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Effects of peripheral sympathetic blockade with dapiprazole on the fear-inhibited light reflex

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Abstract

Fear (e.g. associated with the threat of an electric shock) causes an increase in initial pupil diameter (IPD) and a decrease in the amplitude of the light reflex response. There is evidence for dissociation between the two responses to threat: only the reduction in light reflex response amplitude is sensitive to the anxiolytic drug diazepam. We examined the effects of peripheral sympathetic blockade with the α_1-adrenoceptor antagonist dapiprazole on both responses to threat on the basis of the hypothesis that only the response of the IPD will be affected, whereas the response of the light reflex will remain unaffected. Twelve healthy volunteers (Experiment 1) and eight healthy volunteers with smaller pupils (Experiment 2) participated in one experimental session. Dapiprazole 0.5% (two drops of 20 μl, three times) was instilled in the subjects' right or left eye while the contralateral eye was treated with placebo eye drops (artificial tear, two drops of 20 μl, three times) according to a single-blind balanced design. Pupil diameter was monitored by infrared binocular television pupillometry. At the point of maximum dapiprazole-evoked miosis, the light reflex was elicited three times in each of three Safe blocks (no possibility of electric shock), alternating with three Threat blocks (possibility of electric shock). At the end of each Safe and Threat block, subjects rated their mood and

feelings on the Visual Analogue Scales. In Experiment 1, dapiprazole caused significant miosis. Threat increased subjectively rated anxiety and inhibited the light reflex. The inhibition of the light reflex was unaffected by dapiprazole. The threat-induced increase in IPD was also unaffected by dapiprazole, probably due to a ceiling effect curtailing the threat-induced increase in IPD. In the smaller pupil group in Experiment 2, where the possible contribution of a ceiling effect was minimized, dapiprazole suppressed the threat-induced increase in IPD. The inhibition of the light reflex by threat is likely to reflect central parasympathetic inhibition and is unlikely to involve the peripheral sympathetic innervation of the iris. The threat-induced increase in IPD is likely to reflect mainly central sympathetic excitation. The different central autonomic mechanisms underlying the two pupillary responses to threat may explain the dissociation between the separate effects of threat on IPD and light reflex amplitude.

Keywords

Introduction

The light reflex consists of a brisk and transient contraction of the smooth iris sphincter muscle in response to rapid increments in light flux of the retina, thus reflecting the amount of light captured by the eye. The light reflex is a primitive, cross-species reflex mediated by a mesencephalon-based, simple neural circuit, which has been specified well in animals and humans. Important synapses include the retinal ganglion cells, the olivary pretectal nucleus, the Edinger–Westphal (EW) nucleus, the ciliary ganglion and the

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sphincter iris muscle (Gamlin *et al*., 1997; Kardon, 1998). The light reflex can be elicited and recorded accurately in the laboratory by means of computerized infrared television pupillometry (Loewenfeld, 1999).

It has been shown that the amplitude of the pupillary light reflex is reduced when the light stimulus is presented in the presence of a cue (e.g. a tone) that has been previously associated with an electric shock. These changes in pupillary activity are accompanied by increases in subjective alertness and anxiety. In this test, the conditioned response is considered to be a state of fear and, for this reason, this phenomenon was termed 'fear-inhibited light reflex'. The threat-induced decrease in light reflex response amplitude was proposed as a potential laboratory model for human anxiety (Bitsios *et al*., 1996). Conditioned fear in humans can thus be operationally defined as the inhibition of the light reflex in the presence of a cue associated with a shock. The fear-inhibited light reflex is based on fear conditioning which is similar to that for the fear-potentiated startle reflex, a well-known anxiety model in animals (Davis, 1992; Davis *et al*., 1993) and humans (Grillon *et al*., 1991). Indeed, during simultaneous recording of the startle and the light reflexes, the cue signalling the possibility of the delivery of a shock modifies both reflexes in the predicted direction (Bitsios *et al*., 1999). Moreover, the fear-inhibited light reflex, in common with the fear-potentiated startle reflex, is dose-dependently sensitive to the anxiolytic drug diazepam (Bitsios *et al*., 1998b, 1999), suggesting that a common mechanism may mediate the effect of fear in the case of both reflex paradigms (Bitsios *et al*., 1999).

Following the administration of the threat-signalling cue, apart from a reduction in light reflex amplitude, there is also an increase in initial pupil diameter (IPD) (Bitsios *et al*., 1996). Despite the close temporal proximity of the two pupillary changes, there is increasing evidence that the two effects of threat on the pupil may reflect the operation of separate neural mechanisms (Bitsios *et al*., 1996, 1998a,b, 1999, 2002, 2004). For example, the threat-induced increase in IPD and the threat-induced reduction in light reflex amplitude do not covary, and only light reflex amplitude correlates with subjective anxiety (Bitsios *et al*., 1996, 2002). An easy, alerting, but non-anxiety provoking, attention task increases only the IPD and does not affect the light reflex amplitude (Bitsios *et al*., 2004). Similarly, easy tasks requiring minimal mental effort increase only the IPD without affecting light reflex amplitude, whereas tasks requiring more effortful processing result in greater increases in IPD, as well as in a reduction in light reflex amplitude (Steinhauer *et al*., 2000). Anxious patients matched for age and IPDs have smaller light reflex amplitudes across a range of light intensities compared to sex- and age-matched healthy controls (Bakes *et al*., 1990). Finally, the anxiolytic drug diazepam reduces the effect of threat on the light reflex response amplitude but does not affect the threat-induced increase in IPD (Bitsios *et al*., 1998b, 1999).

It has been postulated that the effect of threat on light reflex response amplitude is due to central parasympathetic inhibition (Bitsios *et al*., 1996, 1998a,b, 1999). To explain the dissociation between the two pupillary responses to threat, we hypothesize that the effect of threat on IPD may reflect, more preferentially, activation of the central sympathetic, whereas the effect of threat on light reflex amplitude may be mediated by the parasympathetic input to the iris.

Dapiprazole is an α_1-adrenoceptor antagonist, which can be administered locally in the conjuctival sac in the form of eyedrops. It produces miosis by preventing the effect of endogenously released noradrenaline on α_1 adrenoceptors in the iris dilator muscle, thus allowing the parasympathetically innervated iris sphincter muscle to predominate. Dapiprazole is used clinically to reverse pharmacologically-induced diagnostic mydriasis caused by sympathomimetic agents, such as phenylephrine, parasympatholytic agents, such as tropicamide, or their combination (Doughtry and Lyle, 1992). Reversal of pharmacologically induced mydriasis by dapiprazole is evident 30 min after instillation and reaches significant levels at 60 min after instillation (Connor *et al*., 1993). Following its introduction, dapiprazole 0.5% has been used in a 2 + 2 drops regimen, two drops followed by two more drops 5 min later; however, a lower dosage (one drop) is also equally efficacious (Wilcox *et al*., 1995).

The aim of the present study was to examine the effects of peripheral sympathetic blockade with dapiprazole on both responses to threat. The rationale of the experiment was to test the hypothesis that peripheral sympathetic blockade will affect only the sympathetically mediated threat-induced increase in IPD, wheras the threat-induced reduction in light reflex amplitude will remain unaffected because it is mediated via central parasympathetic inhibition.

Experiment 1

Subjects

Twelve healthy male volunteers (six male, six female), mean ± SD (range) age 22.6 ± 2.3 (20–25) years, participated in the study. The instructions given to the subjects before the experiment are described in detail under Procedures (see below). They were all tested in the morning (09.00–13.00 h). The study protocol was approved by the University of Crete Medical School Ethics Committee. All volunteers provided their written informed consent following a verbal explanation of the study and after reading a detailed information sheet.

Drugs, tests and apparatus

Dapiprazole chloride (Glamidolo S01EX02 Angelini ACRAF, SpA, Ancona, 2 × 20 µl of a 0.5% solution, repeated three times at 5-min intervals) was instilled in subjects' right or left eye. The contralateral eye was treated with placebo eye drops (artificial tear 2 × 20 µl, repeated three times at 5-min intervals). Treatments were administered according to a single-blind, balanced design.

Pupillometry

The recordings took place in a dark, sound-attenuated room. A binocular infrared television pupillometer (Procyon, P2000D, Procyon Biopharma Inc., Dorval, Canada) was used to elicit and record the light reflex response in darkness in previously dark-

adapted eyes. The sampling rate of the pupillometer was 25 Hz, the spatial resolution was better than 0.05 mm and the accuracy was better than ±3%. The stimuli were weak light flashes of 200 ms in duration (stimulus luminance: 0.35 cd m^{-2}), delivered via a light emitting diode, presented to the subject's placebo-treated eye as a white disk of 8° diameter, providing 'full field' light stimulation while the unstimulated eye was fixating a target dot projected at a distance of approximately 10 m. Stimulus presentation was computer controlled, and pupillary measures were digitized and stored for offline analysis. The parameters studied were: IPD (i.e. the mean pupil diameter recorded over 500 ms before the onset of the light stimulus) and light reflex response amplitude (i.e. the difference between the IPD and the diameter reached at the trough of the pupillary response to the light stimulus).

Subjective ratings

The subjects' mood and feelings were self-rated on a battery of visual analogue scales (VAS) (Norris, 1971) on several occasions throughout the session (for details see Procedures). For each subject, the raw values (mm) 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 and Lader, 1974). The average of the weighted values for each factor was entered in the statistical analysis.

Procedures

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

Training session

Subjects received a training demonstration of apparatuses and procedures to familiarize them with pupillometry. They were then exposed to a mild electric stimulus (constant current square pulse 1.5 mA, 50 ms) delivered to the skin overlying the median nerve of their left wrists, through disposable silver surface electrodes by a Grass stimulator (SD 9) (Grass Instruments, Quincy, MA, USA). This stimulus is known to cause negligible or only minimal discomfort (Bitsios *et al*., 1996). They were informed at this point that the shock in the experimental session would be 50-fold greater, and therefore the discomfort would be greater than the one they had just experienced. There was no further demonstration of electrical stimuli in the training session.

Experimental session

This took place 1 or 2 days after the training session. Figure 1 shows the time course of the experimental session. Following 15 min of dark adaptation, a baseline measurement of resting pupil diameter in darkness was calculated by taking the mean of three 20-s pupil diameter measurements (at time 0), followed by instillation of the eye drops. Thereafter, the resting pupil diameter in the dark was recorded every 15 min for 60 s at a time. At the point of

maximum response to dapiprazole, the light reflex was elicited and recorded (for fear testing, see below). At the end of the testing, as well as 30 min later (150 min post-instillation), two further recordings of resting pupil diameter in darkness were taken to ensure that the treated pupil was still at the same plateau, as during the fear testing (Fig. 1).

The procedures of the elicitation and recording of the light reflex under safe and threat- of-shock conditions have been described in detail elsewhere (Bitsios *et al*., 1996, 1998a,b). Briefly, subjects were exposed to the same non-painful shock as in the training session (see above) and then a pseudo-switch was emphatically switched on to a 'fifty-fold' shock intensity. This was followed by attachment of electrodes on subjects' left wrists, and the elicitation of the light reflex in seven blocks of three identical light flashes each. The first block was discarded and the remaining six blocks were recorded under regularly alternating Safe and Threat conditions, with the order of their presentation being counterbalanced between subjects. The duration of a block was 20 s and the inter-stimulus interval within a block was kept constant at 6 s. Each block ended 6 s after delivery of the third light flash. To investigate rapid 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 VAS (Bond and Lader, 1974). The interblock interval was 90–120 s, to allow sufficient time for the completion of the visual analogue scales. Thus, the elicitation and recording of the light reflex response lasted for 15 min.

In the Safe condition the subjects were instructed to relax and 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 20 s duration of the block, while a continuous low intensity warning tone (conditioned stimulus) was heard. The subjects did not know the exact number of electric shocks or in which Threat block(s) this would occur. The shocks were described by the experimenter as painful stimuli inducing a short-lived localized unpleasant sensation on the wrist. With these procedures, all subjects were successfully conditioned to be apprehensive in the presence of the warning tone. In reality, no shock was administered during testing.

Data reduction and analysis

The pupillary measures (IPD and light reflex response amplitude) were obtained from both eyes. The computerized average of the three within-block elicited light reflexes was taken as the response of a block. Data for each pupillary measure were collapsed across blocks for the two conditions (Safe, Threat), and the two treatments and the collapsed data entered into the statistical analysis. Separate mixed model analysis of variance (ANOVA) with treatment (placebo, dapiprazole) as the between- and condition (Safe, Threat) as the within-factor were used to analyse the pupillary data. The relationship between IPD and light reflex response amplitude was examined with analysis of covariance (ANCOVA) of the amplitude data with the same factorial design as above, taking IPD as the covariate. Furthermore, for each pupillary measure, the within-

Design of session
Experiment 1
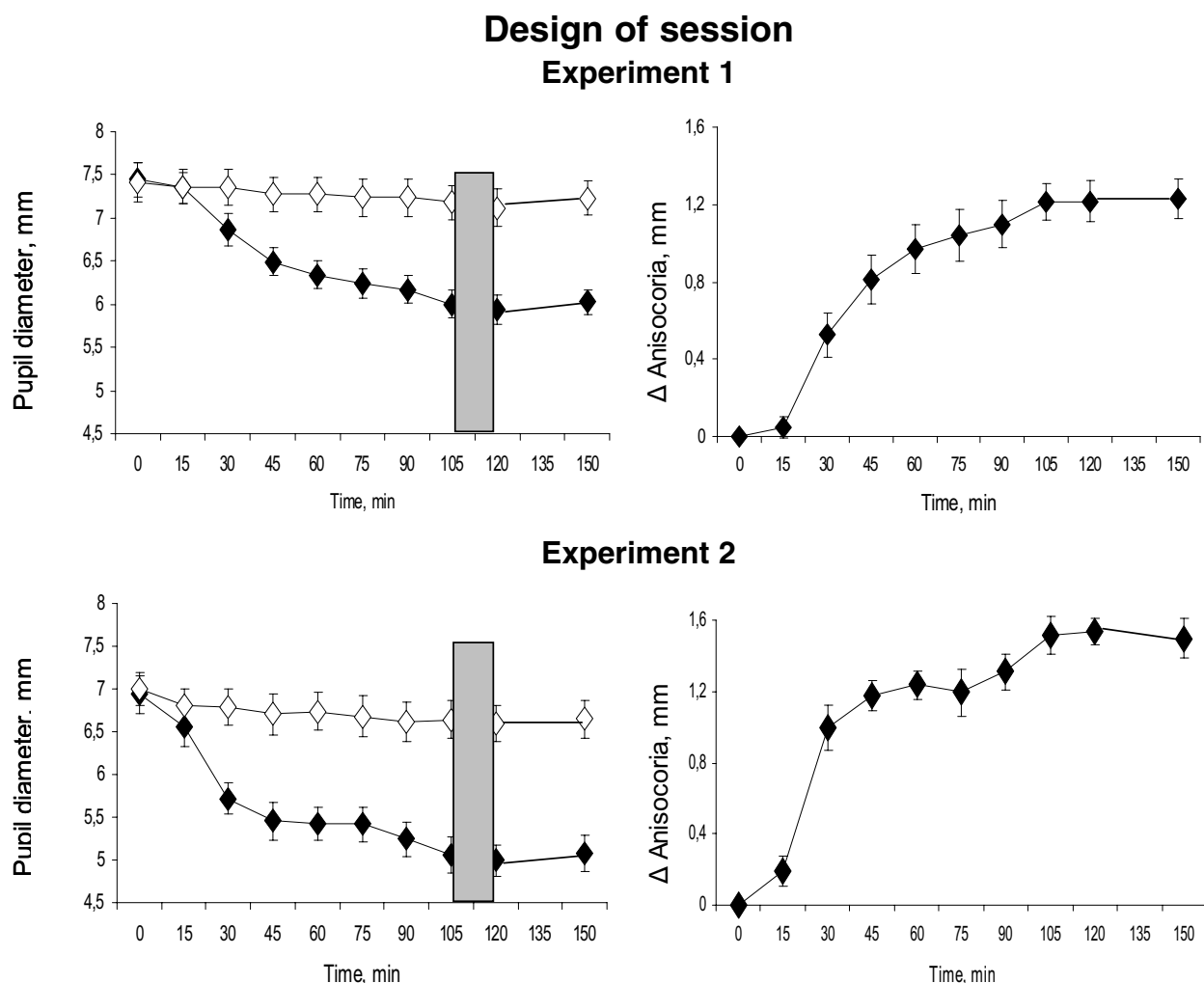
Experiment 2

Figure 1   Experiment 1 is shown in the top panels; Experiment 2 is shown in the bottom panels. Left: Time course of the change in the diameter (mm) of the two eyes, measured in darkness at 15-min intervals between time 0 (pre-treatment baseline) and 150 min following instillation of dapiprazole and placebo eye drops. Open diamonds: placebo-treated; closed diamonds, dapiprazole-treated. Shaded column represents the period of fear testing. Right: time course of anisocoria calculated as the difference from pre-treatment anisocoria. Miotic response to dapiprazole plateaued at 105 min post-instillation. Vertical bars indicate SEM

subject Threat–Safe differences were calculated and they were defined as the individual's response to threat of shock. One-way ANOVAs with treatment (placebo, dapiprazole) as the between factor were used to analyse these data. The VAS measures were obtained as described above (see Subjective ratings), and the average of the weighted values for factors 'alertness' and 'anxiety' was entered in the statistical analysis. Two-way ANOVA (condition × block) with repeated measures was used to analyse the VAS data.

Results

Effects of dapiprazole on resting pupil diameter

Figure 1 (top left) shows the time course of the change in the diameter (mm) of the two eyes, measured at 15-min intervals

between time 0 (pre-treatment baseline) and 150 min following instillation of dapiprazole and placebo eye drops. There was a clear miotic response to dapiprazole already evident at 30 min post-instillation, which reached a maximum at 105–120 min post-instillation, when the elicitation and recording of the light reflex was started. To better display the point of maximum response to dapiprazole, the time course of anisocoria was calculated and shown in Fig. 1 as changes from pre-treatment baseline anisocoria (top right). These were considered a more accurate indicator of the time course of the miotic response to dapiprazole because they take into account the small reduction in pupil diameter of the placebo-treated eye in darkness, which is related to the reduction in the level of arousal during the course of an experimental session involving repeated testing (Loewenfeld, 1993a). Figure 1 shows that a maximum response to dapiprazole occurred at 105 min post-

Table 1 Experiment 1: change in resting pupil diameter from pre-treatment baseline, measured in darkness

Δ Pupil diameter (mm)	Placebo-treated eye			Dapiprazole-treated eye		
	Mean ± SD	Range	95% CI	Mean ± SD	Range	95% CI
105 min	−0.23 ± 0.25	0.88	−0.39, −0.07	−1.45 ± 0.48*	1.57	−1.75, −1.14
120 min	−0.29 ± 0.40	1.41	−0.55, −0.04	−1.51 ± 0.59*	1.88	−1.89, −1.14
150 min	−0.19 ± 0.34	1.15	−0.40, 0.03	−1.42 ± 0.54*	1.83	−1.76, −1.08

*Difference from placebo, $P < 0.001$. 95% CI, 95% confidence interval.

instillation and that the pupil diameter remained at a plateau from that point onwards. Indeed, individual *t*-test comparisons showed that there was no difference in anisocoria at 105, 120 and 150 min. Pre-treatment resting pupil diameters in the dark (mean ± SEM) were 7.45 ± 0.19 mm for the dapiprazole- and 7.41 ± 0.22 mm for the placebo-treated eye. One-way ANOVA revealed no significant difference between the pre-treatment values in the two eyes. The differences from pre-treatment in resting pupil diameter of the placebo- and the dapiprazole-treated eye, measured at 105, 120 and 150 min are shown in Table 1.

Effects of dapiprazole on light reflex and subjective measures

One subject was removed from the analyses due to excessive blinking, which interfered with the scoring of the amplitude of the light reflex. Data from the remaining 11 subjects are therefore presented.

Subjective ratings

The group means of subjective anxiety (left) and alertness (right) obtained with the visual analogue scales across the three safe/threat paired blocks for the two conditions are shown in Figure 2. It is apparent that 'anxiety' but not 'alertness' was greater under the Threat than under the Safe condition. ANOVA of the 'anxiety' data revealed a significant main effect of condition [$F(1,10) = 29.8$, $p < 0.001$] but not a main effect of block ($F < 1$), while there was a trend for a condition × block interaction [$F(2,20) = 3.1$, $p < 0.067$]. ANOVA of the 'alertness' data did not reveal any significant main effects [condition: $F(1,10) = 1.1$, $p > 0.1$, block: $F < 1$] or interaction ($F < 1$).
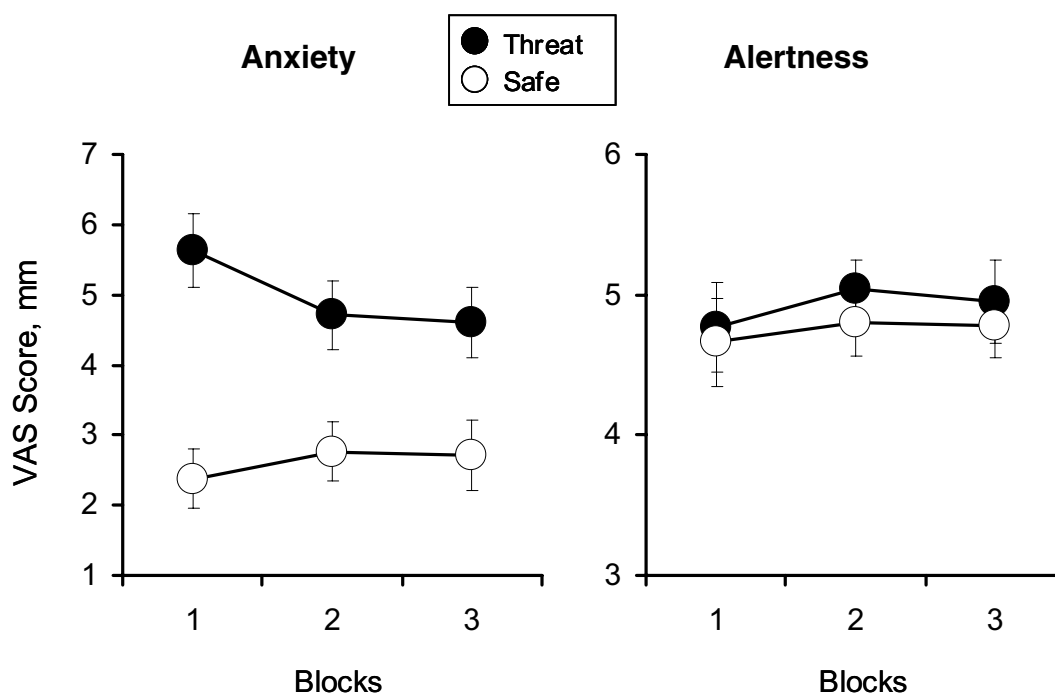
Figure 2 Experiment 1. Results of subjective ratings (mm) of anxiety (left) and alertness (right) obtained on a battery of visual analogue scales. The data points correspond to group means obtained in the group ($n = 11$) across the three Safe and three Threat blocks. Open circles, Safe; closed circles, Threat. Vertical bars indicate SEM
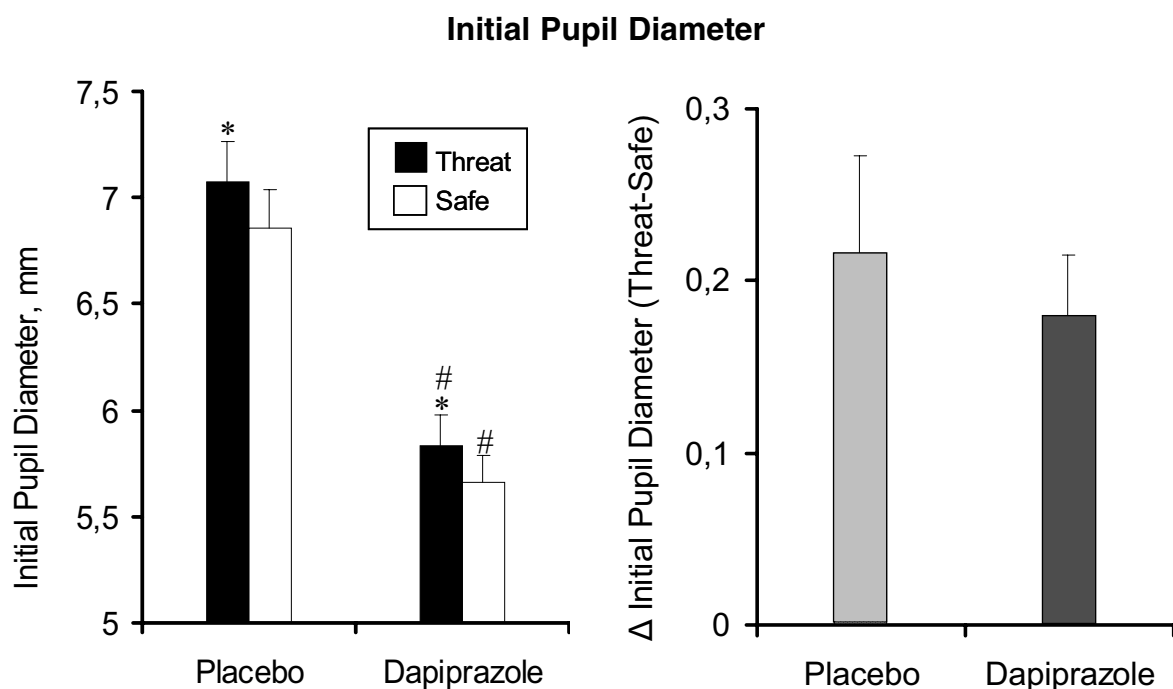
Table 2 Experiment 1: initial pupil diameter and light reflex amplitude (mm) (mean ± SEM) obtained in the dark-adapted pupils of the subjects for the three threat/safe paired blocks in the placebo- and dapiprazole-treated eyes

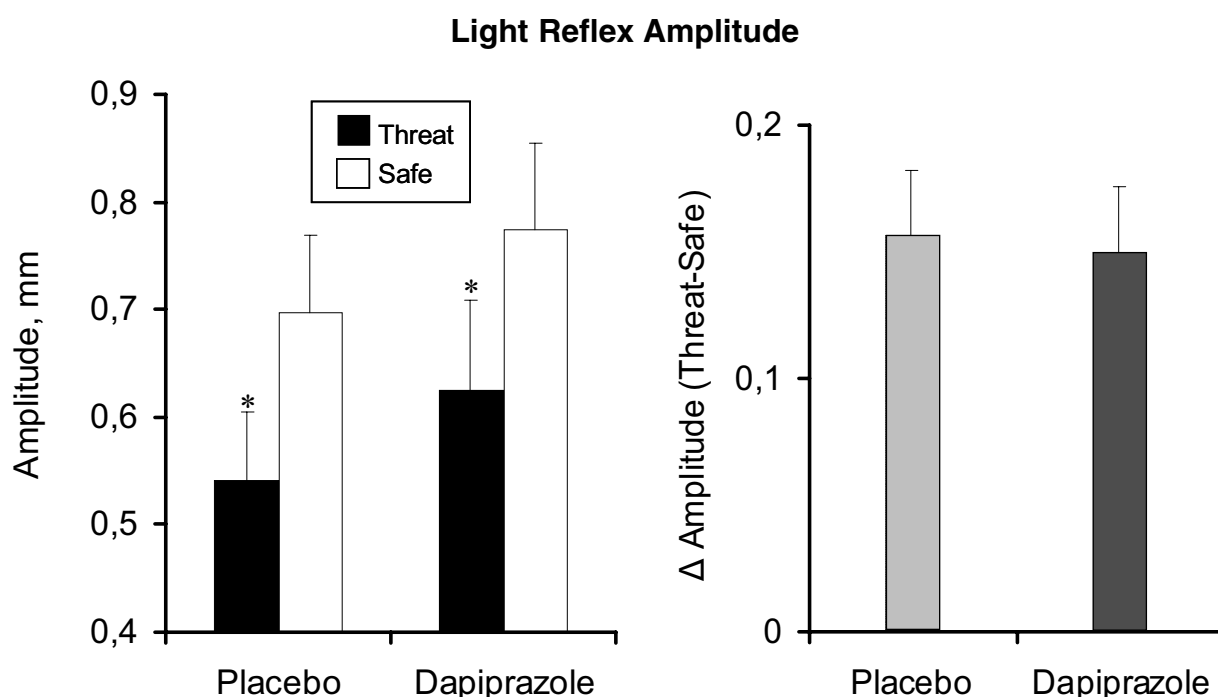|	Placebo		Dapiprazole	
	Threat	Safe	Threat	Safe
IPD				
Block 1	7.18 ± 0.20	6.91 ± 0.20	5.92 ± 0.18	5.68 ± 0.18
Block 2	7.06 ± 0.19	6.85 ± 0.17	5.82 ± 0.14	5.68 ± 0.13
Block 3	6.98 ± 0.20	6.81 ± 0.17	5.77 ± 0.12	5.68 ± 0.12
AMP				
Block 1	0.52 ± 0.06	0.76 ± 0.08	0.60 ± 0.08	0.85 ± 0.08
Block 2	0.53 ± 0.007	0.67 ± 0.07	0.62 ± 0.09	0.76 ± 0.09
Block 3	0.57 ± 0.007	0.66 ± 0.08	0.65 ± 0.09	0.71 ± 0.08

Pupillary measures

IPD was considerably reduced in the dapiprazole-treated eye in both conditions, but it was greater in the Threat condition in both eyes (Table 2 and Fig. 3, left). ANOVA showed a significant treatment [$F(1,20) = 28.3$, $p < 0.001$] and condition [$F(1,20) = 35.2$, $p < 0.001$] main effects, but no significant interaction ($F < 1$). Figure 3 (right) also shows the effect of threat on IPD expressed as the within-subject Threat–Safe difference for the placebo- and the dapiprazole-treated eyes. One-way ANOVA showed that the effect of threat on IPD was not different in the dapiprazole- compared to the placebo-treated eye ($F < 1$).

Light reflex amplitude was slightly greater in the dapiprazole-treated eye in both conditions, but it was smaller in the Threat condition in both eyes (Table 2 and Fig. 4, left). ANOVA revealed a significant main effect of condition [$F(1,20) = 68.8$, $p < 0.001$]. The treatment main effect and the interaction were not significant ($F < 1$). Figure 4 (right) also shows the effect of threat on light reflex amplitude expressed as the within-subject Threat–Safe difference for the placebo- and the dapiprazole-treated eyes. One-way ANOVA showed that the effect of threat on light reflex amplitude was not different in the dapiprazole- compared to the placebo-treated eye ($F < 1$).

Initial Pupil Diameter

Figure 3 Experiment 1. Left: Initial pupil diameter obtained in the dark-adapted pupils of the subjects in the placebo- and dapiprazole-treated eye for the two conditions (Threat, Safe) collapsed across the three Threat and three Safe blocks, respectively. Right: Effect of Threat (Threat–Safe difference) on initial pupil diameter obtained in the placebo- and the dapiprazole-treated eye. The height of the columns corresponds to the mean obtained in the group ($n = 11$). Vertical bars indicate SEM. *Significantly different from Safe; #significantly different from placebo

Light Reflex Amplitude

Figure 4 Experiment 1. Left: Light reflex amplitude obtained in the dark-adapted pupils of the subjects in the placebo- and dapiprazole-treated eye for the two conditions (Threat, Safe) collapsed across the three Threat and three Safe blocks, respectively. Right: Effect of Threat (Threat–Safe difference) on light reflex amplitude obtained in the placebo- and the dapiprazole-treated eye. The height of the columns corresponds to the mean obtained in the group ($n = 11$) . Vertical bars indicate SEM. *Significantly different from Safe

To address the possibility that the changes in amplitude were secondary to the changes in IPD, an ANCOVA of the amplitude data (treatment × condition with IPD as the covariate) was carried out. This analysis revealed a significant effect of the regression in the case of condition [$F(1,19) = 12.8$, $p < 0.005$] but the condition main effect remained significant [$F(1,19) = 11.8$, $p < 0.005$].

Discussion

Consistent with the peripheral blockade of the sympathetic input to the iris dilator muscle, dapiprazole caused significant miosis. The dapiprazole-evoked miosis was present at 30 min post-instillation, reaching a plateau at approximately 105 min post-instillation, when testing was started. One further recording of resting pupil diameter in darkness 30 min after the end of testing (150 min post-instillation) ensured that the treated pupil was still at the same plateau, as during the fear testing (Fig. 1). At the start of testing dapiprazole-evoked miosis measured 1.45 mm in darkness (Table 1) and the anisocoria at that point was 1.22 mm. Most likely, this was a maximal dapiprazole-evoked miosis considering that, in patients with complete unilateral sympathetic paralysis (i.e. Horner's syndrome), the anisocoria between the normal and the defective pupil measures from 0.5 to at most 1.5 mm, even in darkness, when it is most marked (Loewenfeld, 1993b). Moreover, the dose of dapiprazole used in this study (2 × 20 µl, three times) was greater

than the dose (2 × 20 µl, two times) known to effectively reverse (in 120 min) a maximal mydriasis produced by a combination of 2.5% phenylephrine and 0.5% tropicamide (Wilcox *et al*., 1995).

Consistent with its miotic effect, dapiprazole reduced IPD during testing both in the threat and the safe conditions (Fig. 3, left). Dapiprazole also caused a slight but non-significant increase in light reflex amplitude both in the threat as well as in the safe conditions (Fig. 4, left). This suggests that, physiologically, the tone of the dilator muscle may oppose the constriction of the pupil, and the removal of the dilator's tone in the dapiprazole-treated eye is responsible for this effect.

Threat of electric shock was associated with a significant increase in subjective anxiety and a reduction in light reflex response amplitude in the placebo-treated eye, replicating previous results (Bitsios *et al*., 1996, 2002, 2004). Threat of electric shock was associated with a significant reduction in light reflex response amplitude in the dapiprazole-treated eye as well. The lack of interactions involving treatment suggests that threat reduced light reflex amplitude in an identical manner in both eyes, irrespective of treatment. Threat of electric shock was also associated with a significant increase in IPD in both eyes. The lack of interactions involving treatment suggests that threat increased IPD in an identical manner in both eyes, irrespective of treatment. Although both pupillary responses to threat occurred consecutively, changes in amplitude cannot be attributed to changes in IPD as, in agreement with previous studies (Bitsios *et al*., 1996, 1998a,b, 1999),

the analysis of covariance showed no relationship between the two variables. This is consistent with recent results showing that the two responses to threat are not merely different expressions of the same central event but, instead, that the threat-induced reduction in light reflex amplitude is a better correlate of anxiety, whereas the threat-induced increase in IPD reflects general and non-specific arousal mechanisms (Bitsios *et al*., 2004).

The threat-induced reduction in light reflex amplitude (Safe–Threat difference) was identical between the treated and untreated eye (Fig. 4, right). This suggests that blockade of the peripheral sympathetic does not affect the inhibition of the light reflex by threat. Therefore, the latter must be mediated purely through central inhibition of the parasympathetic. We have previously argued that this response to threat is integrated in the pupillary neural circuitry, possibly at the level of the EW pupilloconstrictor nucleus, where it inhibits the firing of its neurones during conditioned fear (Bitsios *et al*., 1996).

In Experiment 1, the threat-induced increase in IPD was identical in the two eyes (Fig. 3, right), which strongly suggests that dapiprazole treatment failed to suppress the effect of threat on IPD. One explanation for this could be that the threat-induced increase in IPD is mediated mainly through central parasympathetic inhibition and that the role of sympathetic excitation in the mediation of this response is absent or negligible. However, this is unlikely because our study was carried out in darkness, and therefore the threat-induced pupillary dilatation must have been due to sympathetic excitation; it is well established that central EW parasympathetic neurones are already switched off in darkness (Lowenfeld, 1958; Nisida *et al*., 1959; Cavaggioni *et al*., 1968; Smith *et al*., 1968; Sillito and Zbrozyna, 1970; Lowenfeld, 1993). Another explanation for the failure of dapiprazole to suppress the threat-induced increase in IPD is the possibility of the operation of a ceiling effect in the placebo-treated eye. Physiological–pharmacological studies have determined that, for most human subjects, the upper end for linear pupillary dilatation (ceiling) is approximately 6–6.5 mm (Newsome and Loewenfeld, 1971; Szabadi, 1977; Longmore *et al*., 1987). Inspection of our data (Table 2 and Fig. 3, left panel) shows that, in the placebo-treated eyes of our subjects, IPD was above the limit of 6–6.5 mm in the safe as well as the threat condition, whereas this was not the case in the dapiprazole-treated eyes. Taken together, this suggests that mechanical limitations of the iris could have curtailed the effect of threat in the placebo-treated eye and thus could have masked an effect of dapiprazole on IPD.

Therefore, we hypothesize that a possible effect of dapiprazole on the IPD may be revealed if the dark-adapted pupils of the placebo-treated eyes were sufficiently small in the safe condition to allow more scope for an increase in the threat condition. To explore this possibility, we repeated the study with another group of subjects with smaller dark-adapted pupils, using identical procedures.

Experiment 2

This experiment examined the effects of dapiprazole on resting pupil diameter and the IPD measure of the light reflex. Eight healthy volunteers (four male, four female), mean ± SD (range) age 24.5 ± 2.5 (20–25) years, participated in Experiment 2. Drugs, stimuli and apparatus, procedures and data reduction and analysis were identical to those reported in Experiment 1. We report effect sizes (η^2) because of the small number of subjects participating in this experiment.

Results and Discussion

This group had a smaller mean pre-treatment resting pupil size in darkness (by almost 0.5 mm) compared to the group of healthy subjects participating in Experiment 1. Indeed, pre-treatment resting pupil diameters in the dark (mean ± SE mean) were 6.93 ± 0.21 mm for the dapiprazole- and 7.00 ± 0.19 mm for the placebo-treated eye. One-way ANOVA revealed no significant difference between the pre-treatment values in the two eyes. Dapiprazole-evoked miosis and the resulting anisocoria in this group of subjects had an almost identical time-course to that observed in Experiment 1 (Fig. 1, bottom left and right panels). It is evident that a maximum response to dapiprazole occurred at 105–120 min post-instillation, when the elicitation and recording of the light reflex was started. Individual comparisons (*t*-test) showed that there was no difference in anisocoria at 105, 120 and 150 min, suggesting that the miotic response of the pupil had reached a plateau. The differences from pre-treatment in resting pupil diameter (mm) of the placebo- and the dapiprazole-treated eye, measured at 105, 120 and 150 min are shown in Table 3.

IPD was much reduced in the dapiprazole-treated eye in both conditions but it was greater in the Threat condition in both eyes. However, it can be seen that the threat-induced increase in IPD was suppressed in the dapiprazole-treated eye (Table 4 and Fig. 5, left). These impressions were confirmed by two-way ANOVA, which

Table 3 Experiment 2: change in resting pupil diameter from pretreatment baseline, measured in darkness

Δ Pupil diameter (mm)	Placebo-treated eye			Dapiprazole-treated eye		
	Mean ± SD	Range	95% CI	Mean ± SD	Range	95% CI
105 min	−0.36 ± 0.19	0.66	−0.52,−0.20	−1.87 ± 0.21*	0.60	−2.05,−1.70
120 min	−0.41 ± 0.17	0.55	−0.55,−0.26	−1.94 ± 0.20*	0.63	−2.11,−1.77
150 min	−0.36 ± 0.19	0.66	−0.52,−0.20	−1.86 ± 0.20*	0.60	−2.02,−1.69

*Difference from placebo, *P* < 0.001. 95% CI, 95% confidence interval.

Table 4 Experiment 2: initial pupil diameter (mm) (mean ± SEM) obtained in the dark-adapted pupils of the subjects for the three threat/safe paired blocks in the placebo- and dapiprazole-treated eyes

	Placebo		Dapiprazole	
IPD	Threat	Safe	Threat	Safe
Block 1	6.89 ± 0.18	6.45 ± 0.17	5.07 ± 0.16	4.79 ± 0.13
Block 2	6.85 ± 0.19	6.51 ± 0.17	5.09 ± 0.15	4.91 ± 0.13
Block 3	6.84 ± 0.14	6.47 ± 0.10	5.17 ± 0.13	4.98 ± 0.12
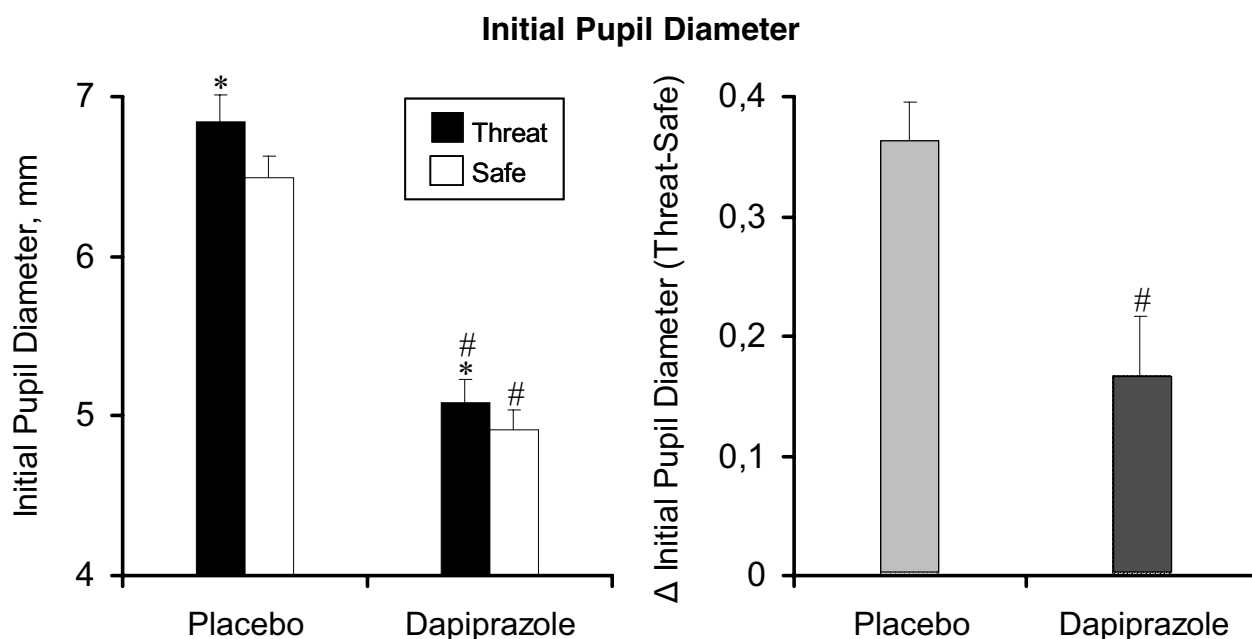
Figure 5 Experiment 2. Left: Initial pupil diameter obtained in the dark-adapted pupils of the subjects in the placebo- and dapiprazole-treated eye for the two conditions (Threat, Safe) collapsed across the three Threat and three Safe blocks, respectively. Right: Effect of Threat (Threat–Safe difference) on initial pupil diameter obtained in the placebo- and the dapiprazole-treated eye. The height of the columns corresponds to the mean obtained in the group ($n = 8$). Vertical bars indicate SEM. *Significantly different from Safe; #significantly different from placebo

revealed significant treatment [$F(1,14) = 68.4$, $p < 0.001$, $\eta^2 = 0.834$], and condition [$F(1,14) = 58.53$, $p < 0.001$, $\eta^2 = 0.807$] main effects, but also a significant treatment by condition interaction [$F(1,14) = 7.4$, $p < 0.05$, $\eta^2 = 0.345$]. Most importantly, the effect of threat on IPD, expressed as the within-subject Threat–Safe difference was significantly smaller in the dapiprazole- compared to the placebo-treated eye (Fig. 5, right) as revealed with one-way ANOVA [$F(1,14) = 10.4$, $p < 0.01$]. The threat-induced increase in IPD in the untreated eye in Experiment 1 was much smaller than that observed in Experiment 2 (Fig. 3, right and Fig. 5, right), consistent with our hypothesis of a ceiling operating in Experiment 1, which was effectively removed in Experiment 2 by choosing subjects with smaller pupils. Accordingly, the results of Experiment 2 confirm our original hypothesis that the threat-induced increase in IPD would be suppressed by dapiprazole, and also the hypothesis for the masking of this effect in the previous group of subjects due to the operation of the 'ceiling'.

Conclusion

The present study confirms a dissociation between the threat-induced decrease in light reflex amplitude and the threat-induced increase in pupil diameter. The inhibition of the light reflex by threat was unaffected by dapiprazole whereas the threat-induced increase in IPD was suppressed when care was taken to avoid the confounding influence of a ceiling effect. Therefore, the inhibition of the light reflex by threat is likely to reflect central parasympathetic inhibition and is unlikely to involve the peripheral sympathetic innervation of the iris. Moreover, the threat-induced increase in IPD is likely to reflect mainly central sympathetic excitation. Although closely related, parasympathetic inhibition and sympathetic excitation may be dissected by way of their influence on pupillary movements. The different central autonomic mechanisms underlying the two pupillary responses to threat may explain the dissociation between the separate effects of threat on IPD and

light reflex amplitude and may provide the neurobiological basis for our findings.

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