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ORIGINAL PAPER

Effects of *Ignatia amara* in mouse behavioural models

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Background: ***Ignatia amara (Ignatia)*, a remedy made from the *Strychnos ignatii* seeds, is used for anxiety-related symptoms, but consistent evidence of its activity in reproducible experimental models is lacking. An investigation was performed in order to assess on mice, by means of emotional response models, the activity of homeopathic *Ignatia* dilutions/dynamizations.**
Methods: **Groups of 8 mice of the CD1 albino strain were treated intraperitoneally for 9 days with 0.3 ml of five centesimal (C) dilutions/dynamizations of *Ignatia* (4C, 5C, 7C, 9C and 30C). Control mice were treated with the same hydroalcoholic (0.3%) solution used to dilute the medicines. Diazepam (1 mg/kg) was the positive reference drug. Validated test models for locomotion and emotional response, the Open-Field (OF) and the Light–Dark (LD) tests, were employed. Five replications of the same protocol were carried out, in a randomised way using coded drugs/controls.**
Results: **In the OF the general locomotion of mice was slightly decreased by *Ignatia* 4C, but not by *Ignatia* 5C, 7C, 9C and 30C, indicating the absence of unspecific motor impairment or sedation by these dilutions/dynamizations. *Ignatia* and diazepam seemed to decrease the number of urine spots released in the OF during 10 min, with borderline significance ($P = 0.083$). In the LD the tested medicine showed anxiolytic-like activity (increase of time spent and distance travelled in the lit area), though to a lesser extent than diazepam. The highest and most significant difference with untreated controls ($P < 0.01$) was observed with the 9C dilution/dynamization. Among the 5 replication experiments, the best drug effects were obtained where the baseline anxiety of mice was higher.**
Conclusions: **Homeopathic *Ignatia* dilutions/dynamizations (peak at 9C) modify some emotion-related symptoms in laboratory mice without affecting locomotion.**
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Keywords: *Strychnos Ignatii*; *Ignatia*; Strychnine; Animal models of anxiety; Open-Field test; Light–Dark test; Homeopathic dilutions/dynamizations; Nanopharmacology

Introduction

Anxiety and depression are among the symptoms most frequently reported by patients seeking complementary or alternative medical treatments, such as homeopathy and natural remedies.[1–4] However there is a need of pharmacological studies elucidating their indications, limitations and mechanisms of action.[5] With conventional drugs, dosages and adverse reactions are generally studied in animal models prior to undertaking human trials. In homeopathy, the opposite has been true: trials on humans have only recently been followed up with tests on animals.

The past few years have seen an increase in the number of pre-clinical (*in vitro* and animal) studies aimed at evaluating the pharmacological activity or efficacy of some

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homeopathic medicines under potentially reproducible conditions.[6–13] In the field of psychopharmacology, rodent models are the most frequently used for exploring various aspects of anxiety making use of defensive behaviours. Although it cannot be proven that animals experience anxiety in the same way as human beings, it is generally undisputed that certain behaviours of rodents in experimental conditions correspond to forms of central and peripheral anxiety: Hormonal and neuromediator variations are common to humans and animals and, most importantly, drug responses in animals are in many cases predictive of the response of the average population in human clinical studies, or can in any case suggest novel pharmacological approaches.[9] We[14] and others[15,16] have previously found a statistically significant anxiolytic-like effect of a homeopathic medicine, *Gelsemium sempervirens*, using experimental models in rodents. In the present work, we employ the same protocol[14] to test another homeopathic remedy that is widely used for anxiety syndromes: *Ignatia* (also named *Ignatia amara*), obtained from the extract of *Strychnos ignatii* beans.

Strychnos ignatii (Figure 1) is a plant belonging to the Loganiaceae family, native to South East Asia, with long branches and pear shaped fruit that contain hard, 2.5 cm long seeds that are odourless but bitter and very poisonous due to a high strychnine content. Although it is best known as a poison, small doses of strychnine were once used in medicine as a stimulant, as a laxative, and as a treatment for other stomach ailments.[17] The Jesuits valued the seeds as a remedy against cholera and named them Ignatius beans after the Jesuit founder St Ignatius Loyola. Strychnine's stimulant effects also led to its use historically for enhancing performance in sports.[18] The use of strychnine in medicine was, however, eventually abandoned due to its high toxicity and tendency to cause convulsions. Symptoms of mild strychnine poisoning are drooling, nausea, vomiting, delusions, involuntary muscle spasms and twitching, and vertigo.[19]

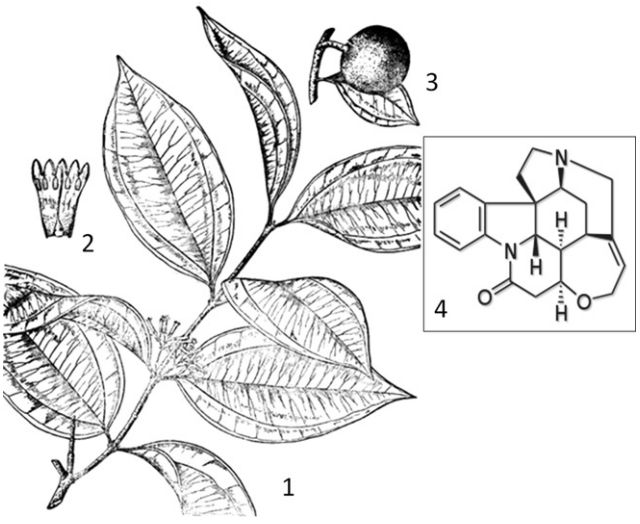

Figure 1 *Strychnos ignatii* plant (1), flower (2) and fruit (3). Insert shows the structure of the principal alkaloid strychnine (4) (molecular formula $C_{21}H_{22}N_2O_2$, molar mass 334.41).

The rationale for testing *Ignatia* is both clinically and experimentally grounded. *Ignatia* is one of the homeopathic remedies most commonly used on patients with anxiety symptoms, depression, manic episodes, emotive urination and diarrhoea, as well as hyperaesthesia and hypersensitivity to emotions.[20–22] It is also one of the first remedies to have been studied in laboratory animals.[19,23,24] However these works were published in non-indexed journals, and consistent evidence for efficacy with validated models is lacking.[9] Recent *in vitro* studies on the rat spinal cord and limbic system have shown that synthesis of the stress-related neurosteroid allopregnanolone is stimulated by *Gelsemium* and blocked by strychnine (the latter in non-homeopathic doses).[16,25] Since strychnine is the major alkaloid of *Ignatia*, it was of interest to determine whether homeopathic dilutions/dynamizations of this plant have some regulating effects on behavioural models in laboratory animals.

Ignatia has been previously investigated by some authors in rodent models, but the results reported are not always consistent, chiefly due to uncertainty connected with the methodology and a lack of statistical evaluations.[9] In 1978 Binsard tested the effect of *Ignatia* 3C, 7C and 30C in the 'hole-board' and 'escape' tests, finding a possible anxiolytic effect for the 3C dilution/dynamization.[23] The same author reports a subsequent experiment in which *Ignatia* 3C, 4C, 5C and *Gelsemium* 3C, 4C, 5C were simultaneously tested in the four-plate test with electric shock, which creates a state of anxiety, due to the conflict between the propensity to explore and the fear of the electric shock.[26] *Ignatia* 3C and *Gelsemium* 5C were reported to have an anxiolytic action (less than that of diazepam, but with the same direction of effect), whereas *Ignatia* 5C was reported to have a sedative action, in that it diminished the movements of the animal. Although the experiments were done in blind, their evidence were treated as preliminary due to the lack of a statistical evaluation of the differences observed.

In the present work, the mice were treated with a number of increasing dilutions/dynamizations of *Ignatia*, representative of those commonly used in humans for treatment of chronic ailments including anxiety symptoms: the dilutions/dynamizations ranged from 4C, which still contains a substantial quantity of molecules of the original active substance, to 30C, which is beyond the Avogadro-Loschmidt limit. Given the extremely low doses used, to increase the probability of inducing an effect, the animals were treated using a chronic regimen of one i.p. injection every day for 9 days. As a positive reference drug to assess the validity of the test we used diazepam (1 mg/kg, the most frequently used dosage in mice, given 30 min before the test), and as negative control we used a treatment consisting of only the solvent. After 1 week of treatment, the behaviour of the animals was assayed using 'ethological' test conditions which elicit a state of anxiety and fear (for example: being kept isolated rather than with other mice, being placed in a brightly lit area rather than in semi-darkness, or finding themselves in a novel environment different from the usual housing cages), and evaluate

the movements and behavioural responses of the animals in a standardised manner, through assignment of point scores. In order to rule out any possible bias due to experimenter interventions or cage effects, a procedure was introduced using randomised and blind conditions. Two validated animal models, the Open-Field test (OF) and the Light-Dark choice test (LD), were used to acquire various behavioural parameters widely employed in neuropsychopharmacology for drug screening.[27,28]

Methods

Animals

All the experiments were performed at the Faculty of Medicine, Verona University, Italy, as previously described,[14] with minor modifications. Male mice 4−5 weeks old of the CD1 strain were purchased from Charles River Laboratories (Lecco, Italy), and allowed to acclimate for 2 weeks before testing, in a controlled animal facility (temperature 22 ± 2°C, humidity 55% ± 5%). The mice were randomly distributed, two per cage, in plastic cages (size: 25 × 14 × 12 cm) and housed with food and water available *ad libitum*, except for during the brief testing periods. Lights were on between 7 a.m. and 7 p.m.

A minimum required sample size of $n = 34$ subjects for each treatment group was determined on the basis of previous experiences with the LD test on CD1 mice,[14] and assuming a pre-established statistical power of 0.8 and an alpha level of 0.05. In order to compare treatment and control groups of sufficient sample size (i.e. a total of 40 animals per treatment group), the study was conducted with 5 separate replications of the same protocol: in each replication experiment, mice were randomised into 8 groups of 8 animals; 5 groups were treated with different *Ignatia* dilutions/dynamizations, one group with diazepam, and two groups with the vehicle. Two groups of control vehicle-treated animals were employed for each replication to obtain highly consistent values, which were used as the reference for calculating percentage effects in the treated groups. Therefore, the total sample size was 40 mice for each drug-treated group and $2 \times 40 = 80$ mice for the vehicle-treated, control group. The mice belonging to each experimental group were randomly distributed among different cages, and the order in which cages were placed in the rack and the mice injected and tested was balanced between all cages and all experimental groups. Each animal was used only once in the same test to avoid the confounding effects of learning and habituation.

Drugs and treatments

The medicines were produced by Boiron Laboratoires, Lyon (F), according to the European pharmacopoeia (Monograph 01/2008:0672Ph.Eu), starting from a Mother Tincture (MT) consisting of a hydroalcoholic extract (60% ethanol/distilled water v/v) of dried and powdered *Strychnos ignatii* seeds. The content of strychnine − the principal alkaloid of *Ignatia* (Figure 1) − in the MT was 0.166% (w/v), corresponding to a concentration of 4.9×10^{-3} moles/l. The MT was diluted 100 times in 30% ethanol/distilled water to obtain the 1C dilution/dynamization. Subsequent serial 100 × dilutions/dynamizations up to 29C were then made up in the same solvent, using glass bottles. After each dilution/dynamization, the bottle was vigorously agitated using a mechanical shaker. The control solution (vehicle) consisted of the same batch of 30% ethanol/distilled water solution used to prepare the *Ignatia* dilutions/dynamizations. All solutions were stored in the dark at room temperature.

Before being used in each experiment, aliquots of the medicines and of control solutions were further diluted by 1:100 in sterile, apyrogenic distilled water and shaken with 20 strokes by hand. In this way, the ethanol concentration of the solutions administered to the mice was reduced to 0.3%. In order to blind the operators with respect to the tested solutions, all the samples were then coded by an independent person and the codes recorded on a sheet that was kept sealed inside an envelope until all the tests and calculations were completed. The solutions were distributed in 15-ml sterile Falcon plastic tubes (3.5 ml/tube), wrapped in aluminium foil and stored at +4°C until the day of use. Prior to administration each tube was again manually shaken with 20 strokes. The drug and control solutions were administered in the morning for 9 consecutive days (including on the last two days, when the tests were carried out) by intraperitoneal (i.p.) injection (0.3 ml) using 1-ml (insulin) syringes. The allopathic drug group (diazepam) received 0.3 ml of control solvent solution (0.3% ethanol/distilled water) for the first 7 days and 0.3 ml of solution containing diazepam (Valium, Roche, final dose 1 mg/kg bw) in the days of experiments only to avoid the well known tolerance to benzodiazepines.[29] Diazepam was administered 30 min before the test, due to its short half-life.[30] The treatments were administered row by row, and all the behavioural tests were started 30 min after the injections.

The treatment and testing procedures were independently approved by the Animal Ethical Committee of the Interdepartmental Centre for Animal Research (CIRSAL) of Verona University, and by the Italian Health Ministry. Aside from the treatment injections and testing, the animals were not subjected to pain or other forms of emotional or physical stress.

Behavioural tests

All testing procedures were performed between 9 a.m. and 3 p.m. The animals were tested individually in 4 separate devices, allowing a complete set of up to 64 mice to be tested during a 5−6 h experimental session. The experiments were performed in the following order: OF exploration test on 8th day of solution administration, LD choice test on the following day (9th day of solution administration). Immediately before testing, the animals were allowed to acclimate to the room inside their cages for 3 min, after being moved there from their customary housing area. The operators stayed outside the testing room during recording of the experimental sessions. The test arenas were cleaned thoroughly with tap water between trials.

The OF behaviour test involves placing an animal in an unknown environment consisting of a 50 × 50 cm black-painted wood platform with 25-cm high surrounding walls, illuminated with white light (100 lux).[31–33] The total distance travelled in the arena reflects general exploratory activity that could be changed by locomotor ability, and is reduced in case of sedation, paralysis, or impairment of movements, and conversely increased in case of excitation. The arena is divided virtually into two parts, with a square central zone having an area corresponding to 25% of the total area. The percentage time spent in the central zone is considered indicative of exploratory behaviour, and could reflect a decrease in anxiety, although this OF parameter is not sensitive to all anxiolytics and may not model features of anxiety disorders.[33] Measurement of various parameters are electronically taken in the same test at two time points, namely 5 and 10 min; at the end of the trial the number of urine spots > 1 cm diameter and the number of defecations are recorded.

The LD exploration test is based on the innate aversion of rodents to brightly lit areas, and their spontaneous exploratory behaviour in response to mild stressors such as novel environments and light.[34–36] Mice tend to prefer dark, enclosed spaces to large, well-lit areas, and the amount of time they spend in the dark zone is sensitive to benzodiazepines and to the agonists of serotonergic receptors, in a manner that correlates well with clinical efficacy in humans.[37] The test apparatus consists of a small, secure dark compartment (15 × 30 cm) and a large, aversive illuminated compartment (30 × 30 cm). The two compartments are separated by a partition with an opening (4 × 4 cm) through which the animal can pass from one compartment to the other. The open arena is brightly illuminated with 200 lux, and the mice are left to explore the space. Measurement of various parameters is taken after 5 and 10 min. An increase in the amount of time spent in the lit compartment is an indicator of decreased anxiety, and the number of light-dark transitions has been reported to be an indicator of activity and of exploration over time. Classic anxiolytics (benzodiazepines) as well as the newer anxiolytic-like compounds (e.g. serotonergic drugs) and natural compounds can be tested using this paradigm.[35,36]

All the sessions were recorded with a video-tracking camera (GZ-MG135, JVC, Japan) and stored on DVD. A software program ('Smart' VTS system from PanLab, Barcelona, E) was used to automatically trace the position and movements of the animals and calculate the time spent in different zones and the distance travelled. All the above described behavioural measurements, including the transitions between compartments in the LD, and the number of urine spots and stools in the OF, were considered as primary outcomes and done with the observer unaware of the treatment group assignment of the mice.

Statistics

Analyses were performed using the SPSS software, version 17 (SPSS Inc., Chicago, IL, USA: http://www.spss.com). The effect of the drugs on each mouse was calculated as a percentage relative to the mean values of the controls (vehicle-treated) in each replication experiment, taken as zero effect, according to the formula:

$$[(\text{Test value of each mouse}/\text{Mean test value of control mice}) - 1] \times 100$$

This standardization allowed the effects observed in all the experiments to be pooled and compared. All data are represented as mean ± SEM values. The Shapiro–Wilk test showed that the data collected were normally distributed. The effects of the various dilutions/dynamizations tested in the different experiments, expressed in standardised form as a percentage of the internal control values for untreated animals in the same experiment, were analysed by two-way analysis of variance, using the treatment group and the experiment as factors, thus correcting for the possible confounding effect of the latter. Post-hoc *t*-tests were performed assuming equal variances with least significant difference (LSD) corrections to adjust for multiple comparisons, as suggested by a consensus report for basic research in high-dilution/dynamization pharmacology.[38]

Results

Open-Field test

Several different behavioural parameters were evaluated in the OF test (Figures 2 and 3). Figure 2, panels A–D, reports the effects of diazepam and *Ignatia* dilutions/dynamizations on the total distance travelled by mice in the open field arena and on their walking speed. These measures were computed from software movement traces after 5 and 10 min of testing. These indexes do not reflect changes in anxiety level, but are important for evaluating the locomotor activity and speed of the animals during the trial.

Ignatia did not change these values as compared with the untreated control animals, except for the 4C dilution/dynamization which produced a small but statistically significant decrease in speed during the first 5 min (panel C) ($P < 0.05$). Diazepam caused a significant increase in both total distance travelled and walking speed during the entire test time ($P < 0.01$), suggesting a possible stimulation of movement or unspecific excitation effect of this drug at the dosage used. There were significant differences between the replications of the experiment performed, but no interaction between groups and experiments.

In the OF test, additional behavioural variables were evaluated (Figure 3), consisting of the percentage time spent in the central zone of the field (panels A–B) and the number of urine spots and defecations detected in the field at the end of the 10-min trial (panels C–D). The experiments did not reveal any clear and reproducible dose-dependence relation with the time spent in the central zone, and the standard drug diazepam likewise had no effect on this parameter, suggesting that this OF variable, in these experimental conditions, fails to detect standard anxiolytic drug effect. Also for these features, there was significant variability between experiments but no

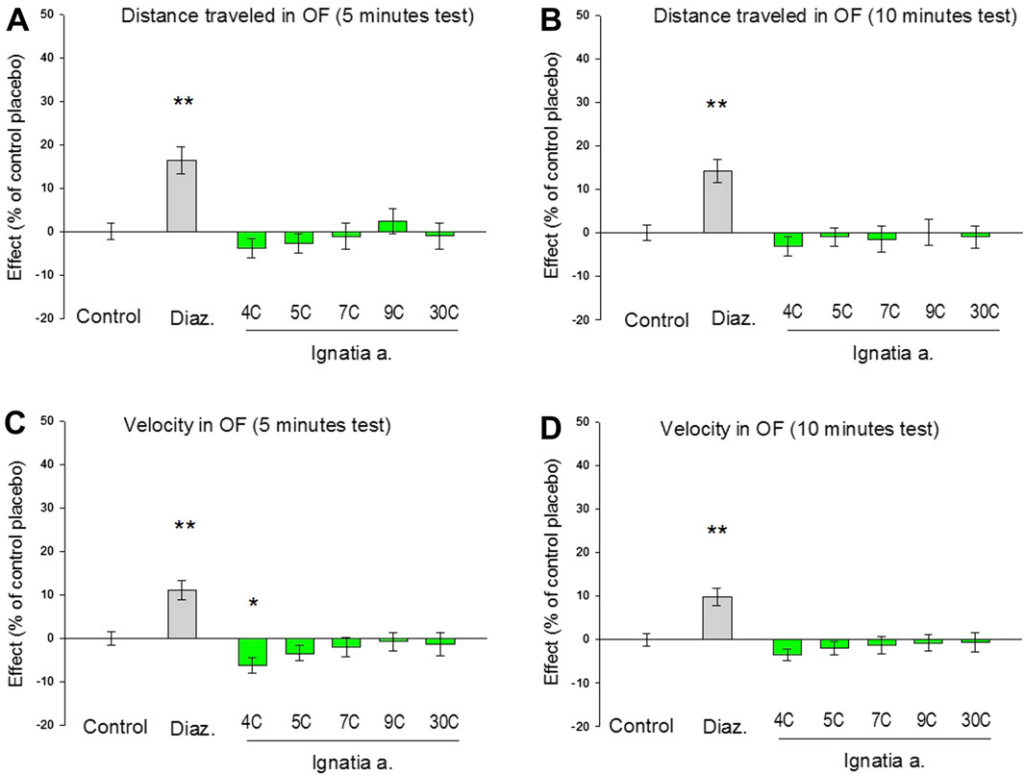

Figure 2 Effects of diazepam and *Ignatia* on the OF locomotion parameters, expressed as percentages ± S.E.M. relative to the mean values for the vehicle-treated, control animals. $N = 40$ mice in diazepam (Diaz.) and *Ignatia* treated groups, $N = 80$ mice in the control group. The mean absolute values for these parameters in control mice were: panel A 3220 cm/5 min; panel B 6228 cm/10 min; panel C 15.6 cm/s; panel D 14.8 cm/s. Global ANOVA values for groups and experiments were $P < 0.01$ in all test systems. The reported P values ($^{*}P < 0.05$; $^{**}P < 0.01$) are from LSD post-hoc analysis by two-ways ANOVA, comparing drug-treated groups with the mean of the corresponding control group.

interaction between experiments and groups. The number of urine spots decreased in treated groups as compared with controls, with an almost significant P-value of 0.083 in global ANOVA for groups. Even if a post-hoc analysis could not be performed due to the absence of global ANOVA significance, these studies indicated the *Ignatia* 5C and 9C as the most active dilutions/dynamizations. The number of stools (panel D) was unaffected by any treatment.

Light−Dark test

Ignatia and diazepam treatment caused a markedly significant increase in the parameters of the LD paradigm (global ANOVA values for groups $P < 0.01$), whose cumulative evaluations and post-hoc statistics are reported in Figure 4. With respect to the percentage time spent in the illuminated compartment (panels A−B), this was increased by diazepam and by *Ignatia*, in a significant way by the 9C dilution/dynamization ($P < 0.01$). This *Ignatia* solution changed the animals' behaviour in the direction of reduced anxiety, by 40−50%.

The percent effects of both diazepam and *Ignatia* were more evident in the first 5 min of the trial (panels A and C of Figure 4), in keeping with the hypothesis that they are really due to a decrease in the anxiety elicited by the novel environment into which the animal is suddenly placed, which would be expected to decrease over time due to habituation. A very similar trend was apparent for the computation of distance travelled in the light compartment (panels C−D). The calculation of the number of transitions between compartments showed a significant stimulating effect with diazepam, while all the *Ignatia* dilutions/dynamizations had only a slight stimulating effect, that however did not reach the threshold of statistical significance (e.g. $P = 0.06$ with Ignatia 7C) (data not shown).

Also in this test, there were significant differences between groups, and a significant interaction between group (treatment) and experimental replications emerged from global statistical analysis for time spent in the lit area (Figure 4A). So, we then analysed all the 5 replications of the LD test, considering the time spent in the lit area during the first 5 min of the trial, which appeared to be the most significant parameter for the purposes of this study. Figure 5 shows the results in both absolute value (left column panels) and percentage terms (right column panels).

Considering the absolute values of time spent in the lit area (which is inversely proportional to the level of anxiety), the untreated control groups exhibited widely differing behaviour in the series of experiments, showing more anxiety in experiments no. 1, 3 and 4 than in experiments 2 and 5. The time spent in the lit area by diazepam-treated animals was higher than for the controls in all experiments, thus confirming the validity of the test and the high significance of the global analysis (Figure 4A), but the difference relative to the control, and thus the

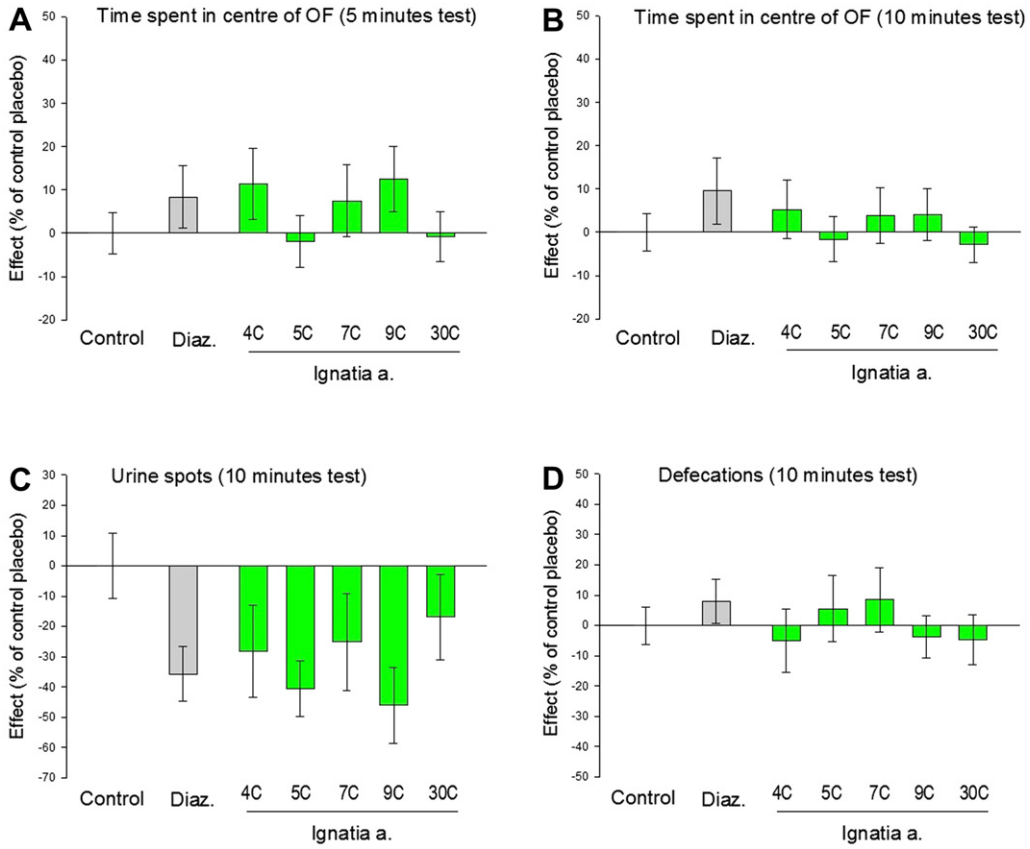

Figure 3 Effects of diazepam and *Ignatia* on the time spent in the centre of the OF (panels A–B), on the number of urine spots (panel C) and on the number of defecations (panel D), expressed as percentages ± S.E.M. relative to the mean values for the vehicle-treated, control animals. *N* of animals per group as in legend of Figure 2. The mean absolute values for these parameters in control mice were: panel A 43.6 s/5 min; panel B 106.2 s/10 min; panel C 1.33 urine spots/10 min; panel D 2.99 defecations/10 min. Global ANOVA values for groups and experiments respectively were: panel A $P = 0.622$, $P < 0.05$; panel B $P = 0.807$, $P < 0.05$; panel C $P = 0.083$, $P = 0.273$; panel D $P = 0.831$, $P < 0.01$.

percentage effect, was variable in the 5 replications of the protocol. The strongest effects of diazepam occurred in experiments no. 1, 3, 4, and in one experiment (no. 5) the drug was almost ineffective. *Ignatia* showed a positive trend (anxiolytic-like) in experiments 1, 3 and 4, while in the other experiments the effects were essentially undetectable or even slightly negative for some dilutions/dynamizations. Since the strongest effects of *Ignatia* were noted in those experiments where diazepam was also most active, this analysis lends support to the hypothesis that the effects of the homeopathic dilutions/dynamizations are due to a true anxiolytic-like activity and not to chance.

Discussion

Use of homeopathic remedies in single-component or complex formulations is frequent among patients seeking relief for anxiety-related symptoms in a variety of conditions [1–3,39,40] and in supportive cancer care,[4,41] but there is currently little evidence for the efficacy of homeopathy in the treatment of those disorders.[42–44] The clinical use of anxiolytic drugs is not without its drawbacks, particularly due to the risk of side effects such as development of tolerance, cognitive and memory changes, physical dependence, and withdrawal reaction on discontinuation. Natural remedies possessing the same efficacy as conventional drugs, but with fewer side effects, would thus be a valuable addition to the treatment options for anxiety-related disorders.

As part of a line of research we have been pursuing for years, our group has recently shown that high-dilutions/dynamizations of *Gelsemium sempervirens* are able to modulate some emotional responses of laboratory mice.[14] The present study was designed to explore the effects of the homeopathic remedy *Ignatia* on animal behaviour, using sufficient sample sizes to detect small difference effects, with validated models and double blinding. According to traditional homeopathic Materia Medica,[20] *Ignatia* has a marked action on various mental conditions such as moodiness, anxiety, anguish, depression, disappointment, melancholy, which can be caused by the ill effects of bad news, fright, grief, anger, disappointed love, or suppressed sexual desire. Other typical symptoms for which *Ignatia* is indicated as a remedy are neuralgic pain, twitching of the face or lip muscles, spasmodic cough, excessive flatulence, griping pains in the abdomen, diarrhoea due to fright, and profuse and watery urine. Ailments which are sensitive to *Ignatia* are typically worsened after drinking coffee or smoking, and relieved by lying on the painful side or by profuse urination.

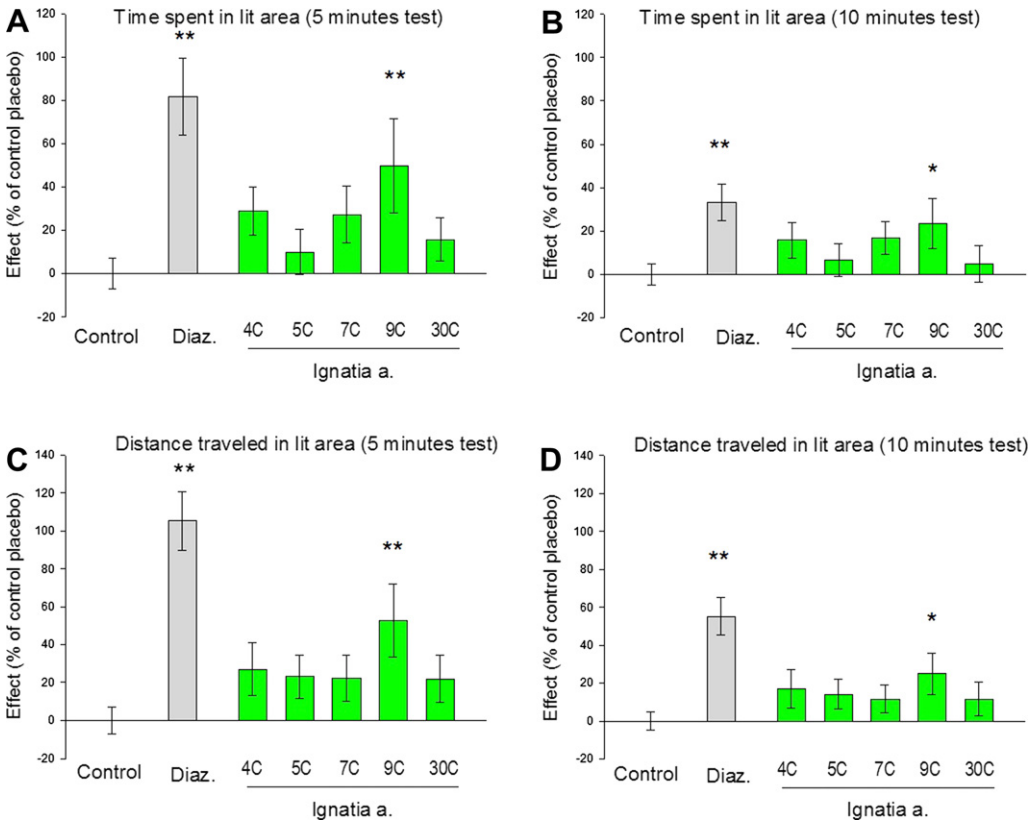

Figure 4 Effects of diazepam (Diaz.) and *Ignatia* on the LD parameters, expressed as percentages ± S.E.M. relative to the mean values for the vehicle-treated, control animals. *N* of animals per group as in legend of Figure 2. The mean absolute values for these parameters in control mice were: panel A 45.0 s/5 min; panel B 135.2 s/10 min; panel C 561 cm/5 min; panel D 1495 cm/10 min. Global ANOVA values for these evaluations were: panel A for groups $P < 0.01$, for experiments $P < 0.01$, interaction $p = 0.025$; panel B for groups $P < 0.05$, for experiments $P < 0.01$, interaction $P = 0.053$; panel C for groups $P < 0.01$, for experiments $P < 0.01$, interaction $P = 0.105$; panel D for groups $P < 0.01$, for experiments $P < 0.01$, interaction $P = 0.085$. The P values (* and **) are as in Figure 2.

The findings of the present study show, in a rigorous manner and using a large group of animals, that *Ignatia* reduced anxiety and fear in a manner comparable to, though quantitatively lower than, the effects of diazepam. The strongest statistical effect ($P < 0.01$) was obtained with the 9C dilution/dynamization, which contained extremely low doses of strychnine in molecular terms. Since the mother tincture contained 4.9×10^{-3} moles/l of strychnine, the theoretical concentration of this compound in the 9C solution was 4.9×10^{-21} moles/l and 4.9×10^{-24} moles/ml, i.e. near the Avogadro-Loschmidt limit. Since the mice received 0.3 ml/day (approximately 1 molecule of strychnine in the 9C dilutions/dynamization), this calculation allows to suggest that the pharmacological action was not attributable to direct action of strychnine molecules on putative receptors but on 'homeopathic' effects involving the participation of 'imprinted' solvent, which remain to be clarified.[45,46] No significant anxiolytic-like effects were observed using low dilutions/dynamizations, so there was no evidence of hormetic mechanisms of very low doses of active principles acting on sensible receptors in the 'molecular' range of concentrations. Including non-successed *Ignatia* dilutions would be a decisive step toward demonstrating the mechanism of action, but due to technical limitations on the number of animals housed and tested, this additional control couldn't be done in our conditions.

Interestingly, the only statistically significant effect of low dilutions/dynamizations (*Ignatia* 4C) was observed in speed in the OF, suggesting this might be a 'pathogenetic' effect, possibly arising from the direct action of strychnine on nerve or muscular function; however it should be pointed out that this *Ignatia* 4C affected total motility (locomotion) and not the anxiety-like responses, which are evaluated according to different parameters. From these results the interesting hypothesis emerges that low dilutions/dynamizations may act at a physical level while high dilutions/dynamizations may regulate behaviour at an emotional level. The stimulating effect of diazepam on locomotion remains unexplained and appears paradoxical, and has also been reported on rare occasions in humans.[47–49]

In the OF, the percentage time spent in the centre of the arena was not affected either by diazepam or by *Ignatia*, indicating that, in the experimental conditions of this investigation, this test may be insufficiently sensitive for detecting drug-related anxiolytic effects. An overview of the literature on the action elicited by effective or putative anxiolytics in animals subjected to this procedure indicates that some compounds (triazolobenzodiazepines such as adinazolam and alprazolam, selective serotonin reuptake inhibitors) that have different spectra of therapeutic efficacy in anxiety disorders such as panic attacks, generalised anxiety disorder or obsessive-compulsive disorder, are poorly effective as anxiolytics in the OF test, suggesting

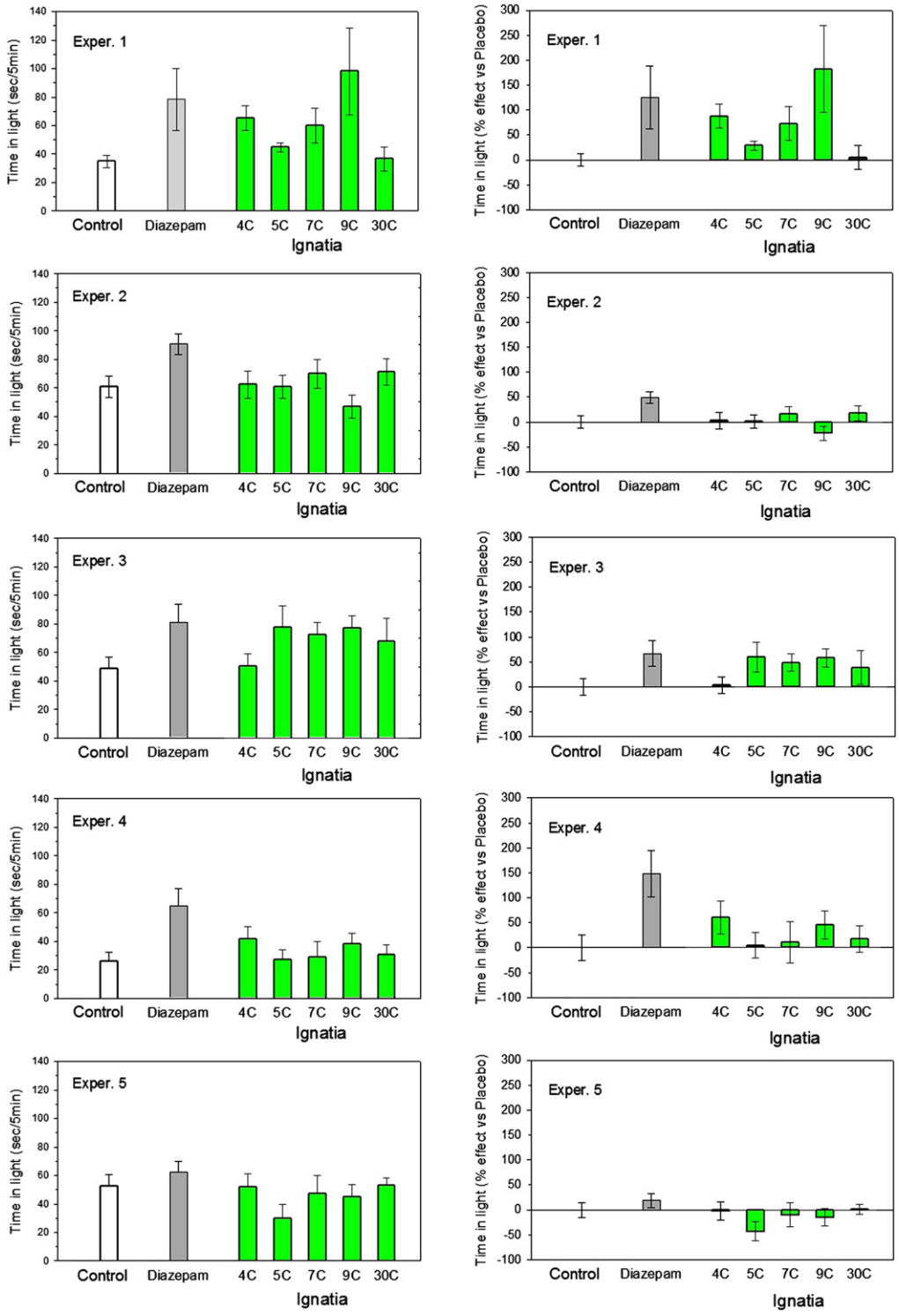

Figure 5 Time spent in the lit area of the LD test in control and drug-treated mice (left column panels) and percent effects of diazepam and *Ignatia* (right column panels) in the 5 replication experiments performed. Values are seconds ± S.E.M. spent in the lit area during the first 5 min of the trial (left column panels) or percentages ± S.E.M. relative to the mean values for the vehicle-treated, control animals of the same experiment (right column panels). For each replication experiment, $N = 8$ mice per group in diazepam and *Ignatia* treated mice, $N = 16$ animals per group in the control mice.

that this paradigm may not model all behaviour disorders.[33] In a previous work, we found a lack of effect also for the partial 5HT-agonist buspirone.[14]

A finding not previously reported concerns the urine spot count, which was reduced by both diazepam and all the *Ignatia* dilutions/dynamizations, although with low statistical significance at the global ANOVA test, which precluded a post-hoc analysis ($P = 0.083$). This parameter has been linked by other authors to changes in the emotional behaviour of rodents, with higher urine counts occurring when the animals are placed in a novel or stressful environment (Henderson and others).[50] Interestingly, in the traditional

homeopathic literature there are reports which include frequent urination among the symptoms that may be cured by *Ignatia*.[20] Therefore, this preliminary evidence, if confirmed, may represent a bridge between the traditional literature (most of which is still awaiting confirmation by statistical evidence) and modern experimental pharmacology.

There was significant variability between experiments. This problem, which affected all the test parameters and has also been noted in previous studies,[14] is possibly attributable to the high sensitivity of the mice and behavioural test systems to peripheral factors like the season, weather, animal breed, etc. We [9,10,51] and others [8,13,52] have previously reported and discussed the methodological aspects of research and theory in the high-dilution/dynamization pharmacology field, particularly with respect to the problem of reproducibility. If a treatment acts by influencing the complex natural healing dynamics of the treated subject as a whole, by means of small doses or highly diluted administrations, this action could, at least in theory, be highly sensitive to even small changes in experimental conditions.[51] This concept is in agreement with our data, which show that the best drug effects in the LD test were obtained in the three experiments where baseline anxiety was higher. This is relevant in the light of homeopathic theory, according to which significant effects of highly diluted drugs are to be expected only in sensitive subjects or in biological systems with primed, upregulated receptors.[51,53] It is also worth noting that our protocol included in the statistical analysis the complete sequence of replications, irrespective of the basal level of anxiety and of the effect of positive control (diazepam). However our results suggest that in future it might be interesting to consider including only those experiments in which the basal level of anxiety is high and the effect of the positive control is higher than a certain predetermined threshold.

On the other hand, the effects of diazepam in all five repetitions also seem to be roughly proportional to baseline anxiety (Figure 5), suggesting that a common environmental factor may be influencing the behavioural responses. In our laboratory, only unconditioned 'ethological' models and spontaneous reactions to non-painful stimuli were used, both for ethical reasons and because our aim was to approximate the natural conditions under which behaviour is influenced by emotional states of fear, curiosity and anxiety. Ethological models are highly sensitive to any influences, and exhibit individual differences and variable behavioural baseline levels.[28] An investigation of neuroendocrine−immune interactions in rats has shown that communication between animals in different cages, by means of noise or body odours, can alter their performance.[54] We were unable to determine such factor(s), despite taking the greatest care to reproduce the experimental conditions of the protocol. In our conditions, cage effects can be ruled out because, in each of the 5 repetitions, 8 animals per treatment group were randomly distributed in 4 cages, which were uniformly distributed in the holding rack. These questions could be further elucidated by future research on these models, repeating the experiments in different settings and rooms.

The mechanisms of action of *Ignatia* on the central nervous system could involve the centres which control pain and anxiety, possibly through interaction with glycinergic receptors, because strychnine at high doses has been found to bind to those receptors. Some authors report indirect evidence for the binding of *Ignatia* with receptors for glycine.[24,55] Glycine is an amino acid that functions as a neurotransmitter with inhibitory effect because it stimulates biosynthesis of the neurosteroid allopregnanolone, a pathway that was proposed as a putative mechanism of action also for *Gelsemium sempervirens*.[9,25] Strychnine is known to block the glycinergic receptors, but it could be hypothesised that in extremely low doses (or at high-dilutions/dynamizations) it might instead hormetically stimulate them, thereby acting in a manner leading to increased neurosteroid synthesis. The hypothesis appears in the recently re-evaluated framework of theories and evidence of a relation between hormesis and high-dilution/dynamization effects.[56,57] The development of interventions that activate hormetic signalling pathways in neurons is a promising new approach for the prevention and treatment of a range of neurological disorders.[58]

In conclusion, *Ignatia* has been shown to be able to modulate the emotional responses of mice in the LD paradigm, and possibly to have some effect in regulating the urination behaviour of the animals. Since the maximum anxiolytic-like activity was exhibited by solutions where the theoretical concentration of strychnine is about 1 molecule/ml, these results are compatible with the hypothesis of specific physicochemical changes of the solvent during dilution/dynamization and suggest non-linear mechanisms of regulation in the animals' central nervous system.

Conflicts of interest

The authors declare that they have no competing interests.

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