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ORIGINAL INVESTIGATION

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Sensitivity of the fear-inhibited light reflex to diazepam

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Abstract We have shown previously that pupil diameter increases and the amplitude of the pupillary light reflex is reduced when subjects are under threat of an aversive event (electric shock), and that light reflex amplitude correlates negatively with subjective anxiety. We have suggested that the "fear-inhibited light reflex" paradigm could be used as a laboratory model of human anxiety. In the present study, we examined whether two doses (5 mg and 10 mg) of the anxiolytic drug diazepam would antagonize the effects of threat on the pupillary light reflex. Twelve healthy male volunteers participated in three weekly sessions, each associated with one of three treatments (diazepam 5 mg or 10 mg or placebo) in a double-blind, balanced, crossover design. The light reflex was recorded during either the anticipation of a shock ("threat" blocks) or periods in which no shocks were anticipated ("safe" blocks). At the end of each "threat" or "safe" block, subjects rated their anxiety using visual analogue scales. Two-factor ANOVA (treatment × condition) showed that diazepam treatment antagonized the effect of threat on light reflex amplitude in a dose-dependent manner but it did not affect the threat-induced increase in pupil diameter. Diazepam had no effect on the pupillary light reflex in the "safe" condition. Diazepam also reduced subjective anxiety and alertness in the threat condition. These results show the sensitivity of the threat-induced reduction of light reflex amplitude to anxiolytic drugs, and provide further evidence for the utility of the fear-inhibited light reflex paradigm as a laboratory model of human anxiety.

Key words Light reflex · Fear · Anxiety · Healthy volunteers · Diazepam · Pupillometry

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Introduction

It has been shown previously (Bitsios et al. 1996a) that the threat of an electric shock increased the diameter of the pupil and decreased the amplitude of the light reflex compared to periods when subjects were resting. The reduction in light reflex amplitude was accompanied by increases in subjective alertness and anxiety, as measured by visual analogue scales. Furthermore, only light reflex amplitude, but not pupil diameter, correlated with anxiety, indicating a possible dissociation between the two pupillary measures. We termed the phenomenon of the attenuation of the light reflex by threat the "fear-inhibited light reflex". We have also shown (Bitsios et al. 1996b) that the aversiveness of the anticipated stimulus is a prerequisite for producing a reduction in light reflex amplitude. When subjects were instructed to attend to and report an emotionally neutral stimulus (i.e. a low intensity acoustic tone), light reflex amplitude was not reduced, whereas their "initial" pupil diameters (i.e. pupil diameters measured immediately before the application of the light stimulus) were increased. The changes in initial pupil diameter were accompanied by increases in subjectively rated alertness but not of anxiety. These results further strengthen the validity of the "fear-inhibited light reflex" paradigm as a potential laboratory model of human anxiety, and confirm our previous findings of a psychophysiological dissociation between light reflex amplitude and pupil diameter.

In the present study, in an attempt to validate further the fear-inhibited light reflex paradigm as a model of human anxiety, we examined whether it can be modified by the anxiolytic drug diazepam.

Materials and methods

Subjects

Twelve healthy male volunteers aged 18–34 years (mean ± SD 22.3 ± 4.6) and weighing 60–80.5 kg (mean ± SD 70.8 ± 6.0) participated in the study. Subjects were all medication-free and were requested to avoid drinking alcohol, coffee and other caffeine-containing beverages for at least 12 h before the experimental session. All of them were occasional caffeine and/or social alcohol consumers. Subjects were tested in the morning (9:00 a.m. to 13:00 p.m.). The study protocol was approved by the University of Nottingham Medical School Ethics Committee. All volunteers gave their written consent following a verbal explanation of the study and after reading a detailed information sheet.

Drugs and design

Diazepam 5 and 10 mg and placebo were administered orally in matching capsules. All subjects participated first in a training session and 1 or 2 days later in three weekly experimental sessions, which were associated with diazepam 5 mg, diazepam 10 mg, or with placebo. Subjects were allocated to drug treatment conditions and experimental sessions according to a double-blind balanced design.

Tests and apparatus

These are described in detail elswhere (Bitsios et al. 1996a). An infrared binocular television pupillometer (TVP 1015B; Applied Science Laboratories, Waltham, Mass., USA) was used for the recording of the light reflex in darkness, in previously dark-adapted eyes. The stimuli were light flashes (green, 565 nm peak wavelength) of 200 ms duration, delivered via a light emitting diode positioned 1 cm from the cornea of the subjects' right eye; the incident light intensity measured 1 cm from the source was 0.43 mW cm^{-2}. The parameters studied were: initial pupil diameter (i.e. diameter of the pupil before the application of the light stimulus) and amplitude of light reflex response (i.e. the difference between the initial and minimal pupil diameters). For electrical stimulation, a constant current square pulse (1.5 mA, 50 ms) was delivered to the skin overlying the median nerve of the left wrist through disposable silver surface electrodes using a Grass stimulator (SD 9). The subjects' mood and feelings were self-rated on a 16-item visual analogue scale (Norris 1971), and pain was rated on a one-item visual analogue scale.

Procedures

Training session

Upon their arrival in the laboratory, the subjects received a detailed description of all procedures and a demonstration of all apparatus. Then the subjects underwent a brief training session (application of a few light flashes in the dark to evoke the pupillary light reflex), in order to familiarize them with pupillometry.

Experimental sessions

At the beginning of each experimental session, after a 10-min rest period, the subjects ingested the capsule. Forty minutes after drug ingestion, the subjects entered the adaptation phase. During the adaptation phase, the subjects first wore red goggles for 15 min in order to adapt to dim red illumination. Following this, the light reflex was elicited in darkness with 12 light flashes, in order to familiarize them with pupillometry (5 min). At the end of the adaptation phase (1 h after the ingestion of the capsule), the skin on the subjects' left wrist was prepared, the electrodes were applied, and the main phase was started.

The main phase comprised nine identical, consecutive blocks of three light flashes of the same intensity and duration (27 light flashes in total, per session). In the main phase, responses in each block were recorded either during anticipation of an electric shock (Threat condition) or without anticipation (Safe condition). The first block was always associated with the Safe condition ("initial" Safe block), responses recorded in this block were not entered in the analysis. After recording responses from the initial Safe block, half of the subjects started with a Safe block, and the remaining half with a Threat block. The Safe and Threat conditions alternated regularly in the remaining 12 blocks. Subjects were asked to rate their mood and feelings during the Safe and Threat blocks retrospectively, at the end of each Safe and Threat block, using the 16 mood and feelings visual analogue scales. The interblock interval was 90–120 s, to allow sufficient time for the completion of the visual analogue scales. The main phase lasted approximately 30 min.

Instructions to subjects

Thirty seconds prior to the onset of each block, the subjects were informed about the nature of the condition with which the block was associated. In the Safe condition, the subjects were told that no electric shocks would be administered. In the Threat blocks, the subjects were instructed to anticipate a total of one to three electric shocks, delivered to their left wrists during the 3 s elapsing between a 500-ms warning tone and a light flash. The subjects did not know the exact number of shocks, or in which Threat block(s) it/they would occur. The shocks were described by the experimenter as mildly painful stimuli inducing a short-lived localized unpleasant sensation on the wrist. In fact, only three single, non-painful 1.5-mA electric shocks were delivered in the entire experiment. It was shown previously (Bitsios et al. 1996a) that it was the threat of the shock, rather than the delivery of the shock, which was responsible for the changes in light reflex amplitude. Therefore, no shock was delivered in the first session. To restore threat in the second session, it was emphasized to the subjects that "although shock delivery had been judged unnecessary the first time, one to three electric shocks", as previously instructed, "would now definitely occur". One 1.5-mA shock was delivered at the end of the second session. It was shown previously (Bitsios et al. 1996a), that subjects did not judge a 1.5-mA shock to be painful. Therefore, in order to restore an effective threat in the third session, subjects were led to believe that this time the shock(s) would be at least 50 times stronger than the shock they had received in the second session. In order to investigate changes in subjects' perception of threat across and within sessions, the subjects were asked to rate themselves with a one-item visual analogue scale for "expected" pain (no pain – extreme pain) on arrival in the laboratory, 1 h after drug ingestion (i.e. immediately prior to the placement of wrist electrodes) and before the onset of the main phase (i.e. immediately after the placement of wrist electrodes).

Data reduction and data analysis

The pupillary measures (initial diameter and light reflex amplitude) for each block were obtained by averaging the three light reflex responses by computer, and taking the measures from the averaged response. The raw values (mm) of the visual analogue scales for each item and each subject were weighted by multiplication with their respective factor loading, and the weighted values for each item and subject were then allocated to "alertness", "discontentment" and "anxiety" factors, based on a principal component

analysis (Bond and Lader 1974). The mean of the weighted group values for each factor was entered in the statistical analysis.

Data for each pupillary and visual analogue scale measure were collapsed across blocks for the two conditions (Safe, Threat) and the three treatments (diazepam 5 mg, diazepam 10 mg, placebo). Two-way analysis of variance with treatment and condition as the within-subject factors was used to analyze the pupillary and visual analogue scales measures. In the case of a significant interaction, the placebo and diazepam 5 or 10 mg treatments were compared with each other under each condition using the least significant difference test (criterion, $P < 0.05$). The relationship between changes in light reflex amplitude and initial pupil diameter (baseline), was analyzed by analysis of covariance (treatment × condition with initial diameter as the covariate).

In order to assess the psychological impact of the threat of shock and to examine whether it diminished across the three sessions, the self-ratings of the one-item visual analogue scales for "expected" pain obtained on three occasions (before drug ingestion, 1 h after drug ingestion and before the main phase; for details see above) within a session were also analyzed separately. Two-way analysis of variance with session and occasion as the within-subject factors was used to analyze these visual analogue scale measures.

Results

Pupillary measures

Initial pupil diameter and amplitude of light reflex (group means) for each of the four Safe and four Threat occasions and the collapsed data (group means) averaged across the blocks for the two conditions and the three treatments are displayed in Fig. 1.

It can be seen that initial pupil diameter was larger under the Threat than under the Safe condition, under all three treatments. Analysis of variance of the initial pupil diameter data revealed a significant main effect of condition ($F = 23.3$; $df = 1,11$; $P < 0.001$) but no significant main effect of treatment ($F < 1$). There was a weak treatment × condition interaction ($F = 3.5$; $df = 1,11$; $P > 0.049$); however, post hoc comparisons with the least significant difference test showed that the slight increase in initial diameter in the Safe condition with both diazepam treatments was not significant.

There was a progressive increase in amplitudes during the experimental session with both treatments, but in each block amplitude was smaller under the Threat than under the Safe condition, under all three treatments. It can also be seen that diazepam increased the amplitude in the Threat condition in a dose-dependent fashion. Analysis of variance of the amplitude data revealed significant main effects of treatment ($F = 21.5$; $df = 2,22$; $P < 0.001$) and condition ($F = 35.1$; $df = 1,11$; $P < 0.001$) as well as significant treatment × condition interaction ($F = 12.6$; $df = 2,22$, $P < 0.001$). Post hoc comparisons with the least significant difference test showed that both diazepam treatments were associated with a significant increase in the response amplitude under the Threat condition, and that the increase in amplitude in the Threat condition was significantly greater with diazepam 10 mg compared to diazepam 5 mg.

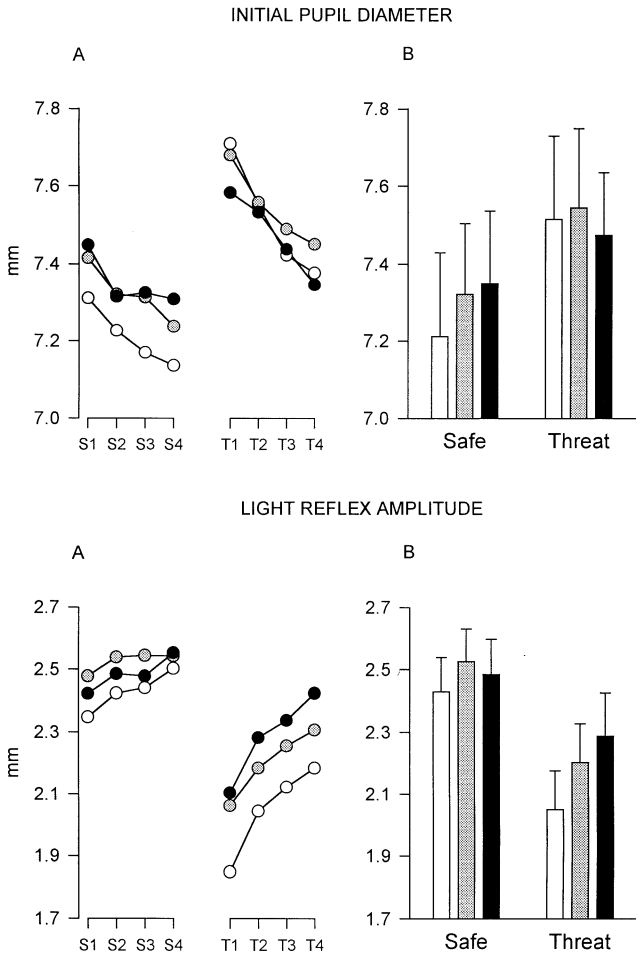

Fig. 1 Initial pupil diameter (*top*), and light reflex amplitude (*bottom*) recorded in the main phase of the three experimental sessions. **A** *Ordinate*: pupil diameter (mm), and light reflex amplitude (mm), respectively; *abscissa*: sequential blocks (*S*: Safe, *T*: Threat). The data points are means ($n = 12$). *Open symbols*: placebo; *light shaded symbols*: diazepam 5 mg; *closed symbols*: diazepam 10 mg. **B** *Ordinates*: as above (**A**). The *bars* represent data averaged across the four blocks for the two conditions (Safe, Threat) and the three treatments (mean ± SEM, $n = 12$). *Open bars*: placebo; *light shaded bars*: diazepam 5 mg; *closed bars*: diazepam 10 mg

In order to address the possibility that the changes in amplitude were secondary to changes in initial pupil diameter, an analysis of covariance of the amplitude data (treatment × condition, with initial pupil diameter as the covariate) was carried out. This analysis showed that there was no significant effect of the regression in the case of treatment ($F < 1$) or condition ($F = 4.9$; df = 1,10; $0.05 < P < 0.1$); there was a significant effect of the regression in the case of the interaction ($F = 4.6$; $df = 1,21$; $P < 0.05$); however, the residual effect of interaction was still significant ($F = 6.9$; $df = 2,21$; $P < 0.005$).

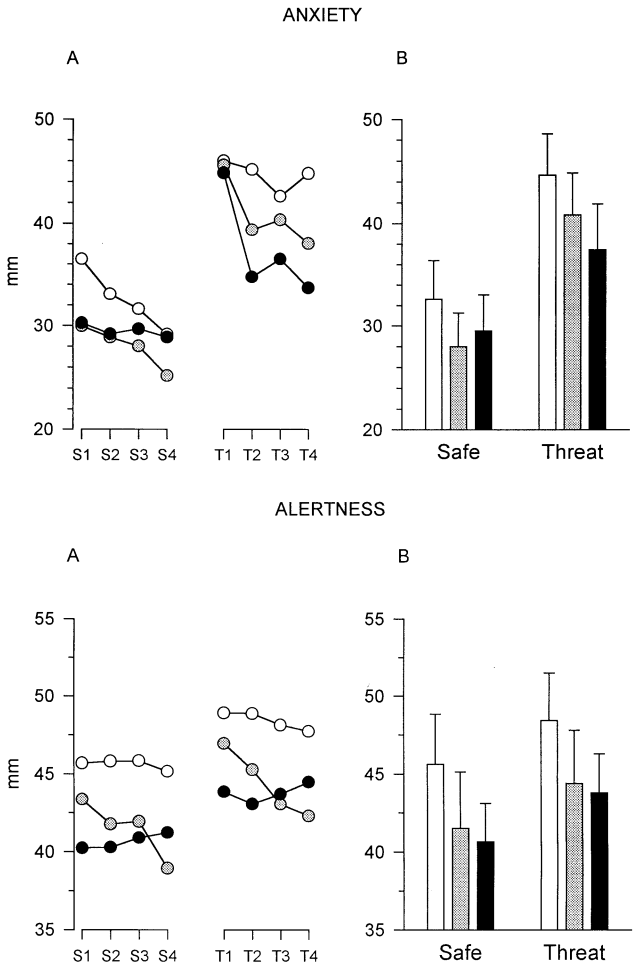

Fig. 2 Subjective ratings on a battery of visual analogue scales of "anxiety" (*top*), and "alertness" (*bottom*) recorded in the main phase of the three experimental sessions. **A** *Ordinate*: score (mm); *abscissa*: sequential blocks (*S*: Safe, *T*: Threat). The data points are means ($n = 12$). *Open symbols*: placebo; *light shaded symbols*: diazepam 5 mg; *closed symbols*: diazepam 10 mg. **B** *Ordinates*: as above (**A**). The *bars* represent data averaged across the four blocks for the two conditions (Safe, Threat) and the three treatments (mean ± SEM, $n = 12$). *Open bars*: placebo; *light shaded bars*: diazepam 5 mg; *closed bars*: diazepam 10 mg

Subjective ratings

The results obtained with the visual analogue scales for "anxiety" and "alertness", are shown in Fig. 2. It is apparent that both "anxiety" and "alertness" were greater under the Threat than under the Safe condition, and that diazepam treatment decreased "anxiety" in the Threat condition in a dose-dependent fashion. Analysis of variance of the "anxiety " data revealed significant treatment ($F = 3.9$; $df = 2,22$; $P < 0.05$) and condition ($F = 16.2$; $df = 1,11$; $P < 0.002$) main effects; however, it did not reveal a significant interaction ($F = 1.9$; $df = 2,22$; $P > 0.1$). Analysis of variance of the "alertness" data revealed significant treatment ($F = 5.04$; $df = 2,22$; $P < 0.05$) and condition ($F = 14.4$; $df = 1,11$; $P < 0.005$) main effects without any significant interaction ($F < 1$). There was no significant difference between the Safe and Threat conditions for the "discontentment" data.

Analysis of variance of the visual analogue ratings of "expected pain", taken before drug ingestion, 1 h after ingestion and after attachment of the electrodes, revealed a significant main effect of occasion ($F = 7.6$; $df = 2,22$; $P < 0.005$) but no significant main effect of session ($F = 2.8$; $df = 2,22$; $0.1 < P > 0.05$), or session × occasion interaction ($F = 1.9$; $df = 4,44$; $P > 0.1$). The mean values of expected pain (mm, ± SEM) averaged across the three sessions were: pre-drug: 53.45 ± 5.2, 1 h after drug: 48.19 ± 5.4, after electrode attachment: 56.94 ± 5.1. A separate analysis of variance was carried out to examine the effect of diazepam on "expected pain" (treatment × occasion); there was no significant effect of treatment ($F < 1$) and no significant treatment × occasion interaction ($F = 2.26$; $df = 4, 44$; $P > 0.05$).

Discussion

The threat of an electric shock reduced the amplitude of the light reflex and increased initial diameter of the pupil, subjective anxiety and alertness, replicating the results of our previous study (Bitsios et al. 1996a). The threat also had consistent effects on the ratings of expected pain in relation to the electric shock, the ratings on this measure increasing as the possibility of receiving the shock became more imminent, thus confirming previous results (Bitsios et al. 1996a). Furthermore, there were no between-session differences in the ratings of expected pain suggesting that, with the present design and procedures, the subjective effects of the threat of shock was equivalent across the three sessions. Thus, it was possible to apply the threat of a shock repeatedly, without actually using multiple or severe shocks. Our protocol also allowed for a within-subjects cross-over design, thus reducing problems that might have arisen with a between-subjects design due to between-subject variability in the effect of threat on the light reflex or in the absorption of diazepam.

While there are previous reports on the effects of benzodiazepines on resting pupil diameter, we are not aware of any studies of the effects of these drugs on the light reflex in man. In agreement with previous reports (Karniol et al. 1976; Safran 1984; Walser et al. 1987; Loewenfeld 1993, pp 683–827), in the present study we found that a benzodiazepine (diazepam) failed to alter resting pupil size. Furthermore, diazepam treatment with either dose had no significant effect on the amplitude of the light reflex in the safe condition.

Diazepam did not block the increase in initial pupil diameter, but antagonized the reduction in light reflex amplitude in a dose-dependent manner in the Threat condition. Diazepam also antagonized the increase in

subjective anxiety and alertness in the Threat condition. These results add further support to the two main conclusions reached in our previous study (Bitsios et al. 1996a): (i) the reduction of the amplitude of the light reflex is a sensitive and reliable correlate of anxiety and (ii) the increase in initial pupil diameter and the decrease in light reflex amplitude in response to the cue signalling threat, may reflect different neural processes.

It is an intriguing possibility that similar mechanisms operate both in the fear-inhibited light reflex and the "fear-potentiated startle reflex" (Grillon et al. 1991; Davis 1992; Davis et al. 1993), another laboratory paradigm of anxiety. It has been shown in rats that diazepam and flurazepam produce a dose-dependent attenuation of the potentiation of the startle response induced by exposure to a stimulus associated with an electric shock (Davis 1979), at doses that do not affect the baseline startle reflex. There is now a large body of evidence which shows that the potentiation of the startle reflex is mediated by the amygdala, a structure implicated in fear and anxiety (Gloor 1960; Mishkin and Aggleton 1981; Kapp et al. 1984; Sarter and Markovwsitsch 1985; Le Doux et al. 1988; Davis 1992). The central nucleus of the amygdala which has direct neural connections with the nucleus reticularis pontis caudalis (an obligatory point of the startle reflex pathway), has been especially implicated in the potentiation of the startle reflex (see Davis 1993 for a review). Thus, lesions of the central nucleus of the amygdala block the fear-potentiated startle reflex, without affecting the baseline startle reflex (Hitchcock and Davis 1986, 1991).

It is known that the pupillary light reflex is under tonic inhibitory control from the hypothalamus (Loewenfeld 1958, 1993, pp 407–480; Koss 1984, 1986) via connections between the hypothalamus and the Edinger-Westphal nucleus (Saper et al. 1976). There is also evidence that stimulation of the amygdala causes pupillary dilatation in the cat (Koikegami and Yoshida 1953; de Molina and Hunsberger 1962), probably via well known excitatory amygdalo-hypothalamic connections (Le Doux 1988; Davis 1992). It is thus possible that stimulation of the amygdala by conditioned aversive stimuli enhances the inhibitory influence of the hypothalamus on the Edinger-Westphal nucleus, resulting in enhancement of the inhibition of the pupillary light reflex. Furthermore, diazepam may antagonize the threat-evoked reduction in light reflex amplitude and the threat-evoked increase in subjective anxiety by preventing the activation of the amygdala-hypothalamus complex by threat, as has been shown to be the case with the fear-potentiated startle reflex.

In conclusion, diazepam, which is thought to reduce fear in other behavioural tests in experimental animals (Gray 1977), attenuated subjective anxiety together with the threat-evoked reduction in light reflex amplitude (fear-inhibited light reflex) in a dose-dependent manner, at two doses (5 mg and 10 mg) which are known to reduce anxiety clinically. Furthermore, the pattern of effect of diazepam on the threat-evoked increase in resting pupil diameter and decrease in light reflex amplitude was consistent with the dissociation between the two pupillary measures, as suggested previously (Bitsios et al. 1996a,b). These results, therefore, provide further support for the validity of the fear-inhibited light reflex as a laboratory model of human anxiety.

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