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ORIGINAL INVESTIGATION

**Johanna M. P. Baas · Christian Grillon
Koen B. E. Böcker · Anouk A. Brack
Charles A. Morgan III · J. Leon Kenemans
Marinus N. Verbaten**

Benzodiazepines have no effect on fear-potentiated startle in humans

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Abstract *Rationale:* Pre-clinical and clinical investigations have provided a great deal of evidence that the fear-potentiated startle paradigm represents a valid model for the objective assessment of emotional states of anxiety and fear. *Objective:* The four studies presented in this report sought to further validate the "threat of shock" paradigm as a human analogue to fear-potentiated startle in rats, by examining the effect of benzodiazepine administration on both baseline and fear-potentiated startle. *Methods:* Three studies, conducted at Utrecht University, evaluated the effects of oxazepam and of diazepam on baseline and fear-potentiated startle, whereas a fourth study, conducted at Yale University, evaluated the effect of diazepam on baseline, contextual and cue-specific fear-potentiated startle. The threat of shock paradigm consisted of verbal instruction about two visual cues (the threat cue predicted the possible administration of electric shock, the other predicted a safe period), followed by a series of presentations of these cues. During these conditions, acoustic startle stimuli were presented in order to elicit startle responses. The magnitude of the startle response was used to index the degree of fear or alarm experienced during the periods of threat and safety. The fourth study examined the effect of IV administration of diazepam in a similar threat of shock paradigm except that there were two additional context manipulations: electrode placement and darkness. *Results:* None of the drug manipulations affected specific threat-cue potentiation of startle. However, reductions in baseline startle were observed. Further, startle potentiation by darkness was inhibited by diazepam. *Conclusions:* At least one type of fear-potentiated startle, i.e. potentiation by a cue-specific fear manipulation, is not susceptible to benzodiazepine treatment. In contrast, effects of manipulations more akin to anxiety (darkness, context) appear sensitive to benzodiazepines. Human experimental models differentiating between these cue specific and contextual responses are needed to shed more light on differences in the anatomy and pharmacology of anxiety disorders.

Keywords Fear · Anxiety · Benzodiazepine · Oxazepam · Diazepam · Startle reflex

J.M.P. Baas · K.B.E. Böcker · A.A. Brack · J.L. Kenemans
M.N. Verbaten
Department of Psychopharmacology, Faculty of Pharmacy,
Utrecht University, P.O. Box 80082, 3508 TB Utrecht,
The Netherlands
e-mail: baasj@intra.nimh.nih.gov
Tel.: +1-301-402-7220, Fax: +1-301-402-6353

C. Grillon · J.M.P. Baas (✉)
Mood and Anxiety Disorder Program, NIMH, NIH,
15k North Drive MSC 2670, Bethesda, MD 20892, USA

C.A. Morgan III
Department of Psychiatry, Yale University School of Medicine,
Connecticut VA Medical Center, 950 Campbell Ave, West Haven,
CT 06516, USA

Introduction

A large body of experimental literature indicates that the startle reflex is a sensitive measure of fear and anxiety. Startle models derive face validity from the clinical observation that the startle reflex is exaggerated in some anxiety disorders (i.e. in post-traumatic stress disorder; Morgan et al. 1995; Grillon et al. 1998; cf. DSM IV, American Psychiatric Association 1994). In addition, the startle reflex is potentiated by fear and anxiety in several animal models, e.g. fear-potentiated startle (FPS) and light-enhanced startle (Walker and Davis 1997a). Further, drugs that increase anxiety (i.e. yohimbine) also increase startle in animals (Davis et al. 1979) and in humans (Morgan et al. 1993). Finally, drugs that reduce anxiety in humans, such as diazepam and buspirone, also decrease the magnitude of fear-potentiated startle in animals (see Davis et al. 1993, for a review). Similarly, buspirone (Walker and Davis 1997a) and chlordiazepoxide (De Jongh et al. 2002) decrease light-enhanced startle. These results suggest that the potentiation or enhancement of startle in these models result from an anxiogenic action. Further support for this view stems from studies

showing that the amygdala, a structure that has been shown to be involved in fear and anxiety, is crucial to the orchestration of startle potentiation (e.g. Hitchcock and Davis 1986).

Although the fear-potentiated startle animal model has generated valuable insight into the pharmacology of fear and anxiety, humans are the ultimate targets for pharmacological treatment. In humans, the eye blink component of the startle reflex has proven to be a reliable probe of emotional reactivity both in terms of interpretation and robustness using procedures such as classical conditioning (e.g. Hamm et al. 1993), threat of shock (Grillon et al. 1991), and emotional valence (e.g. Lang et al. 1990).

The threat of shock paradigm (also called anticipatory anxiety or verbal conditioning) applied in the present experiments was introduced in the literature as a possible human analogue of the animal fear-potentiated startle model (Grillon et al. 1991). In this paradigm, startle is measured during verbally instructed threat of shock and safe conditions. This procedure yields a highly robust startle potentiation in healthy human subjects in the threat compared to the safe condition (Grillon et al. 1991, 1993; cf. Baas et al. 1999). Theoretically, the difference in startle magnitude between safe and threat conditions (i.e. fear-potentiated startle) should provide a crucial test of the anxiolytic properties of psychopharmacological agents.

To date, few studies have investigated the effect of anxiolytics on fear-potentiated startle in humans. Bitsios et al. 1999) reported that 10 mg diazepam blocked startle potentiation by threat of shock. In a related study (Patrick et al. 1996), 15 mg diazepam was found to suppress the increase startle during the viewing of unpleasant/disturbing pictures. Also, very recently, Riba et al. have shown a decrease of startle potentiation by alprazolam (Riba et al. 2001).

There are numerous reasons to regard the potentiated startle reflex as a valuable measure for pharmacological studies of fear. Besides the ones already mentioned, i.e. clear face validity, well-defined neuronal system, and cross-species generalization, both human and animal studies show startle potentiation to be more specific to negative emotional states than to arousal per se (e.g. Lang et al. 1990; Schmid et al. 1995). One characteristic that is especially interesting for pharmacological purposes is that startle has a non-zero baseline. Hence, besides specific drug effects, non-specific effects on baseline startle can be evaluated (Davis 1990).

Non-specific effects, such as sedation or muscle relaxation, might affect the magnitude of startle, as shown in humans in several studies (e.g. Abduljawad et al. 1997; Rodriguez-Fornells et al. 1999). However, recently Guscott et al. (2000) argued that these effects can also reflect a reduction in anxiety to an anxiogenic context. The results from their study are consistent with this view, because these suggest that the effect of the benzodiazepine chlordiazepoxide on baseline startle in a fear experiment is dependent on the presence of an anxiogenic context (Guscott et al. 2000). The implication is that modulation of baseline startle magnitude by drugs in experiments involving fear manipulations cannot automatically be attributed to non-specific effects. Consequently, part of the drug-induced decreases in baseline startle commonly referred to as non-specific effects (e.g. sedation, muscle relaxation), may actually reflect reduction of context-induced anxiety. We employed subjective measures to explore the contribution of anxiolysis to reduction of baseline startle by benzodiazepines.

In this report, the results of four experiments are presented, three of which were conducted at Utrecht University, and the fourth at Yale University. The aim of these studies was to validate the threat of shock paradigm as a model for evaluating the impact of pharmacological modulation on fear and anxiety states in humans. The Utrecht University studies examined the effects of two common benzodiazepine anxiolytics in a threat of shock procedure adapted from Grillon et al. (1991; see Baas et al. 1999). This procedure employed neutral abstract stimuli as threat cues, which induced highly robust startle potentiation in previous studies (Baas et al. 1999). The first experiment tested oxazepam in a between-subjects design. Oxazepam was chosen for its clinical efficacy, its absence of active metabolites and short half-life. Subsequently, because of its reported effectiveness in reducing human fear-potentiated startle, diazepam was tested both in a between (expt II) and a within-subjects (expt III) design. Finally, experiment IV was conducted independently from experiments I–III at Yale University. The effects of diazepam administered intravenously (IV) were tested in an extended design that included additional anxiety manipulations such as instructed threat of shock, application of the shock electrodes, and darkness as context variables (see Grillon and Ameli 1998). In all experiments, contribution of anxiolysis and sedation to drug effects on baseline startle were explored by means of regression analyses.

Experiment I: oxazepam (between-subjects)

Materials and methods

Subjects

Thirty-six healthy volunteers, students at Utrecht University, participated in the study. Subjects were excluded if they had a history of psychiatric or neurologic disorders. Subjects had not taken medication in the week prior to the study, and abstained from alcohol and caffeinated beverages 24 prior to each experimental session. Subjects were paid DFL 100 (approximately $50), for participation. Group statistics were: sex 13 males, 23 females (Placebo 4/8, Low dose 4/8, High dose 5/7), mean age 21.75 year, SD 2.1 (Placebo 21.75, Low dose 22.0, High dose 21.5), mean weight 69.4 kg, SD 10.9 (Placebo 67.3, Low dose 69.8, High dose 70.9), Mean Trait Anxiety Score 32.5, SD 6.6 (Placebo 31.7, Low dose 33.3, High dose 32.5).

Drug manipulation

Oxazepam was studied in two doses (15 mg, 30 mg) in a double blind placebo controlled between-subjects design. Subjects were

assigned to drug groups in such a way that groups were balanced on sex, weight, age, and trait anxiety scores.

Procedure

The studies presented were approved of by the local ethics committee (at Utrecht University the university hospital's committee). During a separate screening session, subjects first provided written informed consent, and were subjected to a medical examination. The Threat of Shock procedure was described in detail in the consent form and indicated to the subjects that they would receive from one to three electric shocks during the test. In addition, subjects were told that the shocks would be harmless but unpleasant and possibly painful.

On the test day, and prior to receiving active drug or placebo, subjects first filled out a state anxiety questionnaire and a sedation scale. Next, they ingested a capsule containing oxazepam or placebo. Subjects paused for 2 h to allow drug absorption, after which the procedure to apply measurement and shock electrodes was started. Instructions concerning the shock procedure were repeated and subjects once again filled out the post-treatment state anxiety questionnaire and the sedation scale (post-ingestion of placebo or active drug), after which they entered the experimental room. During testing, subjects were first presented a habituation block consisting of 12 startle probes. This was followed by the specific instruction about which stimulus would indicate threat and which safe. This instruction consisted of a visual representation of the threat and safe cues (vertical square wave grating patterns with either wide or small bars counterbalanced across subjects), underneath which the words "shock" or "safe" were printed to indicate whether or not electric shocks would be administered during subsequent presentations of that stimulus. After the habituation phase was completed, the experimenter entered the experimental room and announced that the shock electrodes would be connected to the shock stimulator. Subjects watched the experimenters connect the electrodes to the stimulator. The experimental run was started at 2.5 h after drug ingestion, around peak plasma-level of oxazepam. The visual pairing of the cues with the words "shock" and "safe" was repeated before the start of the experiment proper, during which only the cues would indicate the condition. The experimental run consisted of six additional habituation startle probe trials. Subsequently, the shock and safe cues were presented alternating eight times each (cue duration: 50.4 s). Three startle probes were presented during each of the threat and safe periods. Time intervals between probes ranged from 15.2 to 18.4 s (steps of 0.8 s; rectangular distribution). Halfway through the experiment, a startle probe was replaced by a shock. The shock replaced either the last probe of the fourth threat period or the first probe of the fifth threat period, depending on with which condition the experiment had started. The experimental procedure took 13.4 min. Afterwards, subjects were asked retrospectively to rate their subjective feeling during the threat and the safe periods using a shortened version of the state anxiety questionnaire. Finally, 3 h after ingestion subjects rated their degree of sedation for the third time.

Measurement and apparatus

EMG was measured with 2 mm Ag/AgCl Sensor Medics disc electrodes placed on the orbicularis oculi of the right eye, centered under the pupil. The signal was fed through a Tönnies amplifier with a time constant of 50 ms, and low pass cutoff of 300 Hz. AD conversion sample rate was 1000 Hz. One single shock (100 ms duration, 1.8 mA) was administered. It was delivered by a constant current stimulator through tin cup electrodes placed on the inner side of the subjects' right wrist.

Startle probes were delivered by a Neuroscan Stim audio system, through earphones with foam earplugs (Earlink, Aero Company auditory systems). Startle probes were 50 ms duration, 115 dBA broad-band noises with instantaneous rise/fall time. Visual stimuli were presented on a NEC Multisync computer screen.

Physiological data scoring and analysis

Startle reflex EMG was analyzed during epochs of –50–150 ms with respect to startle probe onset. Raw amplifier data were transformed to μV, baseline corrected, rectified, baseline corrected again and subsequently smoothed, using a moving average with a window of 40 ms. A peak was scored between 20 and 100 ms in the smoothed signal. Startle data was averaged per condition (threat and safe). For all statistical analyses SPSS 10.0.5 for windows was used. Overall analyses were repeated measures ANOVAs. Post-hoc tests of significant interaction effects were carried out using *t*-tests. Corrections are indicated by Greenhouse-Geisser epsilon adjusted degrees of freedom. Baseline startle drug effects were evaluated on habituation trials with a repeated measures ANOVA with Phase (separate habituation run, habituation trials in the experimental run) as within-subject and Drug as a between-subject factor. The factor phase was included because between these phases the visual threat instruction occurred and the shock electrodes were connected. Analysis of experimental effects included the within-subjects factor Threat (Threat, Safe) averaged over blocks, and the between-subjects factor Drug (Placebo, 15 mg, and 30 mg). Analyses were performed on eye blink magnitude scores. An alternative analysis was conducted on startle potentiation expressed as percentage of the safe amplitude, to control for some of the variation in startle magnitude between subjects, especially systematic variation caused by the drug treatment.

Subjective ratings

Subjects rated their subjective state of anxiety using the state portion of the State-Trait Anxiety Inventory (STAI, Dutch version; Van der Ploeg et al. 1979) at several intervals during the experimental session. Pre-treatment (upon entering the laboratory) and post-treatment (after electrode application, just before subjects entered the experimental room) ratings were obtained with the complete questionnaire. As indicated above, for retrospective evaluation of subjective feeling during the test, a shortened version (eight of the 20 items) was employed. A measure of sedation was taken at these same times during the test day, as well as after the experimental procedure, by means of a Visual Analogue Scale (VAS). The VAS consisted of a 10 cm long line that indicated on the leftmost end "Very sleepy/drowsy" and on the rightmost end "Very awake/alert".

Results

Startle reflex

Baseline startle magnitude was reduced by the active drug treatment (Fig. 1A), but this effect was not significant [$F(2,32)=1.5$, n.s., linear polynomial contrast $P<0.1$]. The drug did not differentially affect the two phases [interaction with Drug, $F(2,32)=2.0$, n.s.].

Figure 1B shows the startle results for threat and safe in the drug conditions. There was a highly significant main effect of Threat, [$F(1,33)=68.0$, $P<0.001$]. Again, startle magnitude was smaller in the active compared to the placebo condition, there was a significant polynomial linear contrast ($P<0.05$) [main effect $F(2,33)=2.3$, n.s.]. However, the interaction Threat×Drug was not significant [$F(2,33)=0.5$, n.s.], as was the linear polynomial contrast of this interaction ($P=0.4$). When results were expressed as percent scores, similar results emerged [$F(2,33)=0.4$, linear trend $P=0.4$]. If anything, startle potentiation

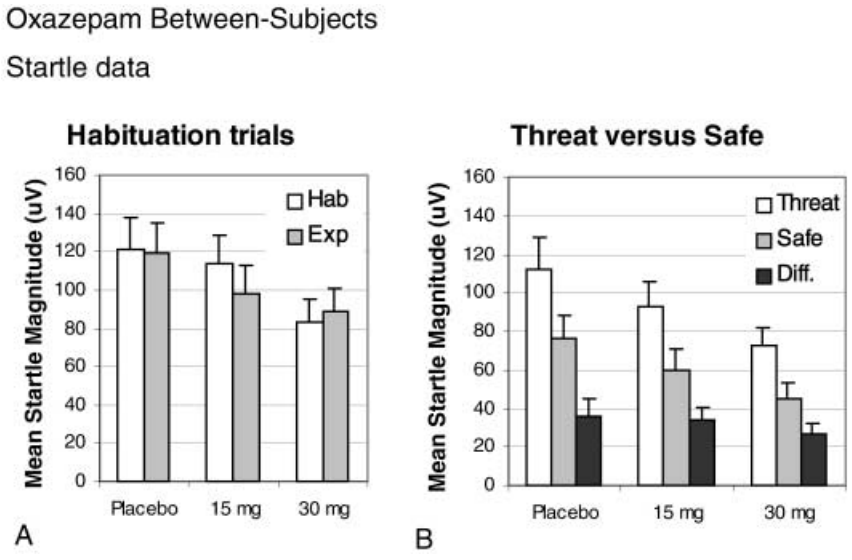

Fig. 1A,B Startle results from experiment I. **A** Habituation trials from the separate habituation run (*Hab*) and from the experimental run (*Exp*). **B** The absolute values for Threat×Drug, along with the difference score for startle potentiation

Table 1 Mean (SEM) values of subjective measures in experiments I–III

	Experiment I Oxazepam			Experiment II Diazepam between		Experiment III Diazepam within		
	Placebo	15 mg	30 mg	Placebo	15 mg	Placebo	10 mg	15 mg
STAI								
Pre	34.5 (2.5)	34.2 (2.6)	32.8 (2.2)	34.4 (2.1)	34.9 (1.7)	32.4 (2.4)	32.3 (2.5)	32.6 (2.7)
Post	34.4 (3.3)	32.2 (2.2)	35.7 (2.9)	33.1 (1.7)	33.9 (1.4)	33.1 (2.2)	31.8 (2.1)	32.9 (2.3)
STAI Retrosp.								
Safe	16.6 (1.4)	14.8 (1.0)	15.7 (0.6)	17.1 (1.0)	15.2 (0.6)	15.8 (0.9)	14 (1.0)	13.6 (1.2)
Threat	22.6 (1.7)	21.8 (1.3)	21.0 (1.8)	23.7 (1.0)	19.9 (1.2)	21.4 (1.5)	17.9 (1.0)	17.7 (1.4)
Sedation								
Pre	3.8 (0.5)	3.9 (0.5)	3.2 (0.4)	4.0 (0.4)	3.9 (0.4)	2.5 (0.3)	3.9 (0.4)	3.1 (0.5)
Post-early	3.8 (0.6)	5.3 (0.6)	6.2 (0.4)	5.0 (0.4)	5.3 (0.3)	3.6 (0.4)	4.8 (0.5)	4.7 (0.6)
Post-late	3.9 (0.6)	5.6 (0.6)	6.7 (0.4)	5.0 (0.4)	6.5 (0.3)	4.4 (0.4)	5.2 (0.6)	6.5 (0.6)

increased (non-significantly) with increasing dose of oxazepam, means (SEM) were Placebo 58% (18), 15 mg 67% (12), and 30 mg 77% (15).

State anxiety

Subjective ratings are presented in Table 1. Subjective anxiety did not differ before and after drug absorption [$F(1,33)=0.5$, n.s.; no interaction with Drug $F(2,33)=2.5$, n.s.]. In the retrospective questionnaires after the threat of shock procedure, subjects reported significantly more anxiety during the shock condition than during the safe condition [$F(1,33)=62.1$, $P<0.001$]. Drug had neither a main effect [$F(2,33)=.4$, n.s.], nor an interaction with Threat [$F(2,33)=0.4$, n.s.].

Correlation between startle and STAI

Because there were no main effects of drug on startle and state anxiety, no analyses on their correlation were performed.

Sedation

Subjects reported moderate alertness before ingesting the capsule (mean of 3.6). An overall repeated measures ANOVA revealed a Time×Drug interaction [$F(2,32)=3.6$, $P<0.01$] and a Time main effect [$F(2,32)=13.2$, $P<0.001$], reflecting an increase in sedation in the drug groups, as compared to the placebo group (Table 1). Because there were no differences between drug groups in the pre-treatment measurement (t-values all <1.2), post-treatment measures could be corrected for pre-ingestion ratings. In the analysis of these corrected values for 2 and 3 h after ingestion, there was an effect of drug group on sedation [main Drug $F(2,33)=8.2$, $P<0.001$, interaction with Time $F(2,33)=0.3$, n.s.]. The linear trend contrast for the effect of drug was highly significant ($P<0.001$), and the quadratic was not ($P=0.9$). These results suggest that oxazepam induced sedation in a dose-dependent manner.

Discussion

Contrary to expectation, there was no anxiolytic effect of oxazepam on the potentiated startle. This result cannot

be attributed to insufficient induction of fear because both physiological measures and subjective reports attested of high anxiety induced by the threat cue. Indeed, startle potentiation was highly significant in each drug group and the subjective ratings of fear/anxiety were greater in the threat than in the safe condition. In addition, the drug was active as shown by the dose-dependent increase in sedation and by the decreasing trend effect of drug on overall startle magnitude.

In summary, oxazepam did not affect our direct measures of fear, i.e. startle and subjective fear ratings, but increased the subjective measure of sedation. Clinically, the initial phase of benzodiazepine treatment is marked by predominant sedative effects, to which tolerance develops within a week (Feldman et al. 1997). This is consistent with the present effects of acute administration that indicate sedative but no anxiolytic effects. Having excluded the possibility that the drug was not physiologically effective, two possible explanations for the absence of anxiolytic effects on startle potentiation may be considered. First, oxazepam might not be the ideal validation drug; it has failed occasionally to show robust anxiolytic effects in animal models (e.g. Joordens et al. 1996). Second, the drug was manipulated between groups of subjects, and individual differences in startle potentiation may have masked differences in startle potentiation between the drug groups. In the Bitsios et al. (1999) study, reduction of fear-potentiated startle was found with diazepam and in a within-subjects paradigm, indicating that either difference might be crucial. In experiment II the drug was changed: we studied diazepam in a between-subjects design comparing a high dose (15 mg) with placebo. We capitalized on sufficient statistical power for the largest effects by increasing group size to 18, as in Patrick et al. (1996), at the cost of not including a low dose.

Experiment II: diazepam (between-subjects)

Materials and methods

Subjects

The sample of subjects was comparable to the oxazepam study. A group of 29 subjects participated in the one-session between-subjects study. The data from the first session of nine subjects from the within-subjects study (see below), who were treated in the first session with either placebo or 15 mg, was included in this sample, yielding data of 38 subjects in total. Inclusion and exclusion criteria were identical to the oxazepam study. Subjects were paid DFL 80 for their participation. Group statistics were: sex 12 males, 26 females (Placebo 5 M/14 F, 15 mg 7 M/12 F), mean age 21.0 year, SD 2.2 (Placebo 21.1, 15 mg 20.9), mean weight 66.1 kg, SD 9.9 (Placebo 65.1, 15 mg 67.0), Mean Trait Anxiety Score 34.8, SD 7.1 (Placebo 34.6, 15 mg 35.1).

Drug manipulation

Diazepam differs from oxazepam in its faster onset of action (higher lipid-solubility results in faster absorption from the gut) and its longer half-life. In addition, diazepam has active metabolites, among which oxazepam (Feldman et al. 1997). Diazepam was studied in a double blind between-subjects design using a high dose (15 mg) and placebo. This relatively high dose of diazepam was chosen to ensure effectiveness. This is the same high dose that was effective in the study by Patrick et al. (1996), who also included a lower dose of 10 mg, while exceeding the dose that proved to be effective in a model similar to the present one (Bitsios et al. 1999). The procedure of assigning subjects to drug groups was identical to that of the first experiment.

Procedure

The screening and experimental procedures were identical to the oxazepam study, except that diazepam reaches peak effectiveness faster (Baldessarini 1996), hence the threat of shock procedure was run 1 h (instead of 2.5 h) after ingestion. Measurement, apparatus, data scoring and analyses were all identical. In the first session in the within-subjects study, no shock reinforcement was given halfway the experimental run. See the procedure section of experiment III for details on these subjects' instruction.

Baseline startle and subjective ratings

Contributions of STAI scores and drug manipulation to explaining the variation in startle magnitude were tested in a multiple regression model. Only residual effects of drug after variation caused by anxiety has been accounted for will be interpreted as non-specific.

Results

Startle reflex

Diazepam reduced baseline startle in the habituation trials (Fig. 2A) [$F(1,35)=5.2$, $P<0.03$]. As in experiment I, the Threat main effect (see Fig. 2B) was highly significant [$F(1,36)=58.7$, $P<0.001$]. The Drug main effect was also significant [$F(1,36)=13.4$, $P<0.001$] (mean Placebo=96 μV, mean High dose=53 μV). However, the Threat×Drug interaction was neither significant in the analysis of the difference in absolute amplitudes threat minus safe [$F(1,36)=2.2$ or $t(36)=-1.5$, $P=0.15$, n.s.], nor in the percentage potentiation [means (SEM) Placebo 44% (10) and 15 mg 47% (12); $t(36)=0.2$, n.s.].

State anxiety

Overall, subjects reported a slight decrease in their subjective anxiety following drug ingestion (34.7 versus 33.5, see Table 1), but this difference was not significant [$F(1,36)=1.4$, n.s.]. Drug treatment had no effect on these state anxiety measures (main Drug $F=0.11$, interaction with pre/post-treatment $F=0.03$, both n.s.). Retrospectively, subjects reported greater anxiety during threat than during safe conditions [Threat: $F(1,36)=90.6$, $P<0.001$]. The overall anxiety level was lower in the diazepam compared to the placebo group [Drug: $F(1,36)=5.0$, $P<0.04$]. However, the Threat×Drug interaction was not significant [$F(1,36)=2.2$, n.s.], indicating that diazepam did not affect specifically anxiety during threat.

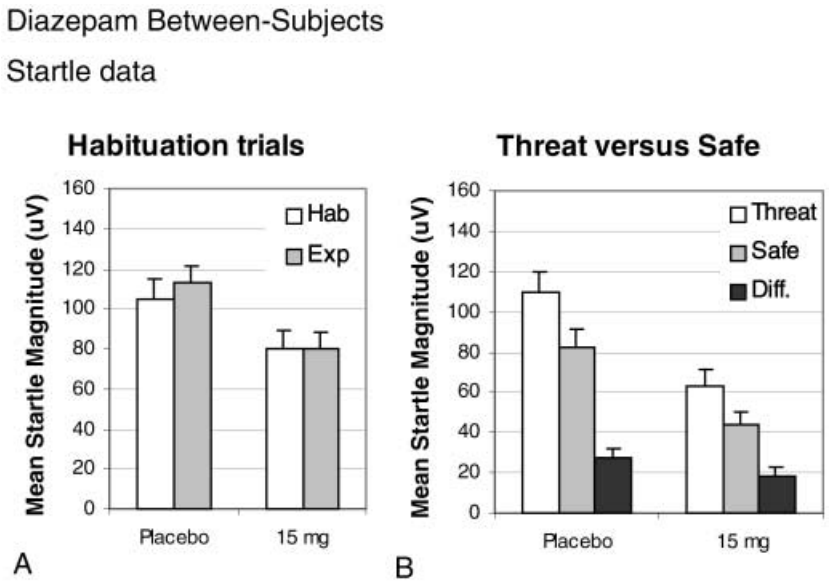

Fig. 2A,B Startle results from Experiment II. **A** Habituation trials from the separate habituation run (*Hab*) and from the experimental run (*Exp*). **B** The absolute values for Threat×Drug, along with the difference score for startle potentiation

Correlation between startle and STAI

Startle magnitudes during the experimental procedure correlated significantly with retrospectively reported state anxiety. When entered in a linear regression model, retrospectively reported state anxiety significantly predicted variation in magnitude of startle (both measures averaged over threat and safe; Beta=0.51; t=4.0, P<0.001). In addition, Drug (Beta=0.34; t=2.7, P=0.01) still accounted for a significant part of the residual variation. Interestingly, entering the subjective rating of sedation (1 h 20 min after ingestion) in the regression explained another portion of the variance in startle magnitude (marginally significant), while this reduced the contribution of Drug to non-significant (State anxiety Beta=0.55; t=4.4, P<0.001; Sedation Beta=–0.23, t=–1.8, P=0.087; Drug Beta=0.23, t=1.6, P=0.108). These results indicate that group differences in startle magnitude were accounted for by the variation in subjective ratings of state anxiety and sedation. Importantly, the contribution of anxiolysis to this effect appeared larger than the contribution of sedation.

Sedation

Pre-treatment alertness was moderate (mean of 3.9 on a scale of 0–10). The overall analysis showed a main effect of Time [F(2,54)=18.4, P<0.001] and a Time×Drug interaction [F(2,54)=3.8, P<0.05]. The placebo and 15 mg group differed neither on pre-treatment sedation scores [t(36)=–0.2, n.s.], nor on the early time of measurement [after 45 min; t(36)=.7, n.s.]. However, by the latest time of measurement (after 1 h 20 min), the 15 mg group reported significantly more sedation than the placebo group [t(36)=2.9, P<0.01]. This finding is consistent with literature reports that diazepam sedation effects peak at a latency of 1.5 h (Grundström et al. 1978).

Discussion

The results of this experiment bear a striking resemblance to the results in the oxazepam experiment. Again, there was a large startle potentiation effect (44% and 47% increased in the placebo and diazepam groups, respectively), which was not influenced by the drug manipulation. Therefore, possible differences in drug action between oxazepam and diazepam must be ruled out as an explanation of the negative results in experiment I.

In comparison with oxazepam, diazepam greatly and significantly reduced baseline startle magnitude. This could reflect an anxiolytic action and/or a non-specific effect of sedation, possibly caused by a comparably more potent dose. Results of the regression analysis argued for an important contribution of anxiolysis to this effect.

In the third experiment, a within-subjects design was employed rather than a between-subjects design, in which a possible drug effect on potentiation might have been masked by inter-subject variability. From a pilot experiment, we concluded that our fear-potentiated startle manipulation could be repeated in a second session a week later and still be effective (Böcker et al., unpublished data). Additional measures were taken to maintain startle potentiation over the three successive sessions.

Experiment III: diazepam (within-subjects)

Materials and methods

Subjects

The sample of subjects was comparable to the oxazepam and the diazepam between-subjects studies. In total, 14 subjects participated in the study. Inclusion and exclusion criteria were identical to the oxazepam and diazepam between-subjects studies. Subjects were paid DFL 200 for participation. Group statistics were: sex, seven males, seven females; mean age, 21.4 year, SD 2.8; mean weight, 68.1 kg, SD 9.4; Mean Trait Anxiety Score, 36.2, SD 7.3.

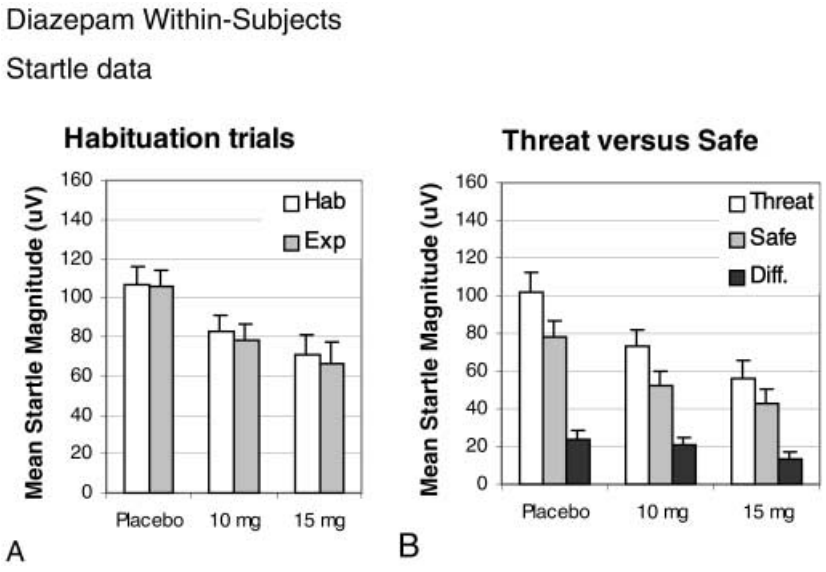

Fig. 3A,B Startle results from experiment III. **A** Habituation trials from the separate habituation run (*Hab*) and from the experimental run (*Exp*). **B** The absolute values for Threat×Drug, along with the difference score for startle potentiation

Drug manipulation

Diazepam was studied in doses of 10 mg and 15 mg compared to placebo in a double blind within-subjects design. Upon completion of a health screening, subjects were matched for sex and weight, and were assigned to a treatment order. Treatment orders were fully balanced over 12 subjects (each order occurred twice), in the two additional subjects orders were mirrored (Placebo, Low, High, And High, Low, Placebo).

Procedure

The procedure used on each test day was identical to the diazepam between-subject study, as far as measurement, apparatus, data scoring and analyses were concerned. Differences in the protocol occurred because the experiment was repeated over three test sessions, one for each treatment. To ensure consistent levels of fear in the three sessions, the shock instruction was modified. The instructions were that one to three electrical shocks would be given over the three sessions. Subjects were also informed that the intensity of the shocks would be increased over the three sessions, regardless of whether or not they received a shock in the preceding session(s). None of the subjects received a shock during the first session or third session, and each subject received one shock during a threat cue near the end of the second session. The time interval between two sessions lasted from 1 to 2 weeks.

Data scoring and analysis

Each experimental session consisted again of eight threat/safe periods. Because the second session differed from the first and third session due to the administration of a shock during one of the last two threat periods, startle trials from these last safe and threat periods were excluded from analysis. In the statistical analyses, Greenhouse-Geisser correction was applied to Drug effects and interactions.

Results

Startle reflex

To check whether the additional instructions resulted in robust startle potentiation over three sessions, startle potentiation was analyzed over sessions. A highly significant main effect of Threat was observed [$F(1,13)=70.2$, $P<0.001$], without a main effect of Session [$F(2,24)=1.4$, n.s.] or an interaction with Session [$F(2,22)=1.1$, n.s.]. Figure 3 shows the startle data separately for each drug treatment. Diazepam reduced habituation startle magnitude significantly [Fig. 3A, $F(2,25)=11.1$, $P<0.001$]. This effect was strictly linear [linear trend $F(1,13)=17.9$, $P<0.001$], suggesting a dose-dependent effect. There was no interaction between the magnitudes in the separate habituation block and in the habituation trials in the experimental run [$F(2,17)=0.1$, n.s.]. During the experimental conditions, the main effect of Threat was highly significant [Fig. 3B, $F(1,13)=70.2$, $P<0.001$]. As in the habituation trials, there was a main Drug effect [mean Placebo=86 µV, mean 10 mg=59 µV, mean 15 mg=46 µV, $F(2,23)=15.3$, $P<0.001$]. As in the between-subjects design, these main effects were not accompanied by a Threat×Drug interaction [absolute amplitudes threat minus safe $F(2,25)=1.9$, $P=0.17$, n.s., linear trend contrast $F(1,13)=3.4$, $P=0.09$, n.s.]. Effect of drug on potentiation expressed as a percentage was also not significant, [means (SEM) Placebo 36% (7), 10 mg 60% (16), 15 mg 47% (14); $F(2,25)=1.5$, $P=0.24$, n.s.]. These results did not change when the factor order was included [Threat: $F(1,8)=56.1$, $P<0.001$, Drug: $F(2,14)=11.7$, $P<0.001$; Threat×Drug: $F(2,10)=2.2$, n.s.]. As a final check, an analysis on normalized z-score data (including six means per condition) was performed. The results from this analysis did also not change the conclusion: main effects of Drug [$F(2,24)=25.0$, $P<0.001$) and Threat [$F(1,13)=60.9$, $P<0.001$), but a non significant interaction Threat×Drug [$F(2,25)=2.0$, $P=0.16$].

State anxiety

State anxiety pre- versus post-treatment was not significant [$F(1,13)=0.04$], and not influenced by drug treat-

ment [$F(2,12)=0.2$; interaction $F(2,12)=0.4$]. There was a decreasing trend of session on the baseline level of state anxiety [means 1=34.5; 2=32.6; 3=30.5; $F(2,26)=6.1$, $P=0.02$, linear trend $F(1,13)=7.8$, $P<0.02$]. There was no interaction of Session with pre/post-treatment.

Retrospective reports showed a significant increase in subjective anxiety during the threat condition [$F(1,13)=33.6$, $P<0.001$]. There was a significant linear trend effect for Session [means per session: 1=18.4; 2=16.6; 3=15.3; linear component $F(1,13)=5.9$, $P<0.03$; quadratic component $F(1,13)=0.1$, n.s.]. There was no Threat×Session [$F(5,6)=1.0$] or Threat×Drug interaction [$F(2,26)=1.4$, n.s.]. There was, however, a marginally significant main Drug effect [$F(2,21.5)=3.3$, $P=0.06$], which consisted marginal differences between Placebo and both active dosages [placebo versus 10 mg $t(13)=2.1$, $P=0.06$; placebo versus 15 mg $t(13)=2.0$, $P=0.07$; 10 mg versus 15 mg $t(13)=0.4$].

Correlation between startle and STAI

To allow evaluation of the contribution of anxiety and sedation to the drug effect on startle magnitude, difference scores of the largest effect (Placebo minus 15 mg) were computed for each of these measures. The regression analysis revealed that both the change in state anxiety (Beta=0.5, $t=2.4$, $P<0.05$) and in sedation (Beta=-0.5, $t=–2.5$ $P<0.05$) from placebo to 15 mg explained a significant part of the variance in startle magnitude. As in the between-subjects design, after these two variables were accounted for the part of the variance explained by Drug was no longer significant ($t=2.1$, $P=0.06$).

Sedation

Pre-treatment alertness was moderate (mean of 3.2 on a scale of 0–10). There was a difference in pre-treatment sedation scores between placebo and 10 mg, which was not caused by a manipulation but did reach significance [means of 2.5 versus 3.9, $t(12)=3.2$, $P<0.01$]. The other pre-treatment comparisons were non significant [$t(12)$ of –1.4 and 1.3, n.s.]. The 15 mg dose increased sedation as compared to placebo only at the late time [early $t(12)=1.9$, $P=0.08$; late $t(12)=–2.9$, $P<0.02$]; this result is consistent with the results from experiment II. Interpretation of sedation in the 10 mg condition is difficult because of the deviation in pre-treatment score [10 mg deviates from placebo at the early time, $t(12)=3.0$, $P<0.02$, but not from 15 mg, $t(12)=0.7$; at the late time the reverse is true, respectively, $t(12)=1.3$, n.s.; $t(12)=–3.1$, $P<0.01$]. Thus, the results for placebo and 15 mg dose replicated the diazepam between-subjects effect, but these results do not allow the conclusion of a dose-dependent effect of sedation.

Discussion

The third experiment replicated the results of the former two. Again, significant increases in subjective fear and in startle magnitude were observed in response to the threat cue. However, benzodiazepine treatment did not significantly decrease either of these threat-specific measures. The three threat of shock studies at Utrecht University yielded similar results, confirming that this type of fear-potentiated startle was indeed unaffected by benzodiazepine treatment. This result stands in contrast with findings from both animal fear-potentiated startle studies, in which benzodiazepine anxiolytics generally test positive (with occasional exceptions, e.g. Joordens et al. 1996), and the reduction of human fear-potentiated startle by diazepam, shown by Bitsios et al. (1999).

What possible causes might explain the difference between the latter study and our results? It is possible that the differences between the results of our studies and that of Bitsios et al. may be due to differences in the methodology employed. Although there were similarities between our threat of shock design and that used by Bitsios, they were not identical. The important similarity is that Bitsios et al. also had subjects expect an electric shock during one threat period but not during a safe period. Yet, a conspicuous difference between the procedures is the way the threat information was communicated to the subjects. In addition, there were other differences. Bitsios et al. used, for example, a lower dose (5 and 10 mg), included light flashes to measure the pupil reflex, included male subjects only, duration and number of threat and safe periods differ and a short 30-s break in which electrodes were (dis)connected separated each threat and safe period. In our experiments, after subjects were seated in the experimental room, the door was closed and subjects received further information by means of a visual display on a computer screen only. Subjects were instructed which of two specific cues, abstract visual stimuli, would symbolically indicate the threat and safe periods during the experiment. They did not receive any additional instruction or information on the occurrence of threat and safe periods. In contrast, Bitsios et al. did not use specific, verbally instructed, cues. Instead, these experimenters entered the experimental room to actively attach or detach the shock electrodes before each new threat or safe period. Though this manipulation clearly constitutes an effective threat, the precise nature of this manipulation is unclear, since attaching the electrodes in itself potentiates startle (Grillon and Ameli 1998). Consequently, in Bitsios et al.'s study, the potentiation effect may be caused by both the instructed threat and by attaching the electrodes. Either of these two effects could be affected by diazepam. In the present procedure, in the safe periods the shock electrodes are still connected (receiving a shock is still possible). Therefore, the threat and safe periods differ only with respect to the instruction that is communicated with abstract cues.

Preclinical and clinical evidence suggests that the nature and characteristics of the threat cue may cause functional differences in the fear responses that it elicits (Morgan et al. 1995; Grillon et al. 1998). Clinically, different anxiety disorders have traditionally been classified based on the specifics of their relationship with

external stimuli (Tyrer 1989). The diagnosis of specific phobia is restricted to fears cued by the presence of a specific object (e.g. spiders), while in generalized anxiety disorder the absence of a specific feared object is characteristic. Recent experimental evidence from startle studies suggested that in contrast to phobic patients, panic disorder, PTSD, and OCD patients do not show increased sensitivity to specific fear-cues, but rather to the general experimental setting (Morgan et al. 1995; Grillon and Ameli 1998; Kumari et al. 2001; see also Lang et al. 1998, for a similar argument). Since these patients exhibit normal fear-potentiated startle, but increased "baseline" startle, the study of the psychopharmacology of contextual manipulations in addition to cue-specific ones is important (Grillon et al. 1999; Ameli et al. 2001). In animal experimental literature, differences between responses evoked by a specific cue versus less specifically defined, e.g. contextual, cues have been observed, and interpreted as differences between fear and anxiety responses (Davis et al. 1999).

Moreover, the idea of different sensitivity of these different fear/anxiety responses to benzodiazepine treatment has received support from similar angles. First, in clinical pharmacology it has been suggested that benzodiazepines have an effect on every anxiety disorder except specific phobia, the only disorder which is truly cue-specific (Tyrer 1989; Sartory et al. 1990). Second, evidence from the elevated plus maze in rats suggest that cue specific fear is not affected by benzodiazepines (File et al. 1998). The manipulation in the present experiments I–III meets the criteria of cue-specificity and duration for evoking fear perfectly. In contrast, the manipulation of Bitsios et al., who conveyed threat not with an instructed cue but by manipulating the context by physically attaching the shock electrodes, fulfills criteria associated with anxiety. This distinction could constitute a framework that allows integration of the apparently discrepant pharmacological results thus far. It could be hypothesized that in humans benzodiazepines do not affect fear (the present cue-specific response), but possess anxiolytic properties on effects constituting instances of anxiety.

Although the dissociation between the effect of drug on baseline startle and the lack of an effect on fear-potentiated startle is in line with the proposed dichotomy, the previous three experiments were not designed to explicitly unravel these effects. In experiment IV, the effects of diazepam on different manipulations that potentiate startle were investigated. Besides a conditioned specific fear cue manipulation, two additional manipulations that may resemble anxiety rather than fear were included.

Experiment IV: diazepam i.V. (between-subjects)

In experiment IV, the effect of diazepam was investigated in a design that included two additional manipulations of anxiety besides a verbal threat instruction coupled to a specific cue (see Grillon and Ameli 1998). First, the application of electrodes was introduced as a context variable, in that they were either present or absent during a block of alternating threat and safe periods. Grillon and Ameli (1998) showed in a threat of shock procedure that attaching the shock electrodes potentiates startle. Potentiation was mostly, but not exclusively, confined to the condition in which both the electrodes and the threat cue were present. According to a recent review of context conditioning (Holland and Bouton 1999), this indicates that electrodes might function as an "occasion setter" context variable. Hence, while in the study by Bitsios the effect of the electrode manipulation cannot be disentangled from the effect of the threat manipulation, in the present design it constitutes a context variable in addition to the instructed threat.

Second, darkness as unconditional anxiogenic manipulation is the human analogue for the animal model employing bright light. While bright light enhances startle in nocturnal species such as laboratory rodents (Walker and Davis 1997a), darkness potentiates startle in humans, a diurnal species, when manipulated either in combination with or without a threat of shock procedure (Grillon et al. 1997; Grillon and Ameli 1998). Based on the resistance to pharmacological treatment of the startle potentiation by a specific cue in the previously presented three experiments, we predicted that diazepam would not affect startle potentiation by the cue-specific threat instruction. Based on human studies showing an effect of diazepam on manipulations that are not so much cue-specific, but may be more contextual (Patrick et al. 1996; Bitsios et al. 1999), i.e. constituting instances of anxiety rather than fear, we expected that dark-potentiated startle and startle potentiated by electrodes might prove sensitive to the benzodiazepine treatment.

Materials and methods

Subjects

Subjects were healthy volunteers, who were determined to be free of 1) major medical illness as determined by physical examination, ECG, laboratory tests of renal, hepatic, pancreatic, hematopoietic, and thyroid function and 2) substance use as determined by urine toxicology screens. In addition, none of the subjects were taking medication nor did they meet criteria for any psychiatric or substance abuse disorder per S.C.I.D.-Non Patient criteria. All of the subjects underwent successful audiologic testing (500, 1000, 2000, 4000 Hz) prior to participation in the study and exhibited a startle response during screening (see below).

A total of 26 paid ($160) subjects participated (17 males). The placebo group consisted of 13 subjects (eight males) with a mean age of 22.8 year, SD 3.5. The diazepam group consisted of 13 subjects (eight males) with a mean age 22.7 year, SD 5.3. Mean Trait Anxiety (Spielberger 1983) did not significantly differ between groups [$t(24)=0.9$]. Trait anxiety scores were 29.5 (SD=6.1) and 32.3 (SD=8.7) in the placebo and diazepam group, respectively.

Drug manipulation

Subjects were randomly assigned to either a placebo or an active IV dose of 4 mg diazepam.

Procedure

Subjects provided written informed consent prior to participating in the study. Subjects participated in two sessions: a screening session and a testing session. These sessions were separated by 1–2 weeks. During the screening session, the psychiatric and medical evaluation was conducted. In addition, subjects' hearing level and baseline startle reactivity were assessed (in the absence of drug). The audiologic exclusion criterion was any hearing loss of more than one frequency band in one ear. The baseline startle assessment consisted of nine startle probes every 18–25 s. Subjects with either a 0 response on one of the nine trials or a mean startle magnitude averaged over the nine trials of less than 50 μV were excluded from the study. In the screening session, subjects were given the state and trait portions of the Spielberger State-Trait Anxiety Inventory (STAI: Spielberger 1983) just prior to the startle test.

One to 2 weeks later, during the testing session, an IV line was placed for drug administration. The eye blink electrodes were attached at that time. Ten minutes later, diazepam (4 mg) or saline was infused IV. Startle testing began immediately after infusion.

Startle testing started with the same baseline startle assessment as in the first session (nine startle stimuli delivered every 18–25 s) in order to assess changes in startle reactivity following drug administration. Following the baseline startle test, a vibrator was placed on subjects' right or left hand (counterbalanced across subjects). Instructions concerning the fear-potentiated startle experiment were given to the subjects. They were told that they would receive at least one shock, but no more than three shocks during the experiment, and that shocks would be delivered via electrodes placed at a later time (on the arm opposed to that on which the vibrator was already attached). The shocks were described as rather unpleasant, producing a very localized and short-lived painful sensation. The subjects were also told that that the shock could be administered only during threat periods signaled by the activation of the vibrator, but not during safe periods signaled by the absence of vibration. Finally, subjects were informed that they would receive startle stimuli from time to time and that the light in the room would alternate between being on and off. It was also indicated that at regular intervals during testing, the shock electrodes would be attached or removed. Subjects were asked to fill out the state portion of the STAI just prior to begin the fear-potentiated startle experiment.

The experimental test was designed after one of our previous studies (Grillon and Ameli 1998). It consisted of three different manipulations (light on/off, electrodes on/off, and threat/safe), which were fully crossed over. The threat/safe conditions were presented in complete darkness or with the light turned on (e.g. light on: threat – safe; light off: threat – safe; light on: safe – threat; light off: safe – threat). The order was counterbalanced across subjects. Each subject was presented with two sequences each containing two experimental runs. Within a sequence, the shock electrodes were connected to the shock stimulator in one run and disconnected in the other run in a manner that was visible to the subjects. The successive runs were presented to the subjects such that there were alternating periods of shock electrodes on and off over successive runs (e.g. shock electrodes on, off, on, off; order also counterbalanced across subjects). For example, for one subject: run 1: shock-electrodes on, light on (safe threat safe threat), light off (safe threat safe threat), light on (safe threat ×2), light off (safe threat ×2). Run 2: electrodes off, same sequence. Runs 3 and 4: same as 1 and 2 but order of light/dark and threat/safe reversed. Finally, each run started with the delivery of four startle stimuli with the light on (referred to as habituation period). These startle stimuli enabled us to compare the effects of placing the shock electrodes before any threat or changes in lighting condition occurred. The last habituation startle stimulus was immediately followed by the presentation of two alternating light and dark phases, each phase lasting 80 s. Light and dark phases were equally divided into a treat and a safe condition, each condition lasting 40 s. Within each threat/safe condition two startle probes were delivered every 17–23 s, starting 8–12 s after the onset of a condition. There was a 5-min rest periods between runs.

After each run (electrodes on or off, not differentiated for light on or off) subjects were asked to indicate retrospectively how they experienced the shock and threat periods with the shock electrodes on and off (without differentiating dark from light conditions) on dimensions of valence and arousal (Russell et al. 1989). Only one shock was administered. It was delivered in the last threat condition with electrodes attached.

Measurement and apparatus

The acoustic startle stimulus consisted of a 40-ms duration 102-dB(A) burst of white noise with a near instantaneous rise time delivered binaurally through headphones. The shock (1.5 mA, 10-ms duration) was delivered by a constant current stimulator. The eye blink reflex was measured by recording activity from the orbicularis oculi muscle underneath the left eye with two disk electrodes (AgAgCl). The ground electrode was placed on the left arm. Impedance level was kept below 5 kOhm. EMG activity was filtered (1–500 Hz), digitized at 1 kHz for 250 ms from the onset of the acoustic stimuli, rectified, and stored for off-line analysis. A 60-Hz notch filter was also used to eliminate 60 Hz interference.

Data scoring and analysis

Following digital filtering of the EMG signal with a 20.9 Hz low-pass filter, peak amplitude and onset latency of the blink reflex was determined in the 21–120 ms time frame following stimulus onset relative to a baseline EMG value. The baseline EMG value was calculated by taking the average of the minimum and maximum values recorded during the first 20 ms for each trial. Trials with excessive EMG activity during the first 20 ms were rejected. Trials which did not reach peak within 95 ms of onset latency were given a peak amplitude value of 0 μV and were included in the analysis.

Startle magnitude data from the baseline startle assessment on sessions 1 and 2 were averaged over successive blocks of three trials. In the fear-potentiated startle test, startle magnitudes were averaged within the habituation period, the light/dark phases, the threat/safe conditions, and the shock electrodes attached/removed for each sequence.

The drug effect on baseline startle magnitudes was evaluated with respect to a baseline measure taken during screening by entering both sessions into a repeated measures ANOVA. The within-subjects factor in this analysis was Session (Screening, Experiment), with Drug as the between-subjects factor. Means per repetition of each condition was entered into the statistical analysis. The statistical design included the within-subjects factors Electrodes (on, off)×Threat (threat, safe)×Light (on, off)×Repetition (sequence 1,2), and the between-subjects factor Drug (placebo, diazepam). Degrees of freedom were all 1,24 (Greenhouse-Geisser corrections not applicable). Selected effects, in comparison with Grillon and Ameli (1998) are reported.

Results

Startle reflex

See Table 2 for details on statistics. Startle magnitude during baseline trials in the screening session compared with baseline trials in the experimental session are depicted in Fig. 4A. These values were entered in a repeated measures ANOVA, which resulted in a highly significant interaction of Session×Drug (F=15.0, P<0.001). This interaction reflected the fact that although the groups were identical during pre-treatment, diazepam, but not placebo, significantly decreased baseline startle during the experimental session (t=2.3, P<0.03).

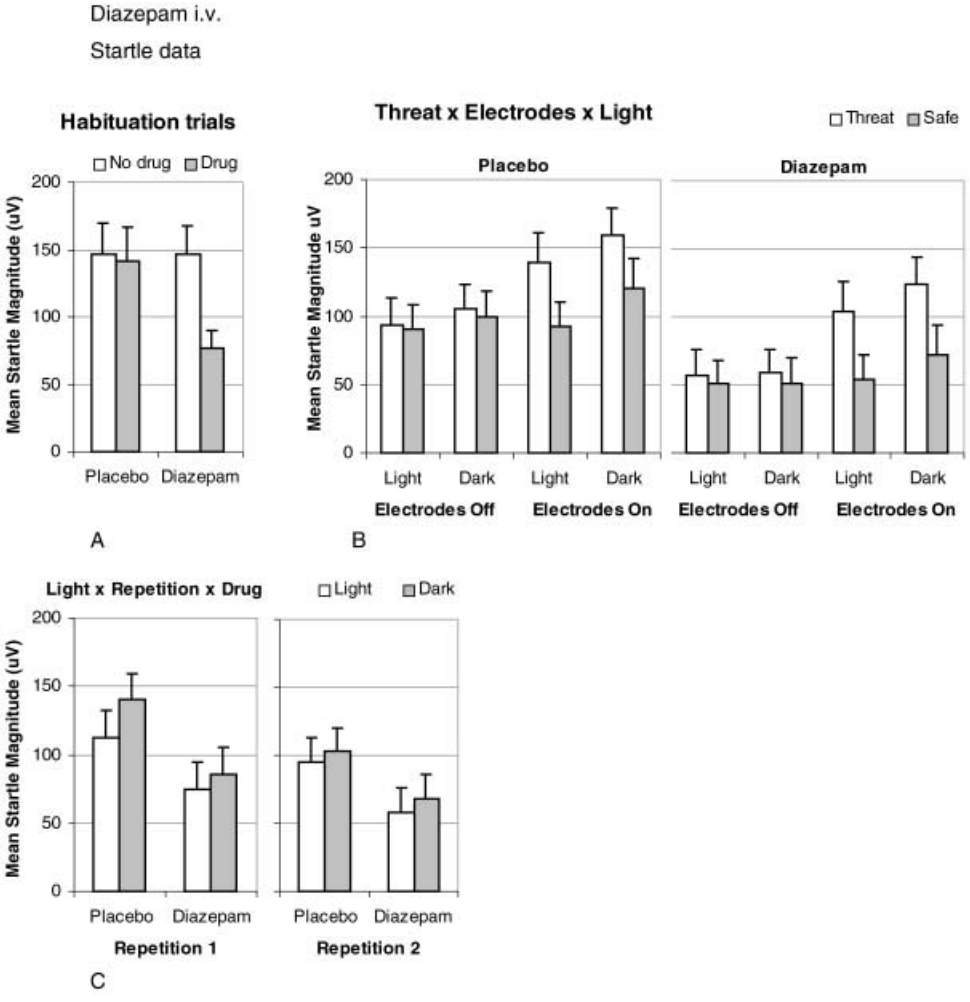

Fig. 4A–C Startle results from experiment IV. **A** Habituation trials from the run in the separate screening session (*No drug*) and from the experimental run (*Exp: drug*). **B** All factors averaged over repetitions for placebo and diazepam, respectively. **C** The significant interaction Light×Repetition×Drug

Table 2 Statistical results of the startle analysis of Experiment IV (all *df* 1,24)

Habituation trials	*F*	*P*<		
Main drug	2.5	0.15, n.s.		
Interaction session×drug	15.0	0.001**		
Experimental factors	Within-subjects		Interaction with drug	
	F	*P*<	*F*	*P*<
Electrodes	40.0	0.001**	<1	n.s.
Light	22.2	0.001**	1.3	n.s.
Threat	45.5	0.001**	<1	n.s.
Repetition	55.0	0.001**	2.6	n.s.
Electrodes×Light	11.3	0.01*	<1	n.s.
Electrodes×Threat	37.4	0.001**	<1	n.s.
Threat×Repetition	6.4	0.02*	<1	n.s.
Light×Repetition	6.8	0.02*	6.3	0.02*
Light; Repetition 1	25.4	0.001**	4.8	0.04*
Light; Repetition 2	7.3	0.02*	<1	n.s.

Results during the habituation periods provided an assessment of the effect of attaching the shock electrodes (another assessment is described below). They were analyzed in a Drug (placebo, diazepam), Electrodes (on, off), Repetition (sequence 1,2) ANOVA. There was a main Electrodes effect (F=10.1, P<0.004) but no significant Electrodes×Drug interaction. These results reflected the fact that attaching the electrodes increased startle magnitude similarly in both groups, means (SD) placebo: 96 µV (79) versus 115 µV (77); diazepam: 61 µV (46) versus 76 µV (49).

The results of the experimental factors averaged over repetitions are compared in Fig. 4B. Each of the experimental factors had a main effect on startle magnitude. However, none of these effects was directly modulated by the drug treatment. The overall effect of Drug (mean Placebo=113 µV, mean Diazepam=71 µV) was not significant. The interaction Threat×Electrodes (threat only potentiated startle with shock electrodes on) was replicated. The effect of Electrodes was smaller in absence of the threat cue as compared to in the presence of the threat cue (safe: electrodes off 73 µV, on 85 µV, t=2.5, P<0.02; threat: electrodes off 79 µV, on 131 µV, t=7.1, P<0.001). For effects of threat in absence versus presence of the threat electrodes a similar pattern emerged (absent: 73 versus 79 µV, t=2.1, P<0.05; present: 85 versus 131 µV, t=7.5, P<0.001). The interdependence is almost complete, in that the combination of electrodes and threat produces a much larger effect than the electrodes and threat cue by themselves. None of these partial

effects was influenced by Drug (all *t*-values <1). The interaction and Electrodes×Light (effects of electrodes greater in dark) was replicated also.

The only effect that was influenced by the drug manipulation was Light×Repetition. The interaction Light×Repetition×Drug is shown in Fig. 4C. In repetition 1, the effect of Light is highly significant, and the interaction Light×Drug is significant: This interaction reflects a significant dark-enhanced startle in the placebo group [$t(12)=-3.4$, $P<0.01$] but not in the diazepam group [$t(12)=-0.7$, n.s.]. In repetition 2, the effect of Light is less significant in the placebo group (and smaller, it decreases significantly from 19 μV in repetition 1 to 9 μV in repetition 2, $t=2.4$; $P<0.03$), and the interaction Light×Drug is no longer significant.

Anxiety, valence and arousal ratings

Subjects' state anxiety was differently affected by the treatment. A Session (1,2)×Drug (placebo, diazepam) revealed a significant Session×Drug interaction ($F=6.5$, $P<0.02$). While state anxiety was non-significantly increased from session 1 (mean=31.0, SD=7.1) to session 2 (mean=32.6, SD=9.1) in the placebo group [$F(1,12)=1.3$], it was significantly reduced over the same periods in the diazepam group [mean=32.2, SD=8.1 versus mean=29.3, SD=8.1, respectively; $F(1,12)=5.2$, $P<0.04$].

The affect-grid was used to assess subjects' subjective reactions to the different conditions on dimensions of sleepiness-arousal (0–9) and displeasure-pleasure (0–9). In the arousal ratings, there were main effects of Electrodes ($F=66.1$, $P<0.001$) and Threat ($F=58.7$, $P<0.001$), as well as a significant Electrodes×Drug interaction ($F=6.5$, $P<0.02$). The increased arousal induced by the placement of the shock electrodes was smaller in the diazepam compared to the placebo condition (placebo on 4.7, off 3.2; diazepam: on 4.0, off 3.2). The Threat×Electrodes interaction was also significant, which is consistent with the startle data ($F=36.6$, $P<0.001$). There was no main effect of drug on arousal ($F=0.8$, n.s.).

The valence ratings also showed main effects of Electrodes ($F=11.1$, $P<0.01$) and Threat ($F=34.5$, $P<0.001$), as well as an interaction Electrodes×Threat (threat: off 5.8, on 4.3; safe: off 6.1, on 6.2; $F=27.1$, $P<0.001$). The interaction of Electrodes×Drug was not significant ($F=0.1$, n.s.), but the interaction Threat×Drug was marginally significant (placebo: threat 4.4, safe 5.8; diazepam: threat 5.7, safe 6.4; $F=3.7$, $P=0.07$). Overall, valence ratings were more positive in the diazepam group (main effect of Drug, $F=6.8$, $P<0.02$).

Correlation between startle and STAI

For both state anxiety and baseline startle magnitude a pre-drug baseline was available from session 1. A regression analysis on percentage change scores from session 1 to session 2 (measured after drug infusion, just prior to startle testing) was performed. When using percent change in state anxiety and drug group to explain variance in percent change in baseline startle magnitude, state anxiety accounted for a marginally significant part of the variance in startle (Beta=0.3, $t=1.7$, $P=0.09$). The effect of drug was still highly significant after accounting for the variance caused by state anxiety (Beta=0.6, $t=4.0$, $P<0.001$). This could mean that the variance in state anxiety that explained variance in startle was not a result of the drug manipulation, but of other unknown causes. These results are different from the results in experiments II and III, probably because in those experiments state anxiety data concerned the experimental conditions, while this measurement was taken before the experiment.

Discussion

The fourth experiment evaluated the effects on startle potentiated by three different anxiogenic manipulations. In accordance with the results from the first three experiments, diazepam was found not to affect fear-potentiated startle to a threat signal, suggesting that cue-specific fear is insensitive to this psychopharmacological treatment.

This is the first study that examined the effects of diazepam on contextual fear. Contrary to our hypothesis, diazepam did not affect the increase in startle induced by the shock electrodes manipulation despite significant potentiation by the shock electrodes. In contrast, diazepam did block the facilitation of startle induced by darkness. This startle facilitation was not very robust, in that it was much smaller in the second repetition, and that drug only had an effect in the first repetition. Yet, we predict that the effect of diazepam on dark-enhanced startle can be replicated in a more robust manner in an experiment specifically designed for this manipulation. The way in which the three manipulations were crossed in the present experimental design may be too complex to robustly measure the effects of the different manipulations by themselves.

Previous results were replicated, showing that the combination of the factors Electrodes and Threat had a much larger effect than one of the two factors by themselves. In terms of associative learning, this form of dependence can be considered as an association between a context and a CS-US relation (i.e. not directly with an US), where one CS (here, the shock electrodes) "sets the occasion" for a relation between another CS (the threat condition) and the US (Holland and Bouton 1999). In this account, startle potentiation by the combination of shock electrodes and threat cue can be interpreted as being evoked by the threat cue, as a direct association with the shocks exists only for that stimulus. In that sense, the absence of a drug effect on the large effect of threat in the presence of the shock electrodes is consistent with the previous results. On the other hand, since the dependence of shock stimulation on the presence of

shock electrodes is generally intrinsic, shock electrodes possess some inherent aversiveness. The present results provide evidence for this, especially on the habituation startle trials (46% potentiation), but also on the safe trials (21% potentiation). Yet, there was no effect of drug on this potentiation, even if tested explicitly.

The present electrodes manipulation was quite different from the manipulation by Bitsios et al. (1999). There, the presence of instructed threat was completely contingent upon the presence of the electrodes, which stresses the intrinsic relation of electrodes with threat. Therefore, one might argue that their drug effect critically depends on that particular contingent threat and electrodes manipulation.

General discussion

The main conclusion based on the present data is that there are instances of fear-potentiated startle that are not sensitive to benzodiazepine treatment. The three replications in one laboratory quite convincingly demonstrated this point in one specific experimental set-up. At the same time, evidence that a baseline effect on startle is due to an anxiolytic drug effect was obtained. The fourth experiment provided evidence that startle potentiated by darkness is sensitive to benzodiazepine treatment. Yet, the negative drug result on cue-specific potentiation with diazepam was replicated in a different mode of administration, different procedures, and in a different laboratory. This negative result conflicts with evidence for the sensitivity of startle potentiation to benzodiazepines from both animal (Davis et al. 1993) and human (Patrick et al. 1996; Bitsios et al. 1999) literature, and thus requires an explanation.

With regard to the animal literature, several studies have reported benzodiazepine-reduced potentiated startle to a threatening explicit cue in rats. Although this is difficult to assess, one possibility is that doses that are used in animal studies are much higher, compared to human studies (Davis, personal communication). Hence, automatic reliance on animal data might not be warranted.

As for humans, it has been argued that qualitatively different emotional responses can be instrumental in startle potentiation. For example, in the emotional valence paradigm it has been shown that pictures of categories ranging from disgust and anger to horror and fear have the quality of facilitating the startle reflex (Lang et al. 1993). Also, in the animal literature, several manipulations that have been proposed to differentiate between fear and anxiety potentiate startle (Davis 1998). These manipulations can even be dissociated with respect to the neural structure critical for their effects (Davis 1998), suggesting that differences in pharmacological effects on potentiated startle might very well be caused by differences in the underlying brain mechanisms.

While the experiments presented in this paper were not designed to provide explanations for the discrepancy between the effects of benzodiazepines on the current fear-potentiated startle and other studies, one factor that may have some explanatory power is that of the distinction between fear and anxiety. In animal experimental literature, the difference between fear and anxiety has been defined mainly by means of evocation, formulated in terms of evocation by a specific cue versus less specifically defined, e.g. contextual, cues (Davis et al. 1999). Also, an unconditioned fearful association is more readily associated with anxiety than a learned association after conditioning (Davis et al. 1999; Lang et al. 2000). Dissociations of functional anatomy supported this dissociation: In the fear response (FPS) the central nucleus of the amygdala plays a crucial role, whereas anxiety manipulations (light-enhanced startle, context conditioning) are critically dependent on the bed nucleus of the stria terminalis (Hitchcock and Davis 1991; Walker and Davis 1997b; Davis 1998; Davis et al. 1999).

There is cross-species evidence that the potentiation of the unconditioned startle response to anxiogenic lighting conditions is sensitive to benzodiazepines. In the present study (experiment IV), diazepam reduced the enhancement of startle by darkness. In the rat, the light-facilitation of startle is blocked by chlordiazepoxide (De Jongh et al. 2002). The exact defining properties that divide fear from anxiety remain unclear. At present, the property that is mostly used to differentiate fear from anxiety is cue-specificity. Our data are consistent with the interpretation proposed in this paper that benzodiazepines would not affect startle potentiated with explicit cues, while affecting contextual manipulations. The results from the present benzodiazepine treatments suggest dissociation between fear-potentiated startle on the one hand and baseline and dark-enhanced startle on the other hand. However, at present the differences between fear and anxiety, and the conditions that evoke these responses, are not well defined. Therefore, more research is needed to delineate these responses and their pharmacological properties.

Another possible interpretation is that diazepam effects on potentiated startle do not depend on the qualitative nature of the induced emotion (e.g. fear versus anxiety or explicit versus contextual cues), but on the intensity of the aversive reaction. According to this view, benzodiazepines are effective in reducing potentiated startle to stimuli or situations that elicit little fear/anxiety, but are ineffective when higher levels of fear/anxiety are involved. This would explain the positive results obtained in mildly fearful manipulation, such as in Patrick et al. using negative slides, and in the dark-enhanced startle in the present experiment IV. Similarly, the manipulation by Bitsios et al. on which diazepam was effective yielded relatively small startle potentiation.

As indicated above, there is evidence for the interpretation that the reduction in baseline startle by benzodiazepines is, in part, due to an anxiolytic effect. The anxiogenic manipulation that induces this anxiolytic action appears to be the experimental context in experiments I–III, rather than the shock electrodes per se, since no differences in drug effects were found on trials before

and after the shock electrodes were attached. Experimental rooms can be a potent anxiogenic context that potentiates startle. For example, we have observed that baseline startle magnitude is greater in an aversive context (threat experiment where shocks were expected) compared to a non-aversive context (attention experiment where button presses were demanded; Böcker et al. (2001). The interpretation that the decrease in startle magnitude is at least partly caused by anxiolytic action is supported by the regression analyses in experiments II and III, in which individual differences in state anxiety appeared to account for a large part of variance in startle magnitude. Subjective sedation accounted for some variance, and the two subjective ratings together reduced the residual contribution of the drug to non-significant. Similarly, in experiment IV the amount of reduction in startle magnitude from session 1 to session 2 was correlated with the reduction in state anxiety. However, in that experiment this correlation did not explain the drug-induced effect of drug on baseline startle. In humans, non-specific effects of benzodiazepines on baseline startle in absence of a threat manipulation have been reported (e.g. Patrick et al. 1996; Abduljawad et al. 1997; Rodriguez-Fornells et al. 1999). Taken together, the conclusion must be that non-specific effects such as sedation can play a role in the startle reducing properties of an anxiolytic drug, but in an anxiogenic context an anxiolytic interpretation of drug effects on baseline startle must at least be considered.

In summary, in the four experiments presented here, no benzodiazepine treatment tried (oxazepam, diazepam, between and within-subjects, administered orally and IV) had any effect on cue-specific fear-potentiated startle. Two important conclusions can be drawn. First, there appears to be at least one type of fear-potentiated startle that is not susceptible to benzodiazepine treatment. Second, experimental models based on refined definitions of the constructs fear and anxiety need to be developed to test their functional and pharmacological properties. These models might shed new light on the relation between these theoretical constructs and the nature and pharmacology of anxiety disorders.

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