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Pharmacological and endocrinological characterisation of stress-induced hyperthermia in singly housed mice using classical and candidate anxiolytics (LY314582, MPEP and NKP608)

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Abstract

The stress-induced hyperthermia test is a paradigm developed several years ago to model the expression of autonomic hyperactivity in anxiety. Whereas in the classical stress-induced hyperthermia, cohort removal was used, in a recently described modification of the stress-induced hyperthermia model singly housed mice rather than groups of mice were used. The modification of this model can be summarized as follows: rectal temperature is recorded in singly housed animals at two consecutive time-points (T_1 and T_2) which are interspaced by a defined time-interval (15 min). Since the value at the second temperature-recording exceeds the value of the initial measure it is the difference between these two core-temperatures which reflects stress-induced hyperthermia. In the present study, the stress-induced hyperthermia paradigm, in its modified design, was evaluated in OF1/IC mice. By comparing the effect of various compounds in both the modified as well as the classical (cohort removal) stress-induced hyperthermia paradigm, a very high correlation was found for the pharmacological sensitivity of the two paradigms. Furthermore, it was demonstrated that other anxiolytics, all known to be active in the classical stress-induced hyperthermia paradigm, such as the benzodiazepines chlordiazepoxide (0.3, 1, 3, 10 mg/kg, p.o.), diazepam (0.1, 0.3, 1, 3 mg/kg, p.o.), clobazam (5 or 10 mg/kg, p.o.) and oxazepam (5 or 10 mg/kg, p.o.) as well as the non-benzodiazepines buspirone (7.5 or 15 mg/kg, p.o.) and ethanol (15% or 30%, 10 ml/kg, p.o.), showed a marked reduction in stress-induced hyperthermia in the modified design. New candidate anxiolytics, i.e. the metabotropic glutamate (mGlu) receptor group 2 agonist LY314582 (1 or 10 mg/kg, p.o.; racemic mixture of LY354740 ((2*S*,4*S*)-2-amino-4-(4,4-diphenylbut-1-yl)-pentane-1,5-dioic acid), the metabotropic glutamate 5 receptor antagonist MPEP (1, 7.5, 15 or 30 mg/kg, p.o.; 2-methyl-6-(phenylethynyl)pyridine) and the neurokinin 1 (NK_1) receptor antagonist NKP608 (0.01 or 0.1 mg/kg, p.o.; quinoline-4-carboxylic acid [*trans*-(2*R*,4*S*)-1-(3,5-bis-trifluoromethyl-benzoyl)-2-(4-chloro-benzyl)-piperidin-4-yl]-amide) also reduced stress-induced hyperthermia in the modified paradigm clearly indicating anxiolytic-like activity for these compounds. Finally, the effects of the classical benzodiazepine chlordiazepoxide (10 mg/kg, p.o.), in parallel with its effect on stress-induced hyperthermia, were also investigated for its effect on plasma concentrations of the two stress hormones, adrenocorticotropin (ACTH) and corticosterone. It was shown that all three parameters were significantly increased 15 min after T_1 in vehicle-treated mice whereas the increase was significantly attenuated following pre-treatment with chlordiazepoxide. In conclusion, all the data presented here indicate that the modified version of the stress-induced hyperthermia-paradigm is a valid and interesting alternative to the classical stress-induced hyperthermia test.

Keywords: Stress-induced hyperthermia; Anxiolytic; Benzodiazepine; Buspirone; Tachykinin; NK_1-receptor antagonist; $mGlu_5$ receptor antagonist; $mGlu_2$ receptor agonist; ACTH (adrenocorticotropin); Corticosterone; (Mouse)

1. Introduction

Stress-induced hyperthermia is an integral part of an individual's response to situations perceived as distressing (Reeves et al., 1985; Marazziti et al., 1992). This phenomenon is mediated by the autonomic nervous system and is well known to occur prior to and during exposure to stress- and/or anxiety-inducing situations. In pathological forms it is considered to represent a symptom of an anxiety disorder. Indeed, autonomic hyperactivity, present in an exaggerated extent, is one of the diagnostic items of generalized anxiety mentioned in DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition). Therefore, it would be

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helpful to have at hand an animal model serving to test drug candidates for their effectiveness in inhibiting this autonomic hyper-reactivity.

The stress-induced hyperthermia test in mice is a paradigm developed several years ago to model the expression of autonomic hyperactivity in anxiety (Lecci et al., 1990; Zethof et al., 1994). In this paradigm, the phenomenon has been exploited as follows: upon removing mice one by one from a stable group of mice within a cage a gradual increase in the animal's core temperature can be observed. This rise in body temperature has been taken as a sign of the anticipatory anxiety experienced by those mice to be removed next. The stress-induced hyperthermia model has been pharmacologically validated and nowadays is considered to represent a robust and reproducible paradigm, which is sensitive to anxiolytic compounds currently on the market (Lecci et al., 1990). Furthermore, it is thought to be sensitive for the identification of potentially new compounds with such an action. The paradigm in its original set-up is, however, rather laborious and even more importantly, it requires a very large number of experimental animals. A wider use of the stress-induced hyperthermia model is probably mainly prevented by this latter requirement. Interestingly, a modification of the stress-induced hyperthermia model in mice has recently been described (van der Heyden et al., 1997) in which individually housed mice rather than groups of mice are used. This would thus make the model more applicable for rapid screening of compounds for potential anxiolytic-like activity. The modification of this model can be summarized as follows: rectal temperature is recorded in singly housed animals at two consecutive timepoints, which are interspaced by a defined time-interval. Since the value at the second temperature recording exceeds the value of the initial measure (which mirrors the stress reaction), it is the difference between these two core-temperatures which is defined as reflecting stress-induced hyperthermia. By varying the inter-recording interval a maximal value for stress-induced hyperthermia was seen around the 10-min interval; when the interval was shortened to 5 min already up to 80% of the maximal hyperthermia was observed (van der Heyden et al., 1997). For pharmacological validation of this modified stress-induced hyperthermia paradigm, Groenink et al. (1996) have demonstrated that benzodiazepines and the 5-HT_{1A} receptor agonist flesinoxan, but not the antidepressant amitriptyline, dose-dependently suppressed stress-induced hyperthermia (see also: Pattij et al., 2001).

In the present study, the stress-induced hyperthermia paradigm, in its modified design, was re-evaluated using OF1/IC mice. Thereby, four main aspects were addressed: (1) a comparison between the original and the modified version of the paradigm; (2) an evaluation of benzodiazepine-like anxiolytics (chlordiazepoxide, diazepam, clobazam and oxazepam) and non-benzodiazepine-like anxiolytics (buspirone and ethanol), all of which are known to be effective in man; (3) testing of novel target-related compounds in the modified design, such as the metabotropic glutamate (mGlu) receptor group 2 agonist LY314582 (racemic mixture of LY354740 ((2*S*,4*S*)-2-amino-4-(4,4-diphenylbut-1-yl)-pentane-1,5-dioic acid); Monn et al., 1997), the $mGlu_5$ receptor antagonist MPEP (2-methyl-6-(phenylethynyl)pyridine; Gasparini et al., 1999) and the neurokinin 1 (NK_1) receptor antagonist NKP608 (quinoline-4-carboxylic acid [trans-(2*R*,4*S*)-1-(3,5-bis-trifluoromethyl-benzoyl)-2-(4-chloro-benzyl)-piperidin-4-yl]-amide; Vassout et al., 2000), which have been recently described to exhibit anxiolytic-like effects in other preclinical models of anxiety (Monn et al., 1997; Spooren et al., 2000a,b; Vassout et al., 2000); (4) a parallel evaluation of the effects induced by the classical benzodiazepine chlordiazepoxide on stress-induced hyperthermia and on plasma concentrations of the two stress hormones adrenocorticotropin (ACTH) and corticosterone.

2. Materials and methods

2.1. Animals and housing

Male OF1/IC mice were obtained several days prior to behavioural testing from Iffa Credo (Les Oncins, France; 18–20 g) and housed in groups of 15 animals per cage (macrolon cages $42 \times 26 \times 15$ cm). When using the classical stress-induced hyperthermia procedure the mice remained group-housed in their home cage until tested (see below). For the modified stress-induced hyperthermia test the animals were individually housed (macrolon cage: $26 \times 21 \times 14$ cm) 24 h before testing. All cages were transferred to the laboratory where the test was performed on the day preceding testing (see below). Both the animal room and the laboratory were temperature controlled and equipped with artificial illumination (L12:D12; lights on 06:00). Mice always had ad libitum access to water and food (Ecosan, Eberle Nafag AG, Gossau, Schweiz). All animal studies were performed in accordance with the local law and were covered by a valid animal permission (No: 150; issued by the Kantonales Veterinäramt Basel-Stadt, Switzerland).

2.2. Stress-induced hyperthermia procedures

2.2.1. Classical stress-induced hyperthermia test

The test procedure for the classical stress-induced hyperthermia paradigm was adapted from Lecci et al. (1990). Briefly, with exactly 1-min intervals one mouse after the other ($n = 15$ in total) was removed from the cage. The difference between the median body-temperature of the final five mice and the median body-temperature of the five initially removed mice was calculated and this difference was defined as the stress-induced hyperthermia of the entire cage.

2.2.2. Modified stress-induced hyperthermia test

The test procedure for the modified stress-induced hyperthermia was adapted from van der Heyden et al. (1997).

Body temperature was measured in each mouse twice, i.e. at $t=0$ min (T_1) and $t=+15$ min (T_2). The difference in temperature (T_2-T_1) was considered to reflect the stress-induced hyperthermia. Pilot studies preceding these experiments showed that a T_2-T_1 interval of 15 min was optimal in terms of stress-induced hyperthermia. In addition, a comparison between T_1 in vehicle-treated mice and in animals treated with a given dose of a test-compound was used, to measure whether a test-compound affected body temperature alone.

Rectal temperature was measured to the nearest 0.1 °C in both the classical and the modified stress-induced hyperthermia paradigm by an ELLAB-instruments thermometer (Copenhagen, Denmark) by inserting a lubricated thermistor probe (2 mm diameter) 20 mm into the rectum; the mouse was hand held at the base of the tail during this determination and the thermistor probe was left in place until steady readings were obtained (± 15 s).

2.3. Pharmacological validation and characterization

2.3.1. Comparison of the classical and the modified version of the stress-induced hyperthermia paradigm

Various compounds or vehicles were tested in two independent studies using the two stress-induced hyperthermia paradigms in parallel. The aim was (i) to ascertain a good correlation of the quantitative effects seen in the two variants of the stress-induced hyperthermia test and (ii) to evaluate inter-experimental variability and reliability of the observed effects. The compounds included in this comparison were vehicle (0.5% methylcellulose), chlordiazepoxide (5 and 10 mg/kg, p.o.), NKP608 (0.01 and 0.1 mg/kg p.o.) and CGP80887 (15 mg/kg p.o.), i.e. a benzodiazepine, a potent NK_1 and a $mGlu_5$ receptor antagonist, respectively.

2.3.2. Validation of the modified stress-induced hyperthermia paradigm with benzodiazepines

Since benzodiazepines are clinically effective anxiolytics and widely used therapeutics, as a consequence, any new model of anxiety needs to be validated with this class of compounds. We tested here both chlordiazepoxide (0.3, 1, 3 or 10 mg/kg, p.o.) and diazepam (0, 0.1, 0.3, 1 or 3 mg/kg, p.o.). In additional studies, selected doses of two other benzodiazepines, namely, clobazam (5 and 10 mg/kg, p.o.) and oxazepam (5 and 10 mg/kg, p.o.), were also tested.

2.3.3. Validation of the modified stress-induced hyperthermia paradigm with non-benzodiazepine anxiolytics

The effects of buspirone (7.5 and 15 mg/kg, p.o.) and ethanol (15% and 30%, 10 ml/kg, p.o.) were evaluated.

2.3.4. Evaluation of novel target-related compounds with potential anxiolytic properties in the modified stress-induced hyperthermia paradigm

The following three novel potential anxiolytics, each of them a representative of a distinct mechanism of action, were tested: LY314582 (racemic mixture of LY354740), a selective metabotropic glutamate group 2 receptor agonist (1 and 10 mg/kg, p.o.); MPEP, a selective metabotropic glutamate receptor 5 antagonist (1, 10 and 30 mg/kg, p.o.); NKP608, a selective neurokinin 1 (NK-1) receptor antagonist (0.01 and 0.1 mg/kg, p.o.). In all of these studies, the experimental conditions were identical to those applied for the benzodiazepines, i.e. the pre-treatment time was always 60 min and the T_1-T_2 interval was fixed to 15 min. In each of these studies, chlordiazepoxide (7.5 or 10 mg/kg p.o.) was tested in parallel and served as a positive standard.

2.4. Neuroendocrinological characterisation of stress-induced hyperthermia in OF1/IC mice

Mice were treated with vehicle (0.5% methylcellulose, p.o.) or chlordiazepoxide (10 mg/kg, p.o.) and 60 min later their rectal temperature was measured (T_1; see above). Subsequently, half of the animals returned to their home cage (group: home cage) whereas the other half of the animals was placed in a novel cage (group: new cage) which was of the same dimension ($26\times21\times14$ cm) as the home cage. Then, the rectal temperature of each animal was re-determined (T_2) either 15, 30 or 60 min later. Immediately following the recording of T_2, the animals were decapitated and the trunk blood was collected in Eppendorf tubes containing EDTA; the tubes were kept on ice until they were centrifuged (3000 r.p.m., 4 °C for 20 min). Plasma was then removed and aliquots thereof stored at −30 °C until further processing. Plasma corticosterone concentrations were determined with a commercially available corticosterone-double antibody [^{125}I]-radioimmunoassay (ICN Biomedicals, Eschwege, Germany). The assay had a sensitivity of 3 ng/ml. Plasma adrenocorticotropin hormone (ACTH) concentrations were determined with a commercially available enzyme-linked immunosorbent assay (Morwell Diagnostics, Egg b. Zurich, Switzerland). This assay had a sensitivity of 1 pg/ml.

2.5. Statistics

For the comparison between the two stress-induced hyperthermia paradigms (nine different treatments), a linear regression was performed and the regression coefficient was considered to be significantly different from 0 if $r>0.798$ ($2P<0.01$).

The effects of the various drugs (see above) on rectal body temperature and stress-induced hyperthermia were statistically evaluated using a Kruskal–Wallis one-way analysis of variance followed post hoc by the Mann–Whitney U test (Bonferroni corrected). $P<0.05$ was considered as statistically significant.

For the evaluation within the stress-induced hyperthermia/neuroendocrinology study, the following statistical methods were used: repeated rectal body temperature measurements (T_1 and T_2) were statistically evaluated by means

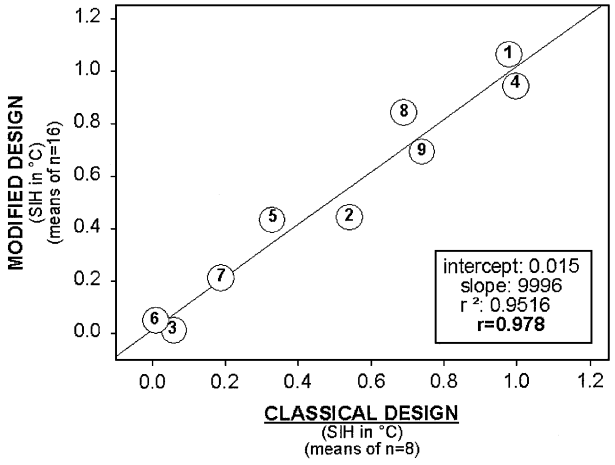


Fig. 1. Comparison of the effect of drug-treatment on their effect in the classical and modified stress-induced hyperthermia (SIH) paradigm in mice. In the classical design, $n=8$ indicates that eight cages each consisting of 15 mice was used whereas in the modified design in total 16 individually housed mice (for 24 h only) were used. The data-points are derived from two independent experiments and represent the following treatments: ①, ④: vehicle in Exp. 1 and Exp. 2, respectively, ②, ⑤: 5 mg/kg p.o. CDZ in Exp. 1 and Exp. 2, respectively, ③, ⑥: 10 mg/kg p.o. CDZ in Exp. 1 and Exp. 2, respectively, ⑦: 15 mg/kg p.o. of the $mGlu_5$ receptor antagonist CGP80887, ⑧, ⑨: 0.01 mg/kg and 0.1 mg/kg p.o., respectively, of the NK_1-receptor antagonist NKP608.

of a repeated analysis of variance (ANOVA) with factors cage (homecage versus new cage), treatment (vehicle versus chlordiazepoxide) and time (T_1-T_2 interval: 15, 30 or 60 min) with temperature as the repeated variable (T_1 and T_2). Temperatures within groups (T_1 versus T_2) were compared using the Wilcoxon paired sign test whereas temperatures between groups were compared using the Mann–Whitney U test. ACTH and corticosterone plasma levels were statistically evaluated using a three-way analysis of variance including the factors cage (homecage versus new cage), treatment (vehicle versus chlordiazepoxide) and time (T_1-T_2 latency: 15, 30 or 60 min). Plasma levels between groups were compared using the Mann–Whitney U test.

3. Results

3.1. Comparison of the classical and the modified version of the stress-induced hyperthermia paradigm

As indicated in Fig. 1, there was a high correlation between the effects seen in the classical and the modified stress-induced hyperthermia design: the regression-coeffi-

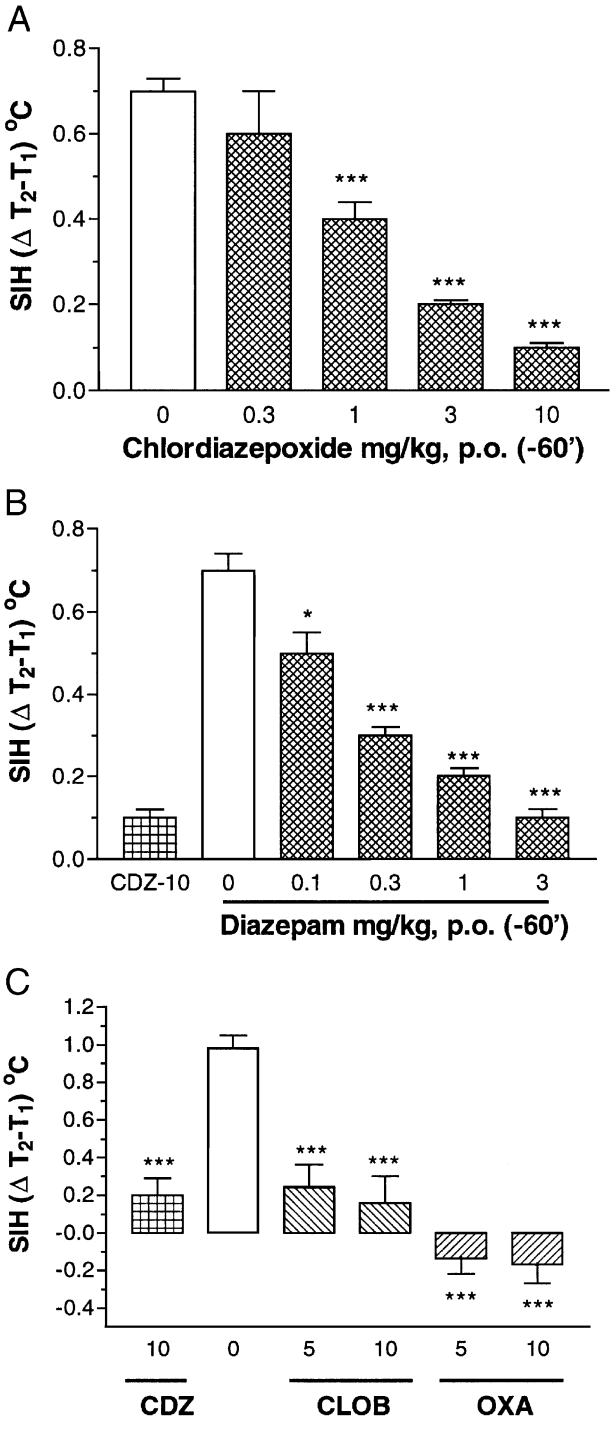


Fig. 2. Validation of the modified stress-induced hyperthermia (SIH) paradigm with benzodiazepines. (A) Effect of chlordiazepoxide on stress-induced hyperthermia in mice. Mice were pretreated (− 60 min prior to the T_1-recording) with 0.3, 1, 3 or 10 mg/kg (p.o.) chlordiazepoxide or vehicle (0.5% methylcellulose). Bars represent means ± S.E.M. of $n=10$ mice.: ***$=P<0.001$ versus vehicle-treated (0 mg/kg) mice. (B) Effect of diazepam on stress-induced hyperthermia in mice. Mice were pretreated (− 60 min prior to the T_1-recording) with 0, 0.1, 0.3, 1 or 3 mg/kg (p.o.) diazepam or vehicle (0.5% methylcellulose). Bars represent means ± S.E.M. of $n=10$ mice. *$=P<0.05$, ***$=P<0.001$ versus vehicle-treated (0 mg/kg) mice. (C): Effect of clobazam or oxazepam on stress-induced hyperthermia in mice. Mice were pretreated (− 60 min prior to the T_1-recording) with 5 or 10 mg/kg (p.o.) clobazam (CLOB) or 5 or 10 mg/kg (p.o.) oxazapam (OXA) or vehicle (0.5% methylcellulose). An additional group of mice was treated with 10 mg/kg chlordiazepoxide (CDZ): this group served as a positive standard. Bars represent means ± S.E.M. of $n=12$ mice. ***$=P<0.001$ versus vehicle-treated (0 mg/kg) mice. ⟶

cient of the linear regression revealed a r-value of 0.978 ($2P < 0.01$). Note that the slope (0.9996) of the regression line was very close to 1.00 and that the value of the intercept (0.015) was very close to zero. In addition, the quantitative comparable effects seen in two independent experiments for vehicle-treated mice (circles 1 and 4 in Fig. 1) and for those animals treated with 5 or 10 mg/kg p.o chlordiazepoxide-treated mice (circles 2 and 5, and circles 3 and 6 in Fig. 1, respectively) point to a high inter-experimental reliability for stress-induced hyperthermia with both designs used.

3.2. Validation of the modified stress-induced hyperthermia paradigm with known anxiolytics

3.2.1. Benzodiazepines

3.2.1.1. Chlordiazepoxide and diazepam. Both chlordiazepoxide (Fig. 2A) and diazepam (Fig. 2B) dose-dependently attenuated stress-induced hyperthermia: the one-way ANOVA on ranks revealed significant intergroup differences for chlordiazepoxide and diazepam, and post hoc comparisons versus the respective vehicle-treated control group indicated that stress-induced hyperthermia was significantly attenuated following 1, 3 and 10 mg/kg chlordiazepoxide and 0.1, 0.3, 1 and 3 mg/kg diazepam (p.o.). Neither of the two compounds affected T_1 at any of the doses tested (data not shown).

3.2.1.2. Clobazam and oxazepam. For clobazam as well as for oxazepam (Fig. 2C), a reduction in stress-induced hyperthermia was obtained. The ANOVA on ranks revealed significant intergroup differences for both benzodiazepines, and post hoc comparisons versus the respective vehicle-treated control group indicated a statistically significant reduction of stress-induced hyperthermia at the 5 and 10 mg/kg doses for both compounds. Neither of the two compounds at any of the doses tested affected T_1 (data not shown). The positive control, i.e. chlordiazepoxide (10 mg/kg, p.o.), also significantly reduced stress-induced hyperthermia.

3.2.2. Non-benzodiazepines

3.2.2.1. Buspirone and ethanol. Both buspirone (7.5 or 15 mg/kg, p.o.; Fig. 3A) and ethanol (15% or 30%, 10 ml/kg, p.o.; Fig. 3B) reduced stress-induced hyperthermia. The ANOVA on ranks revealed significant intergroup differences for both compounds, and post hoc comparisons versus the respective vehicle-treated control group indicated a statistically significant reduction of stress-induced hyperthermia at both tested doses with both compounds. Neither of the two compounds at any of the doses tested affected T_1 (data not shown). The positive control, i.e. chlordiazepoxide (7.5 mg/kg, p.o), significantly reduced stress-induced hyperthermia in both experiments.

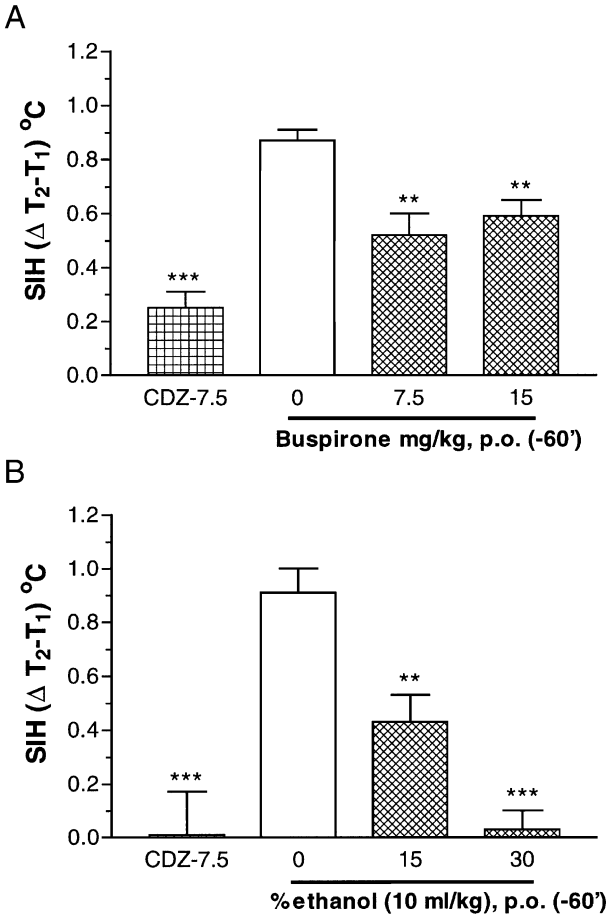


Fig. 3. Validation of the modified stress-induced hyperthermia (SIH) paradigm with non-benzodiazepine anxiolytics. (A) Effect of buspirone on stress-induced hyperthermia (SIH) in mice. Mice were pretreated (− 60 min prior to the T_1-recording) with 7.5 or 15 mg/kg (p.o.) buspirone or vehicle (0.5% methylcellulose). An additional group of mice was treated with 7.5 mg/kg (p.o.) chlordiazepoxide (CDZ): this group served as a positive standard. Bars represent means ± S.E.M. of $n = 12$ mice. ** = $P < 0.01$, *** = $P < 0.001$ versus vehicle-treated (0 mg/kg) mice. (B) Effect of ethanol on stress-induced hyperthermia in mice. Mice were pretreated (− 60 min prior to the T_1-recording) with an aqueous solution containing 15% or 30% or vehicle (water). An additional group of mice was treated with 7.5 mg/kg chlordiazepoxide (CDZ): this group served as a positive standard. Bars represent means ± S.E.M. of $n = 12$ mice. ** = $P < 0.01$, *** = $P < 0.001$ versus vehicle-treated (0 mg/kg) mice.

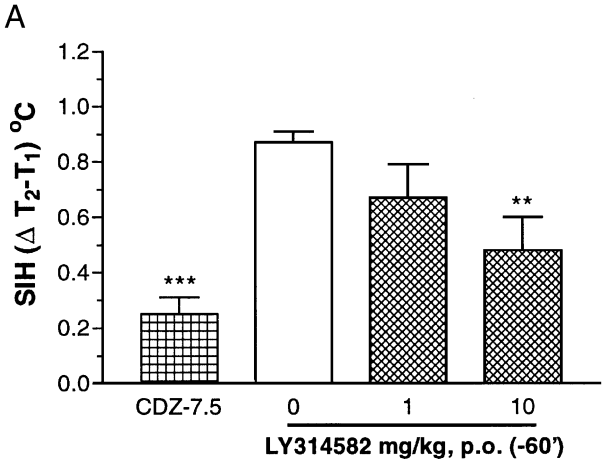
3.3. Evaluation of novel target-related compounds with potential anxiolytic properties in the modified stress-induced hyperthermia paradigm

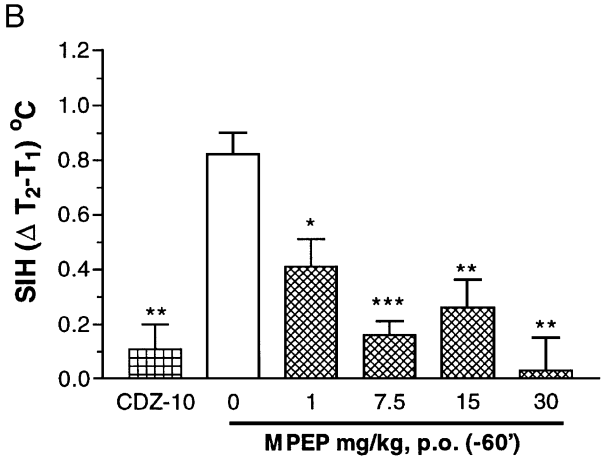
3.3.1. LY314582

The ANOVA on ranks revealed a significant intergroup difference for the metabotropic glutamate receptor 2 agonist LY314582 (1 or 10 mg/kg, p.o.). The post hoc comparison revealed that LY314582 significantly reduced stress-induced hyperthermia at the dose of 10 mg/kg (Fig. 4A). LY314582 had no effect on T_1 at any of the tested doses (data not shown). The positive control, chlordiazepoxide (7.5 mg/kg, p.o.), also significantly reduced stress-induced hyperthermia.

3.3.2. MPEP

The ANOVA on ranks revealed a significant intergroup difference for the metabotropic glutamate receptor 5 antagonist MPEP (1, 7.5, 15 or 30 mg/kg, p.o.). The post hoc comparison revealed that MPEP significantly reduced stress-induced hyperthermia at all doses tested (Fig. 4C). MPEP had no effect on T_1 at any of the doses tested (data not shown). The positive control, chlordiazepoxide (10 mg/kg, p.o.), also significantly reduced stress-induced hyperthermia.





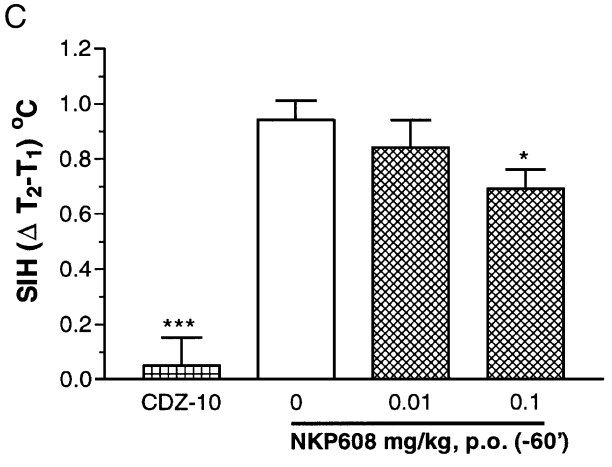


Fig. 4. Effect of new target-related compounds on stress-induced hyperthermia (SIH) in mice. (A) Effect of the metabotropic glutamate receptor 2 agonist LY314582 on stress-induced hyperthermia in mice. Mice were pretreated (− 60 min prior to the T_1-recording) with 1 or 10 mg/kg LY314582 or vehicle (0.5% methylcellulose). An additional group of mice was treated with 7.5 mg/kg (p.o.) chlordiazepoxide (CDZ): this group served as a positive standard. Bars represent means ± S.E.M. of $n=12$ mice. $**=P<0.01$, $***=P<0.001$ versus vehicle-treated (0 mg/kg) mice. (B) Effect of the metabotropic glutamate receptor 5 antagonist MPEP on stress-induced hyperthermia in mice. Mice were pretreated (− 60 min prior to the T_1-recording) with 1, 7.5, 10 or 15 mg/kg (p.o.) MPEP or vehicle (0.5% methylcellulose). An additional group of mice was treated with 10 mg/kg (p.o.) chlordiazepoxide (CDZ): this group served as a positive standard. Bars represent means ± S.E.M. of $n=12$ mice. $*=P<0.05$, $**=P<0.01$, $***=P<0.001$ versus vehicle-treated (0 mg/kg) mice. (C) Effect of the neurokinin 1 (NK-1) receptor antagonist NKP608 on stress-induced hyperthermia in mice. Mice were pretreated (− 60 min prior to the T_1-recording) with 0, 0.01 or 0.1 mg/kg NKP608 or vehicle (0.5% methylcellulose). An additional group of mice was treated with 10 mg/kg (p.o.) chlordiazepoxide (CDZ): this group served as positive standard. Bars represent means ± S.E.M. of $n=12$ mice. $*=P<0.05$, $***=P<0.001$ versus vehicle-treated (0 mg/kg) mice.

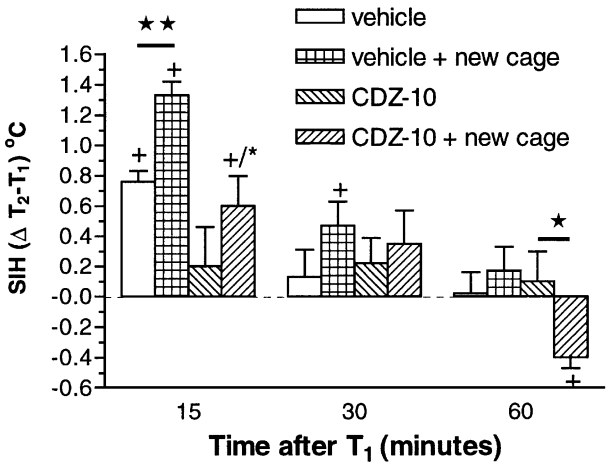


Fig. 5. Temporal characterization of stress-induced hyperthermia (SIH) in OF1/IC mice. The effect of the home cage versus new cage following either vehicle (0.5% methylcellulose) or chlordiazepoxide (10 mg/kg, p.o.; 60 min pre-treatment) on stress-induced hyperthermia (SIH) in mice at distinct T_2 time points (15, 30 or 60 min after T_1). Bars represent means ± S.E.M. of $n=6$ mice.: $+=P<0.05$ (T_2 versus T_1); $*=P<0.05$ versus vehicle-treated mice; ★ $=P<0.05$, ★★ $=P<0.01$ for the indicated comparison.

3.3.3. NP608

The ANOVA on ranks revealed a significant intergroup difference for the NK-1 receptor antagonist NKP608 (0.01 or 0.1 mg/kg, p.o). The post hoc comparison revealed that NKP608 significantly reduced stress-induced hyperthermia at the dose of 0.1 mg/kg (Fig. 4C). NKP608 had no effect on T_1 at any of the tested doses (data not shown). The positive control, chlordiazepoxide (10 mg/kg, p.o.), significantly reduced stress-induced hyperthermia.

3.4. Neuroendocrinological characterization of stress-induced hyperthermia in OF1/IC mice

3.4.1. Stress-induced hyperthermia

The ANOVA indicated a significant temperature effect ($F=42.498$, $P<0.001$) and a significant temperature × time interaction ($F=18.514$, $P<0.001$). Post hoc comparisons indicated that with a T_1-T_2 interval of 15 min, rectal body temperature was significantly increased in vehicle-treated mice in both cage conditions, i.e. 'home cage' and 'new cage' ($P<0.05$ for each condition), and with a T_1-T_2 interval of 30 min for vehicle-treated mice in cage condition 'new cage' ($P<0.05$). Rectal body temperature was significantly decreased at T_1-T_2 interval of 60 min in chlordiazepoxide-treated 'new cage' mice (10 mg/kg, p.o; Fig. 5). Furthermore, the ANOVA indicated a significant temperature × cage × time interaction ($F=3.641$, $P<0.05$). Post hoc comparisons indicated that with a T_1-T_2 interval of 15 min, rectal body temperature was significantly increased in vehicle-treated 'new cage' mice as compared to vehicle-treated 'home cage' mice ($P<0.01$; Fig. 5). In addition, post hoc comparisons indicated that with a T_1-T_2 interval of 60 min, rectal body temperature was significantly decreased in chlordiazepoxide-treated 'new cage' mice as compared to vehicle-treated 'new cage' mice ($P<0.05$; Fig. 5). Finally, the ANOVA indicated a significant temperature × treat-treatment × time interaction ($F=3.302$, $P<0.05$; Fig. 5). Post hoc comparisons indicated that with a T_1-T_2 interval of 15 min, rectal body temperature was significantly decreased in chlordiazepoxide-treated (10 mg/kg, p.o.) 'new cage' mice as compared to vehicle-treated 'new cage' mice (Fig. 5).

3.4.2. Plasma ACTH levels

ACTH plasma levels were significantly increased as compared to baseline values (untreated control animals) with a T_1-T_2 interval of 15 min in vehicle-treated 'home cage' and 'new cage' mice and with a T_1-T_2 interval of 30 min in vehicle-treated 'new cage' mice. The ANOVA indicated a significant treatment effect ($F=21.178$, $P<0.001$) and a significant treatment × time interaction ($F=3.876$, $P<0.05$). Post hoc comparisons indicated that with a T_1-T_2 interval of 15 min, ACTH levels were significantly reduced in chlordiazepoxide-treated mice (10 mg/kg, p.o.) in both cage conditions and also with a T_1-T_2 interval of 30 min in chlordiazepoxide-treated 'new cage' mice as compared to vehicle-treated mice of the respective cage condition (Table 1). ACTH plasma levels were also significantly reduced in mice treated with chlordiazepoxide (10 mg/kg, p.o.) with a T_1-T_2 interval of 60 min as compared to vehicle as well as compared to chlordiazepoxide-treated 'new cage' mice (Table 1).

Table 1
Neuroendocrinological characterization of stress-induced hyperthermia in OF1/IC mice: plasma ACTH levels

Treatment	Cage	Time		
		15 min	30 min	60 min
Vehicle	home cage	38 ± 11^{a}	20 ± 6	9 ± 3
Vehicle	new cage	31 ± 12^{a}	42 ± 9^{a}	8 ± 3
CDZ	home cage	6 ± 2^{b}	8 ± 2	$3 \pm 1^{c,d}$
CDZ	new cage	11 ± 3^{e}	14 ± 3^{e}	8 ± 1

Baseline value (untreated controls) 6 ± 2

The effect of home cage versus new cage and vehicle (0.5% methylcellulose) versus chlordiazepoxide (10 mg/kg, p.o.; −60 min) on plasma ACTH levels (pg/ml) in the stress-induced hyperthermia (SIH) test in mice at distinct T_2 time points (15, 30 or 60 min after T_1). Values represent means ± S.E.M. of $n=6$ mice.

[a] $P<0.01$ versus untreated controls.
[b] $P<0.01$ versus vehicle for the respective cage condition.
[c] $P<0.05$ versus untreated controls.
[d] $P<0.05$ for home cage versus new cage.
[e] $P<0.05$ versus vehicle for the respective cage condition.

Table 2
Neuroendocrinological characterization of stress-induced hyperthermia in OF1/IC mice: plasma corticosterone levels

Treatment	Cage	Time		
		15 min	30 min	60 min
Vehicle	home cage	124 ± 26^{a}	70 ± 18^{a}	17 ± 7^{b}
Vehicle	new cage	136 ± 24^{a}	130 ± 35^{a}	66 ± 40^{a}
CDZ	home cage	$40 \pm 10^{a,c,d}$	46 ± 11^{a}	13 ± 6
CDZ	new cage	92 ± 15^{a}	87 ± 20^{a}	30 ± 15^{b}

Baseline value (untreated controls) 5 ± 2

The effect of home cage versus new cage and vehicle (0.5% methylcellulose) versus chlordiazepoxide (10 mg/kg, p.o.; −60 min) on plasma corticosterone levels (ng/ml) in the stress-induced hyperthermia (SIH) test in mice at distinct T_2 time points (15, 30 or 60 min after T_1). Values represent means ± S.E.M. of $n=6$ mice.

[a] $P<0.01$ versus untreated controls.
[b] $P<0.05$ versus untreated controls.
[c] $P<0.05$ versus vehicle for the respective cage condition.
[d] $P<0.05$ for home cage versus new cage.

3.4.3. Plasma corticosterone levels

Corticosterone plasma levels were significantly increased as compared to baseline values (untreated control animals) in all treatment groups using a T_1-T_2 interval of 15, 30 or 60 min with the exception of chlordiazepoxide-treated 'home cage' mice (10 mg/kg, p.o.; Table 2) with a T_1-T_2 interval of 60 min. The ANOVA indicated a statistically significant treatment effect ($F=10.068$, $P<0.01$). Post hoc comparisons indicated that chlordiazepoxide (10 mg/kg, p.o.; Table 2) reduced corticosterone plasma levels only at the T_1-T_2 interval of 15 min in 'home cage' mice as compared to vehicle-treated 'home cage' mice whose level was also significantly reduced as compared to chlordiazepoxide-treated 'new cage' mice (Table 2).

4. Discussion

An anxiety or fear reaction is critically decisive upon whether an organism's overall subjective perception of a

situation triggers a particular action. Upon becoming anxious, a variety of behavioural, physiological, psychological and endocrinological mechanisms is activated (Ramos and Mormede, 1998). The physical components triggered and unpleasantly experienced in anxiety states or in an anxiety attack consist among others of heart pounding, sweating and an increase in body temperature, and all these body reactions are mediated by the autonomic nervous system. These symptoms are among the most disturbing and distracting epiphenomena of anxiety and anxiety states.

Although various animal models exist to assess the behavioural and psychological components of fear-inducing situations, only relatively few models are available to evaluate (and quantify) the level of autonomic hyperfunction in anxiety states. The stress-induced hyperthermia paradigm is one of the few tests that indeed focusses on the autonomic expression in anxiety (Lecci et al., 1990). The model has been pharmacologically validated and is increasingly used in the preclinical evaluation of potentially new anxiolytic compounds. In the present study, two versions of the stress-induced hyperthermia paradigm have been applied, i.e. (i) stress-induced hyperthermia following cohort removal (=classical stress-induced hyperthermia test; Lecci et al., 1990) and (ii) stress-induced hyperthermia in singly housed animals (=modified stress-induced hyperthermia test; van der Heyden et al., 1997). The direct comparison of the effect of various compounds in both the modified as well as the classical stress-induced hyperthermia paradigm suggests a very high correlation between the findings in the two different stress-induced hyperthermia paradigms with regard to their pharmacological sensitivity as indicated by the following three lines of evidence: (1) benzodiazepines (chlordiazepoxide, diazepam, clobazam and oxazepam) as well as non-benzodiazepines (buspirone and ethanol), i.e. compounds which are all known to be effective anxiolytics in man, are effective in the classical stress-induced hyperthermia test (Lecci et al., 1990; Spooren et al., unpublished data) as well as in the modified stress-induced hyperthermia test (van der Heyden et al., 1997; present study); (2) the response in body temperature and endocrinological parameters, i.e. adrenocorticotropin (ACTH) and corticosterone plasma levels, seen here in the modified version of the stress-induced hyperthermia paradigm, resemble those seen in the classical stress-induced hyperthermia test including temporal aspects (Groenink et al., 1994); (3) some novel anxiolytic candidate compounds, each originating from a different target group, caused comparable effects in both of the stress-induced hyperthermia paradigms.

The present data thus strongly suggest that changes in body temperature are reliably measurable and comparable in magnitude in the classical and modified stress-induced hyperthermia paradigms in OF1/IC mice (the standard mouse strain used in most preclinical studies at Novartis). This is of significance since it has been argued that the change in body temperature induced by either method could be due to different underlying mechanisms (van der Heyden et al., 1997). In the classical design, group-housed mice develop the hyperthermia without being touched, whereas in the modified stress-induced hyperthermia paradigm the individually housed mouse is 'handled' and its rectal temperature is repeatedly determined. Our present data do not support any dramatic influence of the different handling since the pharmacological manipulation of the temperature rise was comparable in both experimental set-ups. For the endocrinological read-out of the stress-induced hyperthermia, there might be, however, a striking difference. Previously, it has been described that diazepam decreased stress-induced hyperthermia but had no effect on stress-hormone levels when using the classical stress-induced hyperthermia paradigm (Groenink et al., 1996). In contrast, we show here in the modified stress-induced hyperthermia design that chlordiazepoxide, another benzodiazepine, clearly reduced ACTH and corticosterone levels in parallel to the stress-induced hyperthermia. Although this discrepancy cannot be explained at present, it would indicate, if at all, that in the modified stress-induced hyperthermia paradigm some better correlation might exist between effects on stress-induced hyperthermia and alterations in stress hormones.

In the present study, we also assessed the effect of a variation of the modified stress-induced hyperthermia paradigm by placing the animal immediately following T_1 into a new cage instead of placing them into the home cage. By this variation, handling- and neophobia-induced stress is thought to be combined resulting in a more general and elevated form of anxiety. Indeed, the findings indicate that this procedure clearly induced a more intense and longer lasting stress response both in terms of additional increases in body temperature and stress hormone levels. Assuming that a concomitant exposure to the novel cage causes a more persistent form of an anxiety response, it will be interesting to see which classes of potential new anxiolytic compounds can counteract the more pronounced autonomic reaction as mirrored by the level of body temperature and blood hormone concentrations.

Focussing next on the effect of the representatives of three novel classes of potential anxiolytic compounds, the following can be concluded: all three, i.e. LY314582, MPEP and NKP608, attenuated stress-induced hyperthermia indicating an anxiolytic-like potential of each of these investigational drugs. The first two compounds target metabotropic glutamate receptors. LY314582 (racemic mixture of LY354740), a metabotropic glutamate group 2 receptor agonist, which probably mediates its effects through $mGlu_2$ receptors (Spooren et al., 2000a), was previously shown to have broad anxiolytic activity (Monn et al., 1997; Klodzinska et al., 1999). The present findings with LY314582 in the modified stress-induced hyperthermia test would thus corroborate and extend the previous notion that stimulation of the $mGlu_2$ receptor can attenuate anxiety. MPEP is a selective $mGlu_5$ receptor antagonist, a representative of a class of compounds for which marked anxiolytic-like activ-

ity in rodents has been described recently (Schulz et al., 2001; Spooren et al., 2000b; Tatarczynska et al., 2001; for review: Spooren et al., 2001). Note that MPEP had previously been shown to be effective in the classical stress-induced hyperthermia test (Spooren et al., 2000b) and that we extend here to the modified stress-induced hyperthermia paradigm. A detail which at present cannot be explained is that MPEP shows anxiolytic-like activity at much lower doses in the modified stress-induced hyperthermia test than previously described in the classical stress-induced hyperthermia design. Finally, NKP608, a selective NK_1-receptor antagonist was found to have a significant, though partial effect in the stress-induced hyperthermia paradigm. This again corroborates the potential of NK_1-receptor antagonists as a promising new class of anxiolytic compounds and is in line with previous anxiolytic-like effects seen with NKP608 in rats using the social interaction test (File, 2000; Vassout et al., 2000) and social exploration test (Vassout et al., 2000). It remains to be determined whether this NK_1-receptor antagonist is more potent in inhibiting anxiety experienced in a social setting as compared to that experienced in a non-social setting such as the stress-induced hyperthermia paradigm. Out of the three new classes of compounds included in the present studies only NK_1 receptor antagonists are currently in clinical evaluation. According to initial data, the NK_1-receptor antagonist MK869 shows antidepressant and anxiolytic activity as reflected by an attenuation in both Hamilton depression (HAM-D) and Hamilton anxiety (HAM-A) scores in patients (Kramer et al., 1998).

Previously, effects on body temperature per se ($=T_1$) following some drug treatments (at higher doses), particularly benzodiazepines, were reported (van der Heyden et al., 1997; Pattij et al., 2001). Although, minor T_1 changes were also noticed in the present study none of these effects reached statistical significance. The different findings by others and by ourselves cannot be explained at present but could be due to numerous factors including differences in mouse strains, metabolism, handling, housing, purity of compound, etc., and is indeed likely to be multi-factorial.

In view of the positive anxiolytic-like effects described in the present study, it is important to consider the following aspect: as with many of the widely used behavioural paradigms designed to positively identify a compound's anxiolytic and/or antidepressant potential, in the present preclinical model, 'normal mice' were used. Accordingly, the animal's reaction in the stress-induced hyperthermia paradigm might be considered as an adequate and normal response. Therefore, it remains to be determined whether the new anxiolytic candidates tested and described here can also indeed effectively attenuate anxiety and autonomic hyperreactivity in man that is generated through psychopathological mechanisms. Although a multistrain comparison in mice (including available strains with different levels of anxiety and/or genetically engineered strains) might shed some additional light on the issue addressed above, it remains obvious that the therapeutic benefit of a new class of compound can ultimately only be demonstrated in humans.

In conclusion, all the data presented here indicate that the modified version of the stress-induced hyperthermia paradigm is a valid alternative to the classical stress-induced hyperthermia test. The modified stress-induced hyperthermia test is pharmacologically equally sensitive and neuroendocrinologically equally reactive to the classical stress-induced hyperthermia test, however, with the great advantage of a much higher throughput and a reduced demand in terms of numbers of experimental animals required. In our view, this, thus, makes the modified stress-induced hyperthermia paradigm a very attractive and valid experimental tool to identify compounds with anxiolytic-like potential.

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