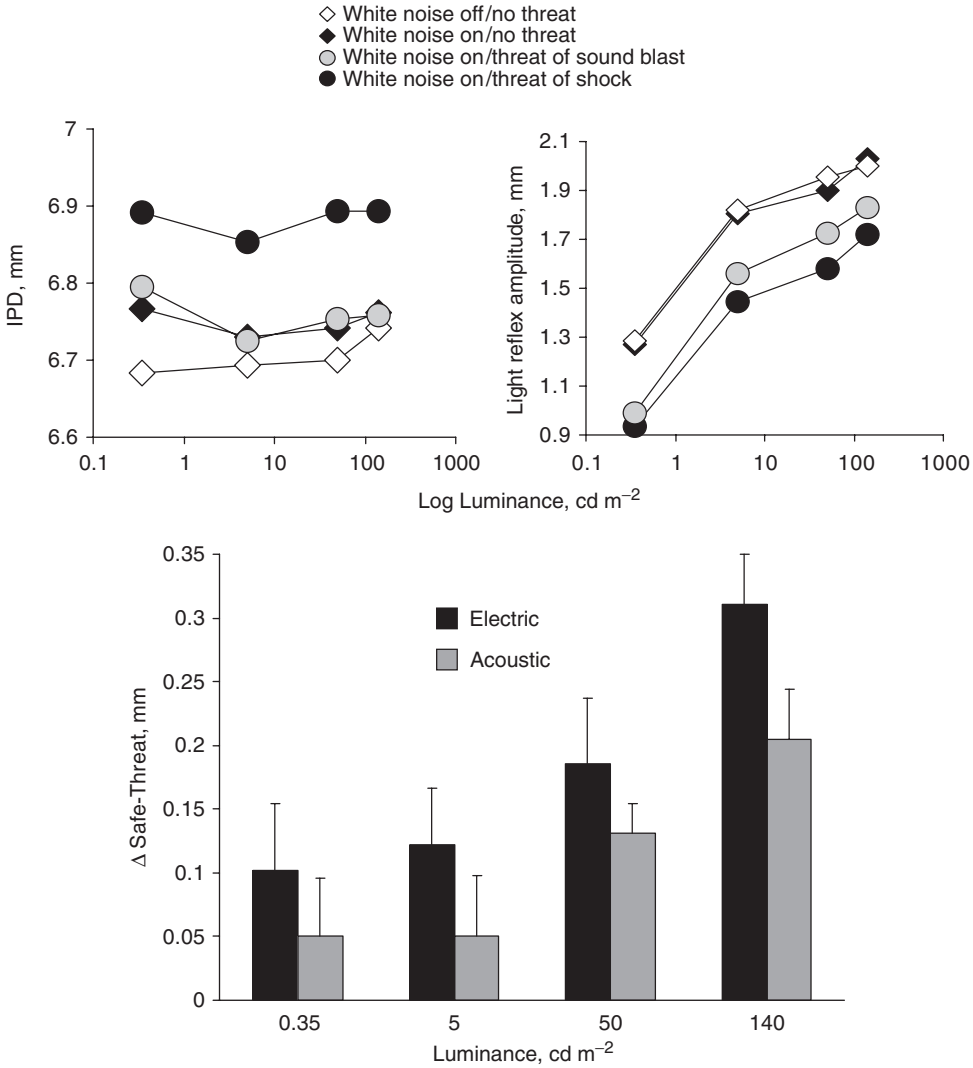

Figure 1. Top: Initial pupil diameter (left) and light reflex amplitude (right) obtained at four graded luminance levels, recorded in Phase 1 (white noise on and white noise off conditions), Phase 2 (anticipation of electric fear stimulus) and Phase 3 (anticipation of acoustic fear stimulus) of the experimental session, in dark-adapted eyes. Note that in Phase 1 the white noise was not warning of a fear stimulus. Ordinate: pupil diameter (mm) and light reflex amplitude (mm), respectively. Abscissa: Log luminance (cd m^{-2}). Data points correspond to the mean obtained in the group ($n = 16$). Bottom: Effect of threat (safe – threat differences) on light reflex amplitude obtained across the four graded luminance levels for the two fear-stimulus types (electric and acoustic) in Phases 2 and 3, respectively. The height of the columns corresponds to the mean obtained in the group ($n = 10$), after exclusion of 6 subjects with a confounding floor effect (see discussion for details); vertical bars are *SEM*.

Finally, comparison of the amplitude data in Phases 2 and 3 revealed that the threat of shock reduced light reflex amplitude significantly more than the threat of sound blast across all luminance levels, $F_{\text{condition}}$ (1,15) = 10.29, $p = .006$, $\eta^2 = .407$; $F_{\text{luminance}}$ (3,45) = 176.92, $p = .001$, $\varepsilon = .60$, $\eta^2 = .922$; $F_{\text{interaction}}$ (3,45) = 1.30, $p > .1$. ANCOVA of the light reflex amplitude data with initial pupil diameter as the covariate revealed a significant effect of the regression in the case of Condition × Luminance interaction, $F(1,44) = 5.75$, $p = .021$, $\eta^2 = .115$, following which the interaction remained nonsignificant. There was no effect of the regression in the case of condition, $F < 1$, or luminance, $F(1,44) = 2.52$, $p > .1$, and both main effects remain significant at the level of $p < .001$.

For each luminance level, the within-subject difference (threat/safe) in light reflex amplitude was taken for each fear-stimulus type, and this was defined as the individual's response to each type of threat. Inspection of these data showed light dependency, as the effect of threat on the light reflex amplitude increased with increasing light probe luminance. This finding however, was not confirmed by a 2 × 4 (Fear-Stimulus Type × Luminance) ANOVA. There were no significant main effects of fear-stimulus type, $F(1,15) = 2.8$, $p > .1$, $\eta^2 = .156$, luminance, $F(3,45) = 1.4$, $p > .1$, $\varepsilon = .58$, $\eta^2 = .085$, or interaction, $F < 1$. Because the effect of threat on the light reflex amplitude is calculated as the safe – threat difference, any factor reducing the light reflex amplitude in the safe condition may mask a true effect of threat. We hypothesized that mechanical limitations of the iris, which come into play when the constricting pupil reaches a critical diameter smaller than 3.5–4 mm (Newsome & Loewenfeld, 1971), could have curtailed the light reflex amplitude in the safe condition for some subjects and thus could have masked a true effect of threat. Indeed, visual inspection of the raw data revealed 6 candidate subjects, whose pupillary diameters at the trough of the constriction were exceeding the 4-mm "floor" criterion when

they were tested with the brightest probes (50 and 140 cd m^{-2}) in the safe condition of Phase 3. A further analysis was undertaken after data for these 6 subjects had been removed from the sample. ANOVA on the data from the remaining 10 subjects, using the same factorial design as above, revealed a significant main effect of luminance, $F(3,27) = 9.5$, $p < .001$, $\varepsilon = .76$, $\eta^2 = .514$. The fear-stimulus type main effect and the interaction were not significant, $F(1,9) = 1.6$, $p > .1$, $\eta^2 = .149$ and $F < 1$, respectively. These data ($n = 10$) are shown in Figure 1 (bottom panel).

Subjective Ratings

The subjective "anxiety" (ratings in millimeters) obtained in Phases 2 and 3 was greater under the threat condition (group means, collapsed across blocks [*SE* mean]) for both fear-stimuli (electric: 49.1 [5.8] and acoustic: 42.3 [5.7]) compared to the respective safe conditions (26.9 [4.8] and 25.1 [3.6]). A significant main effect of condition, $F(1,15) = 18.4$, $p < .001$, $\eta^2 = .550$, was revealed by a 2 × 2 (Fear Stimulus-Type × Condition) ANOVA on the anxiety data, but the main effect of fear-stimulus type, $F(1,15) = 1.4$, $p > .1$, or interaction, $F(1,15) = 1.6$, $p > .1$, were not significant.

The subjective "alertness" (ratings in millimeters) obtained in Phases 2 and 3 was also greater under the threat condition (group means, collapsed across blocks [*SE* mean]) for both fear-stimuli (electric: 52.3 [2.5], acoustic: 51.1 [2.8]) compared to the respective safe conditions (49.2 [2.5] and 49.8 [2.8]). ANOVA with the same factorial design as above revealed a significant main effect of condition, $F(1,15) = 6.3$, $p < .05$, $\eta^2 = .296$, but no significant main effect of fear-stimulus type, $F < 1$. The Fear-Stimulus Type × Condition interaction was significant, $F(1,15) = 7.2$, $p < 0.05$, $\eta^2 = .326$. Post hoc comparisons showed that alertness did not differ significantly in the two safe conditions and that only threat with the electric shock was associated with a significant increase in alertness.

Discussion

Light Reflex Amplitude

Light reflex amplitude across a range of light probe intensities was not affected by the constant white noise when it was presented alone and subjects did not anticipate any aversive stimulation (Phase 1). It, therefore, appears that the white noise alone, and by extension the CS used in previous studies, has no effect on the amplitude of the light reflex and it is unlikely that it may contaminate the results obtained with the paradigm used here.

Anticipation of either the electrical (shock) or the acoustic (sound blast) fear stimulus was associated with significant increases in subjective anxiety and both stimulus types increased subjective anxiety to the same degree, as suggested by the lack of a stimulus type main effect or a significant stimulus by condition interaction of the anxiety data. The experimental design was identical to that of previous studies for reasons of consistency, and ensured that anticipation of a stimulus, rather than its actual delivery, was the relevant independent variable. Anticipation of the electrical stimulus was associated with a significant reduction in light reflex amplitude, replicating our previous results. Anticipation of either the electrical or the acoustic stimulus produced reductions in light reflex amplitude across all light intensities in the same way, as suggested by the lack of a significant stimulus type by light intensity interaction.

Removal of initial pupil diameter influences by means of ANCOVA did not affect the results obtained from any of the ANOVAs of the amplitude data, suggesting that light reflex amplitude may be inhibited by anticipation and that it may not be secondary to baseline (initial pupil diameter) changes. Anticipation of the electric stimulus was more potent than anticipation of the sound blast in inhibiting the light reflex across all light intensities (see Figure 1, top right). This is consistent with the widely accepted notion that an electric shock is a powerful unconditional aversive stimulus with high face validity as an anxiety-provoking condition (Deane, 1969; Reiman, Fusselman, Fox, & Raichle, 1989).

Taken together, these observations suggest that (a) the CS per se does not seem to modify the light reflex and does not contaminate the results, (b) the fear-stimulus modality may not be important for the inhibition of the light reflex as long as the possibility of stimulus occurrence is an adequately threatening prospect for the subjects, and (c) that the magnitude of the light reflex amplitude can be a function of the amount of threat posed by a fear stimulus. The amount of threat may vary between different types of fear stimuli for a given subject, whereas inhibition of the light reflex appears to be sensitive to threat in a "dose-dependent" manner.

Initial Pupil Diameter

The threat of shock increased significantly the initial pupil diameter relative to the baseline condition, whereas the equally anxiogenic threat of sound blast did not (Figure 1, left), although it caused significant reductions in light reflex amplitude across all light probe intensities. Importantly, the threat of shock was rated significantly more alerting compared to the threat of sound blast. These results taken together suggest that the increase in initial pupil diameter may have to do more with the alerting than the anxiogenic properties of a stimulus, a suggestion that is in agreement with previous studies (see discussion below) and consistent with the threat of shock being a powerful aversively arousing as well as anxiety-provoking condition. It is noteworthy, in this context, that presentation of the white noise alone increased initial pupil diameter whereas it had no effect whatsoever on light reflex amplitude (Figure 1, top panels). This effect failed, however, to reach statistical significance, probably because the white noise alone in the present uninstructed paradigm in Phase 1 was not associated with stimulus anticipation, and, therefore, its ability to elicit alertness was weak and quickly habituated.

It has previously been shown that the threat-induced increase in initial pupil diameter and the threat-induced reduction in light reflex amplitude do not covary, and that only light reflex amplitude correlates with subjective anxiety (Bitsios et al., 1996, 2002). Anticipation of a nonaversive, alerting, but non-anxiety-provoking weak auditory stimulus (Bitsios et al., 2004) or execution of an easy task requiring minimal mental effort (Steinhauer, Condray, & Kasparek, 2000), increased the initial pupil diameter but did not affect the light reflex amplitude. Most importantly, the anxiolytic drug diazepam reduced the effect of threat on the light reflex amplitude but did not affect the threat-induced increase in initial pupil diameter (Bitsios et al., 1998a, 1999). Pupil diameter is known to increase in response to any sensory stimuli (with the exception of light), in response to novel or interesting, pleasant or unpleasant stimuli, and finally, in response to guessing, novelty, and uncertainty (for reviews, see Loewenfeld, 1993; Steinhauer & Hakerem, 1992). The magnitude of its increase appears to be a function of the overall

processing load or "mental effort" required to process a stimulus or to perform a cognitive task even when the composition of processing resources differs between tasks (Beatty, 1982; Kahneman, 1973).

It could, therefore, be speculated that the increase in initial pupil diameter may be close to the increase in skin conductance (conditioned skin conductance response), which is thought to reflect nonspecific arousal, orienting to a stimulus (aversive or not) as a function of its relevance but not of its emotional valence (Hamm & Vaitl, 1996; Lang et al., 1990). The threat-induced increase in initial pupil diameter is a sympathetically mediated response, similar to the conditioned skin conductance response and in contrast to the threat-induced inhibition of light reflex amplitude, which reflects central parasympathetic inhibition. Indeed, peripheral sympathetic blockade with dapiprazole eye drops reduced the effect of threat on this measure, whereas reflex inhibition by threat remained unaffected (Giakoumaki et al., 2005).

Unlike baseline pupil diameter changes, the light reflex is not a direct reaction to an emotional, novel, or otherwise significant event. On the contrary, it is an additional homeostatic response to an independent event (i.e., the light probe) and its inhibition by threat could be conceptualized in terms of Lang's theory of emotional responses (Lang et al., 1990), as the result of a mismatch between an aversive ongoing emotional state (e.g., anxious anticipation in the threat condition) and the homeostatic nature of the light reflex.

The Light Dependency of the Effect of Threat

Our main assumption was that the threat-induced reduction in light reflex amplitude reflected an emotionally aversive internal state (i.e., fear), and this assumption was partially validated by the negative correlation between light reflex amplitude and subjective anxiety (Bitsios et al., 1996, 2002) as well as by the sensitivity of this measure to diazepam (Bitsios et al., 1998a, 1999). However, it is still possible that the main causes underlying this phenomenon are the cognitive and attentional mechanisms associated with anxious anticipatory processing rather than the negative emotional valence of aversive shock anticipation per se.

It is interesting that neither startle potentiation (Grillon, Falls, Ameli, & Davis, 1994) nor light reflex inhibition (Bitsios et al., 2004) occurred when subjects were instructed to anticipate and detect weak auditory tones. The possibility of startle and light reflex modulation by attentional processes cannot be ruled out, however, because the weak auditory tone is a low salience stimulus. Indeed, anticipation of either pleasant or unpleasant pictures (Sabatinelli, Bradley, Cuthbert, & Lang, 1996) or a nonaversive reaction time task (Lipp 2002) can potentiate the startle reflex, thus revealing the importance of attentional mechanisms brought about by (not necessarily anxious) anticipatory processing. Equally, when the light probes (four graded intensities) were used concurrently as the imperative signal for a motor reaction time task as well as the light reflex eliciting stimuli, robust inhibition of the light reflex was observed that was much greater for reflexes elicited by the weakest light probes (Gavriysky 1991). This latter study suggests that attentional mechanisms can, indeed, inhibit the light reflex, but this is in striking contrast with our results, which demonstrate that threat-induced light reflex inhibition was greater for reflexes elicited by the more intense light probes (Figure 1, bottom).

The pupillary light reflex is a graded response: Increases in probe intensity cause a graded recruitment and increase in the firing rate of afferent retinal neurones, which in turn, via the olivary pretectal (Gamlin et al., 1997) and the Edinger-Westphal (EW) nuclei, result in increasing the iris constrictor activity and the degree of pupillary constriction. It is generally assumed that the EW nucleus (the motor center of the reflex) acts as a signal generator whose output at any one time is modulated by a number of excitatory inputs, the major one being the afferent projection from the retina via the olivary pretectal nucleus and a number of inhibitory inputs from different brain areas (Barbur, 2004).

Assuming that EW nucleus neurons act as steady spike generators that are modulated by inhibitory inputs, the increased steady-state pupil size observed during the threat condition suggests a small increase in steady-state inhibition (i.e., a small decrease in the firing rate of these neurons). When a light reflex signal is generated, it causes a separate excitatory input that increases the firing rate of EW neurons. The size of this signal must be under some cortical control, because the dynamic pupil light reflex response to small, low contrast stimuli is absent when the stimulus is restricted to cortically blind regions of the visual field (Alexandridis, Krastel, & Reuther, 1979; Barbur, Keenleyside, & Thomson, 1988; Harms 1951; Kardon 1992). Furthermore, the threat-induced changes seem to affect the effectiveness of this control, because the dynamic light reflex amplitude is reduced in the threat condition. The reduction represents an almost constant fraction of the actual response amplitude, because the reduction increases with increasing light probe intensity (see Figure 1, bottom), and this is consistent with a multiplicative gain control and not with an additive mechanism. In contrast, the results of Gavriysky (1991) are consistent with an algebraic summation of excitatory (from the light probe) and inhibitory (from the activation of arousal/attentional circuitries) inputs to the EW nucleus (i.e., consistent with an additive control mechanism).

Our results are important in two ways. First, they suggest that two separate pathways appear to mediate the pupil response to light, one controlling the steady-state size of the pupil and the other mediating the transient constriction of the pupil in response to rapid increments in light flux on the retina (Barbur, 2004). Second, in conjunction with the results of Gavriysky (1991), our results suggest that activation of both attentional- and threat-related circuitries can inhibit the light reflex, but they can be dissected because they affect light reflex amplitude to weak and bright light probes in entirely opposite ways.

One possible reconciliation of these apparently opposite patterns may be that, during anxious anticipatory processing in our threat condition, the more intense light probes become more motivationally relevant (i.e., more aversive), thus multiplying the allocation of attentional resources compared to the weaker probes. This interpretation is consistent with the assumption that the threat-induced reduction in light reflex amplitude reflects fear, although it requires the addition of an intermediate attentional/cognitive component. Early automatic allocation of attentional resources to emotional stimuli can modulate startle within the first 300 ms from presentation of the emotional stimulus (Bradley, Cuthbert, & Lang, 1993), a long enough time period for the cortical processing of light probes in our paradigm.

Furthermore, this interpretation is consistent with neurobiological evidence; activation of the amygdala during conditioned fear (Davis et al., 1993) or verbal threat (Phelps et al., 2001) can trigger, through its well-established connections (Amaral, Price, Pitkänen, & Carmichael, 1992), a specific cortical area processing a relevant stimulus (the light probe in this case), thus directing attention and perception to emotionally relevant stimuli. This

process is facilitated by synchronous amygdala-triggered activation of the brain stem's nonspecific arousal networks, which "prime" the function of relevant cortical areas (see LeDoux, 1999, pp. 284–291). We postulate that, although the threat-induced increase in initial pupil diameter reflects the tonic activation of nonspecific arousal systems, especially that of the noradrenergic locus coeruleus (Bitsios et al., 1998b), the threat-induced light reflex inhibition not only has a tonic component reflecting inhibition from brain stem arousal centers but also a light-stimulus-intensity-specific phasic component that reflects amygdala-driven allocation of attention to that light stimulus.

The effect of threat on light reflex amplitude appears to be light intensity dependent, and this may have important methodological implications in future research involving the fear-inhibited light reflex, as it favors a choice of bright probes for obtaining a larger manifest effect of threat. However, this study also suggests that the choice of bright probes may be associated with a greater likelihood of operation of a confounding floor effect. Future studies should, therefore, carefully weigh the risks and benefits of using very bright probes in order to elicit the fear-inhibited light reflex.

In summary, our results have replicated previous reports showing evidence for dissociation between threat-induced increase in initial pupil diameter and threat-induced reduction in light reflex amplitude. More specifically, we here suggest that (a) the CS per se does not seem to modify the light reflex, (b) the light reflex can be inhibited by anticipation of fear stimuli from different sensory modalities, (c) the light reflex can be sensitive to variations in the amount of fear induced by different fear-stimuli, and (d) the manifest effect of fear on light reflex amplitude increases with increasing light probe intensity suggesting that, compared to the weaker probes, light probes of greater intensity perhaps become more relevant motivationally, thus attracting greater allocation of attentional/cognitive resources.

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