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ORIGINAL PAPER

Assays of homeopathic remedies in rodent behavioural and psychopathological models

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The first part of this paper reviews the effects of homeopathic remedies on several models of anxiety-like behaviours developed and described in rodents. The existing literature in this field comprises some fifteen exploratory studies, often published in non-indexed and non-peer-reviewed journals. Only a few results have been confirmed by multiple laboratories, and concern *Ignatia*, *Gelsemium*, *Chamomilla* (in homeopathic dilutions/potencies). Nevertheless, there are some interesting results pointing to the possible efficacy of other remedies, and confirming a statistically significant effect of high dilutions of neurotrophic molecules and antibodies. In the second part of this paper we report some recent results obtained in our laboratory, testing *Aconitum*, *Nux vomica*, *Belladonna*, *Argentum nitricum*, *Tabacum* (all 5CH potency) and *Gelsemium* (5, 7, 9 and 30CH potencies) on mice using ethological models of behaviour. The test was performed using coded drugs and controls in double blind (operations and calculations). After an initial screening that showed all the tested remedies (except for *Belladonna*) to have some effects on the behavioural parameters (light–dark test and open-field test), but with high experimental variability, we focused our study on *Gelsemium*, and carried out two complete series of experiments. The results showed that *Gelsemium* had several effects on the exploratory behaviour of mice, which in some models were highly statistically significant ($p < 0.001$), in all the dilutions/dynamizations used, but with complex differences according to the experimental conditions and test performed. Finally, some methodological issues of animal research in this field of homeopathy are discussed. The "*Gelsemium* model" – encompassing experimental studies *in vitro* and *in vivo* from different laboratories and with different methods, including significant effects of its major active principle gelsemine – may play a pivotal rule for investigations on other homeopathic remedies. *Homeopathy* (2009) **98**, 208–227.

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Introduction

Anxiety and behavioural disorders have a high prevalence in modern society, and consume significant financial resources. The most well-known tranquilisers or anxiolytics are those of the benzodiazepine family (BZDps), which act by modulating the GABAergic receptors, but many others are known, including Buspirone and other drugs belonging to the class of azaspirodecanedione compounds, which act as agonists of the serotoninergic receptors (5-HT1A). However, the clinical use of conventional drugs is not without its drawbacks, particularly due to side effects such as psychomotor impairment, potentiation of other central depressant drugs, and induction of forms of dependence that are difficult to reverse. Natural

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remedies possessing the same efficacy as allopathic drugs, but with fewer side effects, would be a valuable addition to the treatment options for anxiety-related disorders.

Research in anxiety has a long history of development of animal models. Murine models are the most frequently used, and explore various aspects of anxiety, often making use of defensive behaviours. The measurement of anxiety-related behaviour in animal models is based on the assumption that anxiety in animals is comparable to anxiety in humans. 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 central and peripheral emotional responses to acute or protracted stress. Hormonal and neuromediator variations are common to humans and animals and, most importantly, drug responses in animals are often predictive of the response in human clinical studies, or at least suggest novel pharmacological approaches.

Animal models have greatly assisted in understanding the development of disease at the tissue, cell and molecular level. They have also helped to elucidate the mechanisms of absorption, distribution, transformation and excretion of drugs, thereby allowing the active ingredients of medicinal plants and animal products to be identified. With allopathic 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.

A number of animal models have been used to investigate the action of diluted/succussed (potentised) homeopathic remedies. In this work, we will focus on the experimental (murine) models of anxiety and other neurological or behavioural disorders that are in some way correlated with anxiety. To the best of our knowledge, this is the first systematic review of homeopathic research in this field.

The advantage of animal tests is that they allow testing of multiple dilutions/dynamizations, compared to a vehicle, under reliable, reproducible and valid conditions. Different experimental models can be tried out on groups of animals to identify which one is most responsive under particular conditions. As well as offering a preclinical indication of the possible efficacy of new remedies, another goal of basic research in homeopathy is to study the mechanism of action of remedies. Here, the two major themes under investigation are: (a) the mode of action of the 'simile' effect, i.e., how a substance known to have pathogenetic effects in healthy organisms (cells, animals, humans) can act as a therapeutic agent in diseased organisms and (b) the 'high-dilution' effects, i.e., the question of whether and how substances diluted (and dynamized) to the point that few or no molecules of active ingredient remain can have biological effects. These two themes can in turn be split into a number of narrower questions regarding the methods of dilution, the most valid model for investigating the effect, the type of solvent used (distilled water, saline solutions, water/ethanol mixtures), and other technical details that may often affect the final outcome.

This paper is divided into two sections: the first provides an overview of the international literature on animal models for studying homeopathy in the specific field of anxiety disorders and other correlated behavioural disorders, while the second describes recent experiments conducted in our research laboratory on the behaviour of mice exposed to homeopathic remedies potentially capable of modulating anxiety and emotional responses to novel environments.

Because the existing literature on this subject is very varied and largely preliminary, with many reported observations not subsequently confirmed by other authors, it is important to adopt models that have already been validated by the pharmacological literature, as well as highly rigorous experimental conditions (blind protocols, reproducible preparation methods, sufficient numerosity) and solid statistics.

To provide a meaningful model of human behaviour, an animal test must meet three criteria: predictivity of responses to drugs, face validity (meaning that the phenomenological aspect – e.g., agoraphobia, neophobia, avoidance of threatening places – is observed also in humans) and construct validity (similar aetiology and triggering factors). In experimental tests, animals are placed in situations involving exploration, conflict between desire and punishment, conditioned fear and aggression. Exploring an environment consisting of light and dark zones, or the situation of being suddenly placed in a novel environment, elicits a conflict between approach and avoidance that allows different responses to be measured in a controlled manner. Mice tend to prefer dark, enclosed spaces to large, well-lit arenas, and the amount of time they spend in the dark zone, or the number of transitions between light and dark, are sensitive to benzodiazepines and to the agonists of serotoninergic receptors, in a manner that correlates well with clinical efficacy in humans.[1] There are also many other experimental approaches to studying animal behaviour, some of which will be described in this review, and genetic models (hitherto not used in homeopathy) have also recently been introduced. Animal models of anxiety can be grouped into two main subclasses[2]: the first involves the responses of animals conditioned with stressful and often painful events (e.g., exposure to electric shocks, forceful containment in small spaces), while the second involves the study of unconditioned responses using ethological paradigms and spontaneous reactions to non-painful stimuli (e.g., exposure to a novel highly illuminated test chamber or to an unfamiliar open field (OF)).

Our general hypothesis is that complex models of animal behaviour, in which different symptoms are evaluated in situations where the animal is not particularly stressed, are able to sensitively detect 'pathogenetic' or 'therapeutic' signs. After identifying the remedies that are effective in the chosen model, and ascertaining the reproducibility conditions and the most active dilutions, it is possible to proceed to study the mechanisms of action with a greater likelihood of obtaining consistent results.

Ethological observations show that, though rodents naturally tend to explore a novel environment, OFs are aversive

to them and hence counteract the normal behavioural responses.[3,4] It is conceivable that these two conflicting drives make the test highly sensitive to even extremely weak stimuli, such as one might expect from a homeopathic medicine. Ethological models were chosen both for ethical reasons and because our aim was to mimic the natural conditions in which behaviour is influenced by emotional states of fear, curiosity and anxiety, thus allowing for a comprehensive 'behavioural profiling'. This approach is especially relevant to homeopathic research, where symptoms 'peculiar' to the individual can be as significant as classic responses to pain or inflammatory tissue changes. However, ethological models are subject to inter-individual differences and variable behavioural baseline levels,[2] so that great care must be taken with variable parameters such as environment, handling and testing.

Two validated animal models, namely the light–dark (LD) choice test and the OF test, were used to acquire various behavioural parameters widely used in neuropsychopharmacology for drug screening.[2,5] After an initial screening of a number of potentially interesting remedies, we decided to carry out a series of experiments on dilutions/dynamizations of *Gelsemium sempervirens*, as this appeared to be the most promising and to give consistent responses.

Methods

Literature search

A literature review was carried out of all the relevant experimental trials that we were able to find, spanning the period from 1960 to 2009. Our main sources of information were current reading of the leading CAM journals, scanning of the monthly complementary medicine index (British Library), proceedings from homeopathic conferences, the databases of Central Council for research in Homeopathy, the Hom-inform Information Service, HomBRex-Database literature searches using Medline, CAM on PubMed, and cross-referencing.

Experiments on mouse behaviour in LD and OF models

Subjects and handling: In this section we shall describe our experimental procedures in some detail, to comprehensively illustrate these two widely used tests of animal behaviour as well as to clarify certain technical constraints on this type of research, which will also enable the reader to better interpret the results of the other work reported in the review.

All our experiments were performed at the Faculty of Medicine, Verona University, Italy. Male mice 4–5 weeks old of the CD1 strain were purchased from Harlan Laboratories (Udine, Italy) or from Charles River Laboratories (Lecco, Italy), and allowed to acclimate for two weeks before testing. The mice were socially housed (4 per cage or 2 per cage, see results) in plastic cages with water and food available *ad libitum*. The cages were cleaned and the bottles filled with fresh tap water twice a week. Lights were on between 7 a.m. and 7 p.m. The animals were randomly distributed among different cages; the order in which cages belonging to each experimental group were placed in the rack was balanced between all cages and all experimental groups, as was the order in which were mice injected and tested. Each animal was used only once in the same test to avoid the confounding effects of learning and habituation. All the experiments were conducted in accordance with the Italian National Institute of Health policies on use of animals in research and the 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.

Remedies: The remedies were produced for this study and supplied by Laboratoires Boiron Lyon (F) in 30% ethanol/distilled water at the potencies of 4CH (to obtain a 5CH final working potency), 6CH (final 7CH), 8CH (final 9CH) and 29CH (final 30CH). The gelsemine content of *Gelsemium sempervirens* mother tincture was 0.021%, corresponding to a concentration of 6.5×10^{-4} mol/l. Control solution was provided by Boiron Laboratoires and was the same batch of 30% ethanol/distilled water used to prepare the remedies. Before using in each experiment the remedies and control solution were diluted 1:100 in sterile, apyrogenic distilled water and strongly succussed with 20 strokes by hand. By this way, the ethanol concentration of solutions administered to mice decreased to 0.3%. In order to blind the operators as regards the tested medicines, 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 stored in 15-ml sterile Falcon plastic tubes (7.5 ml/tube), wrapped in aluminium foil and stored at +4°C until the day of use. Before use each tube was again manually shaken with 20 strokes. The 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). Intraperitoneal injection was chosen for this series of experiments because it is much easier to control the dose/volume and it is by far the most commonly used method in neuropharmacological studies on mice. Diazepam (Valium, Roche) was diluted in the control solution (0.3% ethanol/distilled water) and administered as 0.3 ml i.p. at the final dose of 1.0 mg/kg only on the days of the experiment, due to its well-known pharmacokinetic properties. Buspirone (Sigma) was dissolved in the control solution (0.3% ethanol/distilled water) and administered as 0.3 ml i.p. at the final dose of 5 mg/kg to a mice group for all the 9 consecutive days in parallel with the other experimental groups. The operators who performed the injections and the behavioural tests were totally unaware ('blind') of the treatment group to which the animals had been assigned.

Behaviour assessment: In each experiment the behavioural activities of drug-treated animals were compared with the activities of a parallel control group of animals which were treated only with control solution (succussed 0.3% ethanol in distilled water).

The LD exploration test (Figure 1) is based on the innate aversion of rodents to brightly illuminated areas, and their spontaneous exploratory behaviour in response to mild

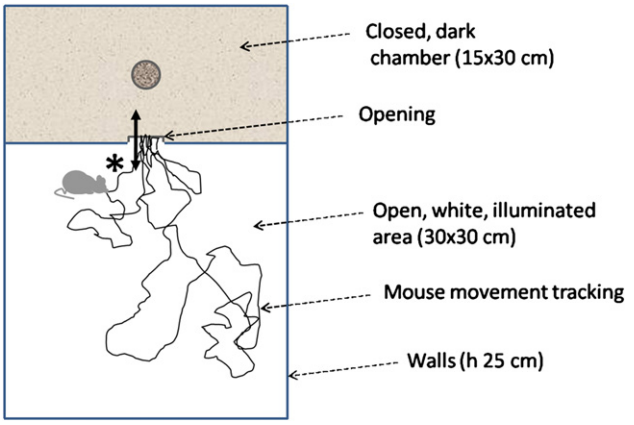

Figure 1 The LD test for assessing the mice behaviour. The asterisk indicates the point of uncertainty and of behavioural choice affected by the anxiety status.

stressors, i.e., a novel environment and light. The test apparatus consists of a small, secure dark compartment (one third) and a large, aversive illuminated compartment (two thirds).[6] The open arena is brightly illuminated at 200 lux, and the mice are left to explore the space for a 5-min testing period. An increase in the amount of time spent in the lit compartment is an indicator of decreased anxiety, and the number of LD transitions has been reported to be an indicator of activity-exploration over time. Classic anxiolytics (benzodiazepines) as well as the newer anxiolytic-like compounds (e.g., serotonergic drugs) and natural compounds[7,8] can be detected using this paradigm.

The OF test (Figure 2) involves placing an animal for 10 min 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). The arena is divided virtually into two parts, with a square central zone having an area corresponding to 25% of the total area. The tendency to stay on the periphery of the field is known as thigmotaxis, and is often considered indicative of highly emotional behaviour.[9] Conversely, the percentage of time spent in the central zone and the distance travelled in the central zone are considered indicative of exploratory behaviour, and should reflect a decrease in anxiety. Moreover, the total distance travelled in the arena (irrespective of the zone) reflects general locomotor ability, and is reduced in case of sedation, paralysis, or impairment of movements.

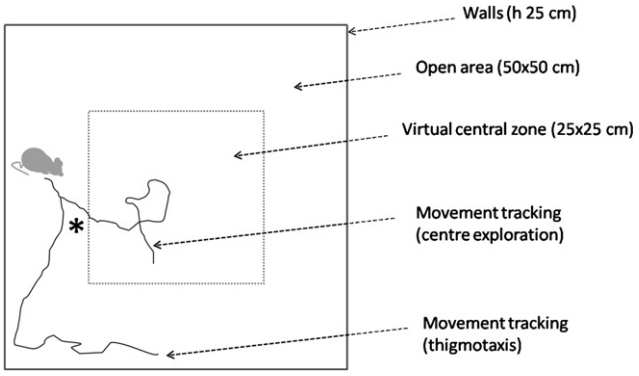

Figure 2 The OF test for mice. The asterisk indicates the bifurcation point of choice in the mouse behaviour.

A video-tracking camera (GZ-MG135, JVC, Japan) and a software program (''Smart'' VTS system from PanLab, Barcelona, E) were used to record the sessions automatically and calculate the time spent in different zones and the distance travelled. The number of transitions between the light and dark compartments in the LD test was evaluated on the screen by an operator who was unaware of the group assignment of the mice. All the sessions were recorded and stored on DVD.

Statistics: The effect of the drugs on the various behavioural parameters was calculated as a percentage relative to the mean control values (taken as 100%) for each experiment, according to the formula:

$$[(\text{Activity of each treated animal}/\text{mean activity of control group}) - 1] \times 100$$

This allowed the effects of the various dilutions tested in the different experiments (expressed in normalised form as a percentage effect of the internal control values in the same experiment) to be compared and statistically evaluated. The groups were compared by two-way analysis of variance (ANOVA SPSS, version 11 for Windows, Chicago, IL), using the treatment group and the experiments as the factors. Post-hoc *t*-tests were performed assuming equal variances with least significant difference (LSD) corrections to adjust for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

Literature review

A number of experimental psychopharmacology models have been used to test the anxiolytic and general effects on behaviour of homeopathic remedies in standardised and controlled conditions. The main results of the published investigations are summarised in Table 1. The quality of these experiments and their descriptions was evaluated semi-quantitatively by assigning scores for a number of criteria, with the results shown in the legend of Table 1. The purpose of these scores is to help the reader judge the reliability of the evidence furnished by each study. However we avoided computing a summary score, as this would have required assigning relative weights to the different criteria, in a necessarily subjective manner.

Current anxiety tests, taken singly, do not provide a clear and comprehensive picture of an animal's emotional profile. Therefore, many authors test their experimental hypotheses using a series of anxiety-related tests, which are thought to reflect different facets of emotionality.[32] The reviewed contributions have been grouped according to the principal experimental models used. Although this distinction is somewhat rigid, given that in many cases multiple models are used, that are different or partially overlap, it can still help us organise a wide array of approaches.

Table 1 Reports on psychopathological and behavioural models of homeopathy in rodents

Date/author/ref	*Publication features*					*Animal*	*Model*	*Remedy*	*Route*	*Main effects*	*Notes*
	A	*B*	*C*	*D*	*E*						
1960/Tetau and Tetau[10]	1	1	0	1	0	Rat	Drinking behaviour (electricity-conditioned labyrinth)	Thuya T.M (20%)	Sub-cutaneous (single injection)	Initial increase of activity, then sedation (loss of conditioning) in 50% of rats	
								Thuya 9CH	Sub-cutaneous (single injection)	Normalization of reflex after intoxication with high-dose Thuya	Test with only 3 rats
1965/66/Julian and Launay[11]	1	0	0	1	1	Mouse	Psychomotor disturbances, catalepsy	Reserpine 7, 9, 15, 30CH *Cicuta virosa* 3DH, 5, 7, 15, 30CH	Sub-cutaneous inject., 3 per week for 3 weeks	Protection against Reserpine-induced catalepsy (only Reserpine 9CH and Cicuta v. 3CH)	Reserpine and Cicuta v. in homeopathic doses induce pathogenesis
		0	0	1	1	Guinea pig				Protection against Reserpine-induced catalepsy (7, 9, 15CH Reserpine)	
1978/Binsard[12]	1	1	1	1	2	Mouse	Hole-board Escape test	*Ignatia* 3CH, 7CH, 30CH	i.p. 3 weeks	Anxiolytic (3CH only)	
1979/Binsard[13]	1	1	1	1	1	Mouse	4 Plates	*Ignatia* and *Gelsemium* 3CH, 4CH, 5CH	i.p. 3 weeks	Anxiolytic (*Ignatia* 3CH and *Gelsemium* 5CH only) or sedative (*Ignatia* 5CH)	
		1	1	1	1	Rat	Staircase	*Ignatia* and *Gelsemium* 3CH, 4CH, 5CH	i.p. 3 weeks	Sedative (*Ignatia* 4CH and *Gelsemium* 3CH and 5CH only)	
1980/Binsard *et al.*[14]	1	1	1	1	0	Mouse	4 Plates Escape test Rota-rod	*Gelsemium* 3CH, 5CH, 7CH	i.p. 3 weeks	*Gelsemium* 3CH reduces exploration, *Gelsemium* 7CH increases it	Difficult interpretation because Librium also decreases exploration (sedative?)
1981/Guillemain *et al.*[15]	1	1	0	1	0	Mouse	Strychnine Induced convulsions	*Ignatia* 3D, 3, 5, 7, 12CH	i.p. 0.5 ml/20 g single dose	Slight protective effect of 3DH and 5CH	Difficult interpretation due to strong toxicity of the model and to different protocols of administration.
1986/Sukul[16]	2	1	0	1	1	Rat	Cataleptogenic effects of restraint	*Gelsemium*, Cannabis Indica, *Graphites* and Agaricus Muscarius (30CH and 200CH)	Per os	Increase cataleptogenic effects of restraint.	Data reported also in a book [17]
1989/Guillemain *et al.*[18]	1	0	0	1	1	Mouse	Behavioural test (4 plates)	*Ignatia* 3, 5, 7CH	i.p. Single dose	Anxiolytic effect	Unpublished data reported in a review
		0	0	1	1	Mouse	Chronic anxiety induced by a 21-day treatment with RO 15-3505 Testing with 4 plates and labyrinth (plus-maze)	*Ignatia* 5 and 15CH *Gelsemium* 5CH Sempervirine 5CH *Coca* 5CH *Argentum nitricum* 9CH	i.p. 1/day for 21 days	Anxiolytic effect of all tested remedies in the plus-maze test, in some but not all experiment	

1991/Sukul *et al.*[19]	1	1	0	1	1	Rat	Electrophysiology of central nervous system	Arnica 30CH, Hypericum 200CH, Arsenic 30CH	Per os (0.5 ml)	Arnica and Hypericum decrease firing rate, Arsenic increase it. Instantaneous effects	Similar results also in cats
1997/Cristea *et al.*[20]	0	1	1	1	1	Mouse	Behavioural tests	*Chamomilla* 5CH and 30CH	Per os 4 times/day for 1 day (5CH) or 2 times/day for 3 days (30CH)	Stimulating effects with 5CH and tranquilising effects with 30CH	Studies with rats are mentioned but not reported. No information about variations.
1999–2001/Sukul *et al.*[21,22]	2	2	0	1	1	Mouse	Loss of righting reflex due to ethanol	*Nux vomica* 30CH	Per os 0.05 ml/2 ml water and given at 0.05 ml/individual.	Protective effect	
2001/Bousta *et al.*[23]	2	2	0	2	1	Mouse	Electric stress Staircase test LD test Blood cell count Gastric lesions	*Atropa belladonna* *Gelsemium sempervirens* *Poumon histamine*	i.p. 30 min before stress and test	Reversal of stress-induced alterations	Difficulties in interpretation of behavioural test parameters
2005/Ruiz-Vega *et al.*[24]	2	2	0	2	2	Rat	Sleeping behaviour	*Coffea cruda* 30CH and 200CH	Per os in feeding bottle	Coffea 30CH changes spectral power of EEG Delta band	No therapeutic effects evaluated
2006/de Paula *et al.*[25]	2	2	2	2	2	Rat	Heat-induced itching and OF	*Dolichos pruriens* 6, 9, 12, 30CH in ascending potency administration	Per os 40 days	Protection against skin lesions, no behavioural changes	No pathogenetic effects
2006/Epstein *et al.*[26]	2	2	0	2	1	Rat	Conditioned learning, inhibitory avoidance	Antibodies to antigen S-100 diluted and successed to 6CH	Per os, 0.5 ml/day for 4 weeks	Increase memory tasks	The same antibodies have neurotrophic properties[27]
2008/da Silva Rocha *et al.*[28]	1	1	1	1	1	Rat	OF	Rhus toxicodendron 200CH	Per os 24 h	Decreases locomotion in hyperactive rats	Small number of animals, high variability
2008/Pinto[29]	2	1	1	1	1	Mouse	OF Forced swimming After cohabitation with sick cage-mate	*Chamomilla* 6CH	Per os 7 days	Prevents decrease of general activity. No changes in forced swimming test	Disturbing effects of vehicle (10% ethanol)
2009/Bellavite *et al.*[30,31]	2	2	2	2	2	Mouse	OF LD test	*Gelsemium sempervirens* 5CH, 7CH, 30CH	0.3 ml i.p. 9 days	Increases exploratory behaviour in OF	No sedative effects

Publication features: (A) type of publication: 0 = book chapter or conference proceeding, 1 = non-indexed, non-peer-reviewed journal, 2 = indexed, peer-reviewed journal; communications reporting single cases or expert opinions have been excluded; (B) description of methods and of statistics: 0 = absent or insufficient, 1 = simple but insufficient to permit replication, 2 = complete; (C) blinding: 0 = not done or not described, 1 = mentioned but not described, 2 = complete description; (D) results: 0 = largely insufficient, 1 = simple description but no statistical evaluation, 2 = complete; (E) clarity of conclusions: 0 = absent or insufficient, 1 = sufficient, 2 = complete.

Catalepsy and convulsion models

Catalepsy is a transitory state of immobility in which animals are unable to correct their posture starting from an abnormal position. This can be induced by restriction of movement or by drugs. Kent's repertory of homeopathic Materia Medica lists a large number of homeopathic remedies under the heading 'catalepsy'.

Among the early 'pioneering' trials, some worth noting are those done in the 1960s by Julian and Launay[11] and by Tetau and Tetau,[10] who conducted a series of studies for testing homeopathic remedies with nervous tropism on murine models. These studies, in themselves of great interest, were however reported on non-indexed journals, with insufficient description of the methodology, and only qualitative reporting of results.

Julian and Launay[11] induced a state of catalepsy (or catatonia) by administering ponderal doses of Reserpine (4.5 mg/kg), and showed the therapeutic effect of Reserpine (in homeopathic dilutions) and of *Cicuta virosa*, a homeopathic remedy that has similar symptoms in its pathogenesis (alternating periods of excitement and depression, mental confusion, to the point of reaching a catatonic state). Reserpine in homeopathic dilutions (with the 9CH dilution/dynamization proving most effective) reduced the speed of onset and the intensity of cataleptic crises provoked by Reserpine in ponderal doses.

In the same series of experiments, the authors show that Reserpine in homeopathic dilutions (7CH and 9CH) by itself induced a state of generalized motor slowing that lasted about 24 h (pathogenetic effect). In mice, *Cicuta virosa* delayed the onset of the effects of Reserpine, but only in the 3DH dilution and not in centesimal dilutions; in guinea pigs, on the other hand, a preventive effect of *Cicuta virosa* was observed even in the 5CH, 15CH and 30CH dilutions. Finally, the authors report that treatment of healthy (non-intoxicated) rats with homeopathic dilutions of *Cicuta virosa* resulted in appearance of some pathogenetic symptoms such as loss of weight and motor stimulation.

Overall, this series of experiments provides preliminary evidence for what the authors call the 'law of identity' and the 'law of analogy': the law of identity is demonstrated by the fact that preventive sub-toxic exposure to Reserpine in a Hahnemannian dilution/dynamization (9CH) increases the onset latency and reduces the duration of catalepsy provoked by injecting a triggering dose of Reserpine; the law of analogy is demonstrated by the fact that chronic sub-toxic exposure to *Cicuta virosa* (which in high doses provokes catalepsy) delays the onset of catalepsy triggered by Reserpine as well as reducing its duration. In rats, only sub-toxic exposure to *Cicuta virosa* had a protective effect. What is more, some (but not all) dilutions, even beyond the Avogadro limit, had a measurable action, though this varied depending on the animal and the products and dilutions used.

Experimental homeopathic research in psychopharmacology can be considered to have started with the work of Tetau and Tetau on Thuja.[10] These authors found that ponderal doses of Thuja alter the psychic equilibrium of rats, causing them to lose their environmental 'conditioning'. In animals intoxicated with material doses of Thuja, a 9CH homeopathic dilution/dynamization of Thuja caused their disturbances to disappear, restoring their normal conditioning.

The group of J Guillemain, M Tetau and G Narcisse, in collaboration with other authors, conducted a lengthy series of studies on the properties of various homeopathic remedies (*Ignatia*, *Gelsemium*, Sempervirine, *Coca*, Strychninum, *Argentum Nitricum*), but these were unfortunately published in non-indexed journals and are therefore difficult to access. In the field of neuropharmacology studies, it is worth mentioning the work on *Ignatia*, a homeopathic remedy whose pathogenesis also includes muscle contractions, spasms and violent convulsions, as well as hyperaesthesia and hypersensitivity to emotions.[15] The experimental model involved convulsions provoked by administering strychnine sulphate. The homeopathic remedy, in a dilution/dynamization of 3DH and 5DH, had a slight protective effect against intoxication with strychnine.

These effects were however minimal and not consistent across the different parameters considered (mortality, latency of mortality, onset time of convulsions), probably due to the intense toxicity provoked by the high dose of strychnine used, which makes the results difficult to interpret. The authors formulated the hypothesis that the protective effect of *Ignatia* against convulsions triggered by strychnine could be due to a specific interaction at the level of the glycinergic receptors. These receptors, which bind the neurotransmitter glycine, are known to have an inhibitory, anxiolytic and sedative effect, and moreover strychnine is an antagonist of glycine at the level of these receptors. In an experiment in which synaptic membranes of rat brain were incubated with radioactive strychnine, *Ignatia* 3DH proved capable of significantly blocking this binding, whereas it had no effect on the binding of radioactive GABA and Diazepam.[33] Computing the experimental dilution factors, the authors maintain that the concentration of *Ignatia* in the test corresponds to 4CH of strychnine, and is therefore sufficiently low to suggest a high affinity of the glycinergic receptor for some of the active ingredients of the plant.

Other interesting and varied studies have been reported by the group of Sukul,[16] involving the use of four homeopathic remedies (*Gelsemium*, *Cannabis* indica, *Graphites* and *Agaricus muscaris*) administered orally to albino rats in potencies of 30CH and 200CH. All four remedies augmented the cataleptic effect induced by restriction of movement (restraint) in a similar manner to the reference compound haloperidol (i.p. injection at 5 mg/kg), an antagonist of dopamine receptors. The same authors also observe that Agaricus 30CH can reverse catalepsy due to haloperidol, but only when administered orally,[17,34] suggesting that the effect of the homeopathic potencies may be mediated by receptors situated on the oral mucosa. This conclusion is not in agreement with the fact that various authors, including ourselves, have found significant effects of homeopathic remedies even when they are administered intraperitoneally.

In addition to the catalepsy model, Sukul's group has developed other models for evaluating the effect of homeopathic remedies in behavioural models, on animals treated

with ethanol. These involve the righting reflex, by which animals recover and maintain their normal erect posture through a series of integrated responses especially at the level of the mesencephalon. Ethanol can induce loss of the righting reflex in mice, and the effect can be measured in terms of the amount of time the animal remains as if asleep.

Nux vomica is a homeopathic remedy traditionally said to be effective for treating the effects of alcoholism. Sukul *et al*.[21,22] observed that *Nux vomica* 30CH, administered orally, significantly reduces the duration of ethanol-induced loss of the righting reflex in albino mice. The mice were intraperitoneally administered a 25% ethanol solution, in a dose of 4 grams per kilogram of body weight, 6 h after treatment with Nux 30CH. The duration of sleep time with ethanol was recorded in four sessions for the same group of mice, with an interval of 10 days between sessions. In addition to using 90% ethanol, the authors prepared Nux 30CH using other diluents such as pure ethanol and pure water. In this series of experiments. They observed that the most effective vehicle was ethanol in the aqueous phase, whereas neither pure ethanol nor water Nux vom potencies showed an anti-hypnotic effect in albino mice. This result indicates that ethanol in the aqueous phase is the best vehicle for preparing an effective homeopathic potency. It should nevertheless be borne in mind that, because the potentised remedies had been stored in the laboratory for at least six months before being tested on the animals, it is possible that the pure water preparations may have lost their efficacy during storage.[17] The experiment therefore needs to be confirmed using other models. The results have important implications for the hypotheses of a physical basis for homeopathic potencies.

The same group, in collaboration with Russian researchers, has obtained interesting results on the mechanism of action of the remedy at the level of the rat central nervous system.[19] In male albino rats, a glass-coated silver micro-electrode was inserted into the medial frontal area, for electrophysiological recordings. In this model, *Arnica montana* 30CH, administered orally, caused a decrease in the firing rate of the neurons, and the effect lasted for over 20 min with a latency ranging from 1.5 to 6 min. *Hypericum perforatum* 200CH caused a decrease in the firing frequency in neurons, and the effect lasted between 1.5 and 14 min with a latency ranging from 1 sec to 2 min. *Arsenicum album* 30CH produced a marked increase in the firing rate. An interesting observation is that the action of homeopathic potencies begins within a few seconds and lasts for a maximum of a few minutes after their intake. The same remedy can have inhibitory and excitatory effects on different neurons. Some neurons may also be completely unresponsive to a remedy. The authors suggest that the homeopathic remedies exert their effect through oral receptors and interact with different neurons in different areas of the brain.

Anxiety models

Anxiety is not a unitary disease, but a complex phenomenon that probably involves different neurochemical systems with varied aetiological origins, and can be divided into various forms including 'state' anxiety (excess anxiety that a subject experiences at a particular time in the presence of a stimulus) and 'trait' anxiety (which does not vary from time to time). The classification of anxiety disorders is based on symptom clusters and therapeutic responses.[35] For this reason, there are also many tests which investigate anxiety-like behaviour in animals. In this section, we have included the homeopathy studies that meet one of the following criteria: (a) they use behavioural tests commonly employed by conventional laboratories for studying the effects of anxiolytic drugs, or (b) they use an anxiolytic drug such as benzodiazepine or 5-hydroxy tryptamine (5HT) receptor agonists as a positive control.

Ignatia is one of the homeopathic remedies most commonly used in patients with anxiety symptoms, nervous depression, insomnia, emotive diarrhoea, etc. It is also one of the first remedies to have been studied in experimental models. In 1978 Binsard reported an experiment[12] which tested the effect of *Ignatia* 3CH, 7CH and 30CH (0.5 ml/20 g of weight, administered intraperitoneally, for 5 days out of 7 for 3 weeks) in the 'hole-board' test, which involves the animal exploring the holes on a flat surface, and in the 'evasion test', which involves measuring the time taken by the animal to leave an enclosed space through a single exit opening. These are therefore tests of exploration and locomotion. Binsard's results show that *Ignatia* 3CH improved the performance of mice in these two tests, suggesting an anxiolytic effect. Although the experiments were done in blind, the evidence must be treated as preliminary due to the lack of a statistical evaluation of the differences observed, and of a comparison against a reference anxiolytic drug.

The same author reports a subsequent experiment in which *Ignatia* 3, 4, 5CH and *Gelsemium* 3, 4, 5CH were simultaneously tested in comparison with reference drugs and the solvent (water). The first part of the work involved submitting mice to the four-plate test,[13] which involves observing the movements of the animal in a square enclosure having a base made up of 4 steel plates that can be electrified to 'punish' the animal when it moves from one plate to another. Putting the animal in this situation creates a state of anxiety, due to the conflict between the propensity to explore and the fear of the electric shock.

In this test, *Ignatia* 3CH and *Gelsemium* 5CH showed an anxiolytic action (less than that of Diazepam, but having the same direction of effect), whereas *Ignatia* 5CH showed a sedative action, in that it diminished the movements of the animal. The treatment in both cases was 0.4 ml/20 g of weight intraperitoneally, 5 days out of 7 for 3 weeks. The same publication also reports an experiment on rats of the Wistar strain, treated with 1 ml of remedy/100 g of weight and subjected to the 'staircase' test, where the animal is left free to explore a space that includes a staircase which it can decide to ascend or not.

The exploratory propensity was assessed based on the number of steps and on 'rearing' behaviour (standing in an erect position, on the hind legs). In this case, the reference anxiolytic drug was chlordiazepoxide (Librium),

which produced a notable increase in the number of steps ascended and a reduction in rearing. The two homeopathic remedies instead produced a marked diminution in the number of steps ascended – the opposite action to that of chlordiazepoxide – which was therefore interpreted as a sedative, rather than anxiolytic, effect.

A further report in a homeopathic journal[14] utilised the hole-board test – to study the effects of *Gelsemium* 3CH, 5CH and 7CH (0.5 ml/20 g, intraperitoneally, 7 days out of 7 for 3 weeks) comparing it with chlordiazepoxide (50 mg/kg) and a control solution (distilled water). In this test, the reference drug produced a notable reduction in exploration, an effect that was also observed with *Gelsemium* 3CH, and in part with *Gelsemium* 5CH. *Gelsemium* 7CH instead enhanced exploration, thus suggesting a two-phase effect dependent on the dilution/dynamization. In this same study, a slight 'anxiolytic' effect was observed for *Gelsemium* 3CH in the evasion test, and for *Ignatia* 3CH in the operant conditioning test (which involves rewarding the animals with access to food and water when they press a pedal). However the results are difficult to interpret, both due to the lack of statistical evaluations, and because the reference drug was administered in a very high dose that resulted in prostration, loss of motor coordination and marked hypotonia of the animals.

Guillemain *et al*.[18] report an overview of their previous research, including some previously unpublished findings, which we summarise here. According to their review, *Ignatia* 3CH, 5CH and 7CH (administered intraperitoneally, 1 h before testing) increased the number of transitions in the four-plate test (see description above), where nitrazepam (control drug) was also active in the same direction. No significant effects were obtained with *Ignatia* 3DH and 12DH or with Strychninum (homeopathic dilution of strychnine) 3CH and 4CH. Moreover, *Gelsemium* 5CH, Sempervirine nitrate 5CH (one of the active principles of *Gelsemium*) and *Argentum nitricum* 9CH appeared to contrast, in a few experiments, the effects of the anxiogenic compound RO 15-3505 (inverse agonist of benzodiazepines) in the labyrinth (plus-maze) test.

The same authors[18] made an interesting attempt to investigate the neurobiological mechanisms, and report that RO 15-3505 slightly decreased the affinity of radioactive (3H) benzodiazepine in the mouse cortex, and that this effect was contrasted and reversed by Sempervirine 5CH in one experiment and by *Ignatia* 15CH in another. Moreover, RO 15-3505 slightly decreased the affinity of the radioactive (3H) GABA in the mouse cortex, and this effect was reversed by *Ignatia* 15CH and *Argentum nitricum* 9CH (single experiment). These findings would seem to suggest a possible action of Sempervirine on the benzodiazepine receptors, and of *Ignatia* on GABAergic inhibitory transmission, but the lack of statistical evaluations and replications of the results make any firm conclusions about the mechanism impossible.

Cristea *et al*. conducted an extensive investigation into the effects of homeopathic dilutions of *Matricaria chamomilla* on the central nervous systems of mice.[20] Interestingly, the approach in this work involved a preliminary actometric test for assessing spontaneous motility, in order to classify individuals into groups of animals with hypomotility or hypermotility. Mice with hypomotility were stimulated by *Chamomilla* 5CH in a number of behavioural parameters, including motility, evasion test, righting reflex and nimbleness (behaviour in rotational test). Some specific differences between the stimulating effects of *Chamomilla* and those of amphetamine, the reference substance, suggest that the homeopathic effect was not an amphetamine-like effect. Mice with basal, spontaneous hypermotility were tranquilised by *Chamomilla* 30CH, based on the above behavioural parameters, in the same way and to the same extent as by allopathic doses (5 mg/kg) of Diazepam. The authors suggest that these pharmacotherapeutic effects experimentally prove the validity of the homeopathic therapeutic principle of similitude.

More recently, Bousta *et al*. report that in some – but not all – experimental conditions, *Belladonna*, *Gelsemium* and *Poumon histamine* (5CH, 9CH, 15CH) reduce stress-induced behavioural alterations of mice in the staircase test and LD test.[23] The work, published in a major international journal, is highly complex in its possible interpretations. The mice were subjected to stress consisting of a series of electric shocks according to a standardised procedure, with the remedies administered intraperitoneally 30 min prior to the stress, after which various behavioural parameters such as the LD test and the staircase test were immediately measured. Finally, the animals were sacrificed and their blood tested for certain immunological parameters, and their stomachs examined for possible gastric lesions. It appears that the homeopathic remedies were prepared in alcohol dilutions, while the control group was treated with NaCl 0.9%, which raises some concerns as to the methodology. Another flaw of the protocol was the absence of a positive reference drug, which would have aided in interpreting the experimental data. In fact, one of the most peculiar aspects of the results is that, in comparing stressed and unstressed animals, it is reported that stressed animals reduced their number of transitions between the light and dark compartments in the LD test, but increased the amount of time spent in the illuminated compartment (a parameter that conventionally denotes a reduction in anxiety). This latter observation is especially surprising because it would appear to indicate a reduction in the anxiety of mice following stress. From the laboratory analysis results, it was found that stressed mice had a general reduction in leukocyte count (lymphocytes, neutrophils, basophils and monocytes) and an increase in gastric lesions. Given the many parameters analysed and the different remedies tested in various dilutions/dynamizations, the results of these experiments are particularly complex to describe. The data pertaining only to the main positive results obtained with homeopathic remedies are reported below:

(a) *Atropa belladonna* reversed the effects of stress on the behavioural parameters. The active dilutions/dynamizations were 15CH in the staircase test and all (5, 9, 30CH) in the LD test. The remedy (5CH and 15CH) increased the number of monocytes in the blood of stressed animals and, in the 9CH dilution/dynamization, reduced the number of gastric lesions.

(b) *Gelsemium sempervirens* in 5 and 15CH improved the performance of stressed mice in the staircase test, while the same dilutions reduced the activity of unstressed mice. In the LD test, the remedy in the 5, 9 and 15CH dilutions/dynamizations increased the number of transitions but reduced the amount of time spent in the light compartment. This result indicates that it reversed the effects of stress, but is nevertheless strange because it suggests, according to the conventional interpretation of this test, that the remedy produced an increase in anxiety. *Gelsemium* did not have significant effects on unstressed animals, except for a reduction in rearing. In stressed mice, *Gelsemium* (9 and 15CH) significantly increased the number of neutrophils and basophils and, in all dilutions, protected against development of gastric lesions.

(c) *Poumon histamine* (5, 9, 15CH), in the staircase test, reduced the activity of unstressed mice and increased it in stressed mice compared to untreated animals. In the LD test, all dilutions/dynamizations reduced the amount of time spent in the light compartment, thus reversing the experimental effect of stress. The same remedy, in stressed mice, notably increased the counts of the various types of leukocytes, and especially of neutrophils (in all dilutions) and reduced the gastric lesions. That said, a significant increase in gastric lesions was observed in unstressed mice.

The authors conclude that the tested remedies generally have protective effects on the parameters altered by stress, both in terms of behaviour and at the level of the immune and gastrointestinal systems. These effects are probably associated with their neurotrophic and anxiolytic effects. However, the above results were obtained as reversals of the effects of severe stress, and the findings varied greatly depending on the potency used and the test performed. Therefore, further studies exploring the neurotrophic and behavioural effects of *Gelsemium*, *Belladonna* and *Poumon histamine* are necessary before any firm conclusions can be reached.

Pinto and colleagues investigated the effect of homeopathic dilutions of *Chamomilla* (6CH, administered orally each day for 7 days) on a murine experimental stress model consisting of forced cohabitation of a healthy mouse with one suffering from a tumour.[29] Mice that cohabitated with a sick cage-mate showed a decrease in their general activity in an OF arena, but those treated with *Chamomilla* 6CH were less severely affected ($p = 0.0426$). No haematological changes were observed.

In a second experiment, a stress based on forced swimming was applied to mice pre-treated with *Chamomilla* 6CH (administered orally 24, 5 and 1 h before the test). The controls were: water, 10% ethanol or amitriptyline. Then the immobility time (a sign of anxiety and depression) was measured. Only the amitriptyline and ethanol treated groups showed significant decrease in immobilisation time ($p = 0.0020$). The scores of animals treated with *Chamomilla* 6CH were midway between those for animals treated with water control and ethanol or amitriptyline. A reduction in the leukocyte count was observed in the amitriptyline and *Chamomilla* 6CH treated groups ($p = 0.039$). These results suggest that treatment with *Chamomilla* 6CH is related to the recovery of basal behavioural conditions in mice subjected to stressful conditions. However, the finding that ethanol (the vehicle used for the homeopathic remedy) has an effect on the immobilisation time in the forced swimming test makes it difficult to interpret these last results, and further studies are required.

Da Silva Rocha *et al*. tested Rhus toxicodendron 200CH (administered orally, added to the drinking water for 24 h) in a rat anxiety model based on locomotion (distance travelled in an OF) and other behavioural parameters such as rearing, grooming (motion of front legs directed at the head or body), immobility time and defecation.[28] The rationale for testing *Rhus tox* was that, according to the Materia Medica, this remedy has an inhibitory action on the nervous system, as well as having effects on rheumatic symptoms, particularly in mice subjected to stress. A group of 16 rats was divided into two subgroups characterised by high and low locomotor tendency (hyperactive and hypoactive). The hyperactive animals showed a significant reduction in locomotion following the homeopathic treatment, and also after treatment with Diazepam, the anxiolytic control drug. Treatment with water (the vehicle of the homeopathic remedy) also produced some diminution in activity, but not to a statistically significant extent. All the other behavioural parameters did not show significant differences between before and after treatment, neither with *Rhus tox* nor with Diazepam. The work is interesting, but must be regarded as preliminary because it was conducted on a small number of animals, and because careful inspection of the data in the tables reveals a very high inter-individual variability (coefficient of variation often equal to or greater than 100%), that might have masked a possible efficacy of the tested substances.

Other behavioural and memory models

The therapeutic and pathogenic effects of *Dolichos pruriens* were evaluated in experimental models in rats, based on the evaluation of behavioural parameters and skin lesions.[25] In the therapeutic experiment Wistar rats were housed in a heated environment ($25 \pm 3°C$) to induce itch, and treated with *Dolichos pruriens* (ascending potencies of 6CH, 9CH, 12CH and 30CH diluted 1:500 in the drinking water, each administered for 10 consecutive days). The positive control group received the vehicle (30% ethanol diluted 1:500 in water). The experiments were performed blind.

The results point to the existence of therapeutic effects, with inhibition of the itching, skin lesions and fur thinning produced by heat, more evident in later observations, with the 9, 12, and 30CH potencies ($p = 0.001$). No changes were observed in the behavioural parameters such as OF activity (grooming and rearing) and laterality of the itching. In the pathogenetic experiment, all animals were kept at a temperature of $20 \pm 3°C$ and treated for 30 consecutive days with *Dolichos pruriens* 6 or 30CH, or ethanol vehicle, or no treatment; no changes were observed in any of the parameters examined. The authors conclude that this experimental model demonstrates the therapeutic effect of *Dolichos pruriens*, but not its pathogenetic effects.

The effects of *Coffea cruda* 30CH and 200CH and caffeine on the sleep pattern of rats were widely investigated by the group of Ruiz-Vega *et al*., by recording the EEG from the parietal region.[24,36,37] Treatments were administered orally at the beginning of the sleeping period. In synthesis, the spectral power of Delta band (0.5–2.5 Hz) was significantly higher than baseline for Coffea 30CH and caffeine (15.5 mg/kg). Coffea 30CH and caffeine have similar effects on sleep pattern, enhancing delta power, *Coffea cruda* 200CH appeared to affect only the synchronization.

Besides being a inflammation mediator, histamine is a central neurotransmitter, it increases arousal *via* H1 receptors. Even the pure histamine is not used as a homeopathic medicine, several studies investigated the effects of high dilutions/dynamizations of this substance *in vitro* and *in vivo*. A study examining the effect of ultra-diluted histamine on arousal through changes in the sleep pattern of rats[38] is worth of mentioning in this context. The spectral density in delta (0.5–2.5 Hz) band, one of the three major spectral components of the sleep-electroencephalogram, was lower in rats receiving histamine 30CH (0.05 ml every 20 min during the first 2 h orally) than the control group. Significant differences between histamine 30CH and baseline during the first 2 h imply an immediate effect. Subsequently the same group tested different potencies and found highly non-linear patterns, with peaks of activity at 15, 21 and 30 centesimal shake/dilution steps.[39]

Antigen S-100B of nervous tissue, according to data from numerous studies, participates in the mechanisms of nervous system plasticity and memory. The influence of ultra-low doses (6CH) of antibodies to S-100B has been studied on behavioural models in rats: learning – inhibitory avoidance, choosing of bowls with sucrose, and cessation of feeding behaviour after an auditory signal.[26] For all three tasks, an improvement in the parameters of reproducing the learned skills was observed following oral administration of potentiated antibodies against S-100B antigen immediately after learning. In discussing the possible mechanisms of the effect of potentiated antibodies on memory, it should be borne in mind that the amount of S-100B protein in the brain increases during learning, and the anti-S-100 antibodies in normal doses disturb the process of memory consolidation. However, in ultra-low doses, antibodies to S-100B may have a diametrically opposite effect to what they have in normal doses, instead improving reproduction of the learned skills. More recently, the same research group has shown that antibodies against S100 protein, administered in high and ultra-high dilutions, possess neuroprotective and neurotrophic activity on neuroblastoma cells under conditions of oxygen and glucose deprivation.[27]

Experimental studies on mouse behavioural responses

Screening of six homeopathic remedies

In a preliminary trial,[30] we tested the effects of *Aconitum*, *Belladonna*, *Gelsemium*, *Nux vomica*, *Argentum nitricum* and *Tabacum*, all in the 5CH dilution/dynamization, on the main behavioural responses of mice. The choice of substances was suggested in part by the homeopathic Materia Medica, which cites a number of neurological and anxiety-related symptoms for those remedies, and in part by the pre-existing literature. Each remedy was tested on 12 animals of the CD1 strain, which is one of the most commonly used in behavioural studies. This initial screening yielded the following results for the four main behavioural parameters:

(a) The time spent in the open, illuminated (white), compartment in the LD test was increased by the benzodiazepine standard drug Diazepam (0.5–1 mg/ml). Of the six homeopathic remedies tested, only *Gelsemium*, *Aconitum* and, to a lesser extent, *Argentum nitricum* showed a stimulating (anxiolytic-like) effect.
(b) The number of transitions between the two compartments was increased by Diazepam and by *Gelsemium*, but not by the other remedies.
(c) In the OF test, *Gelsemium*, *Tabacum* and, to a lesser extent, *Argentum nitricum* appeared to have a stimulating effect on the amount of time spent in the centre of the arena, indicating an increased tendency to explore the environment and a decrease in thigmotaxis.
(d) An increase in the distance travelled in the centre was observed only with *Gelsemium*. Diazepam proved ineffective in this test, possibly due to a sedative effect on locomotion or for other reasons that remain to be elucidated (see also below).
(e) *Nux vomica* decreased the time spent in the centre and the distance travelled in the OF, suggesting a pathogenetic, anxiogenic effect.
(f) *Argentum nitricum* had contradictory effects, since it increased the time spent both in the LD white zone and in the centre of the OF, but this effect was not accompanied by an increase in the number of transitions and distance travelled, suggesting that its activity is not truly anxiolytic-like and possibly attributable to other factors, or to statistical variability.
(g) In general, we found there to be high inter-individual variability.

In summary, from this preliminary study it emerged only that *Gelsemium* had an effect on all the parameters considered. The high experimental variability – a factor well known to researchers in this field – prompted to concentrate on only one remedy, *Gelsemium*, leaving to future work the investigation of responses to other potentially interesting remedies (*Aconitum*, *Tabacum* as anxiolytics, *Nux vomica* for its possible pathogenetic effect). Another reason for undertaking a systematic experimental trial of *Gelsemium* was that this remedy is frequently used in humans for anxiety symptoms, and has already been investigated in studies by other authors which do not, however, always report consistent results.[13,14,16,18,23] In experimental research in homeopathy it is especially important to consolidate new and preliminary data – which can often be unexpected or even contrary to the beliefs of official pharmacology – through statistically solid evidence. We conducted two complete series of experiments testing various *Gelsemium* dilutions,

with small changes and adaptations of methodology: in the first series – the results of which have been recently published[31] and here are further detailed – the LD test was performed the day before the OF on animals (from Harlan Laboratories), which were housed 4 per cage; in the second series of studies (Magnani *et al*., manuscript in preparation, whose preliminary data are here reported) the LD test was performed the day after the OF test on animals (from Charles River Laboratories), which were housed 2 per cage.

Effects of *Gelsemium sempervirens*

A series of eight experiments was carried out on *Gelsemium* using CD1 mice, administering in all cases the 5CH dilution/dynamization as well as the control solution, and in some experiments also adding higher dilutions to the experimental setting. In this series of experiments, the animals were housed 4 per cage, the LD test was performed the 8th day and the OF test was performed the 9th day of drug administration. The results are summarised in Figure 3 (LD test) and Figure 4 (OF test).

Figure 3 shows the mouse behaviour data in the LD test, in terms of the two main parameters: percentage of time spent in the illuminated compartment and number of transitions between the two compartments. The mean time spent in the light compartment and the number of transitions were significantly increased by Diazepam, as expected. These two parameters were also slightly increased in *Gelsemium*-treated animals (three experiments with 5CH and one with 30CH), but due to the high inter-assay variability the overall differences between the *Gelsemium*-treated animals and controls were not statistically significant. In this series of experiments the global *F*-test of ANOVA for *Gelsemium*-treated groups was 0.909 (not significant) and 0.229 (not significant) for the mean time spent in the light compartment and the number of transitions respectively. No interaction between experiments and groups was found.

Figure 4 shows the results for the OF tests. In the *Gelsemium*-treated groups, the time spent in the central zone of the arena increased significantly in all but one experiment, while Diazepam was ineffective in this test. The distance travelled by mice in the central zone of the arena was also positively influenced by treatment with *Gelsemium* at all dilutions/dynamizations. In this series of experiments the global *F*-test of ANOVA for groups was 0.006 for both the mean time spent in the centre and the distance travelled in centre. No interaction between experiments and groups was found.

It is interesting that the efficacy seems to be higher with the highest potencies of the remedy, and is highly significant both with 7CH and with 30CH. Nevertheless, it should be underlined that all potencies proved significantly active, and that the differences between the groups treated with the homeopathic remedy did not prove statistically significant in a post-hoc analysis. During the OF test the total distance travelled by the mice in the entire arena was also analysed. No significant effect was found in drug-treated *vs*. control animals, indicating that the observed differences in time spent and distance travelled in the central zone were not due to changes in the general, unspecific, locomotor activity of the mice (data not shown). The reference benzodiazepine drug was inactive in the OF test, indicating that, at least in our experimental conditions, OF and LD explore different emotional responses, with different sensitivity to drugs and neurological mechanisms.

In a second series of experiments we used animals from a different producer (Charles River Laboratories) and slightly changed the experimental protocol, housing the mice 2 per cage and performing the OF test the 8th day and the LD test the 9th day of drug administration. Six independent experiments using 8 animals per group were performed, assessing increasing potencies of Gelsemium *sempervirens*. In these experiments a significant anxiolytic-like effect of *Gelsemium* (5CH, 9CH and 30 CH, not 7CH) was observed also in the LD test. The global *F*-test of ANOVA for groups was highly significant: $p = 0.001$ and $p = 0.002$ for the mean time spent in the light compartment and the number of transitions respectively. No interaction between experiments and groups was found. Post-hoc analysis indicated a significant effect with *Gelsemium* s. 5CH ($p < 0.05$), 30CH ($p < 0.05$) and particularly 9CH ($p < 0.001$) potencies (Figure 5). It is conceivable that factors linked to methodology like animal breeding, housing condition, or re-testing the second day induced a higher state of anxiety and this could have made the animals more sensitive to the effects of the homeopathic medicine in the LD test. The positive results with *Gelsemium* 9CH were the most reproducible and were comparable or even higher than those of the conventional drug of reference Buspirone, which in this series was administered daily in a parallel group. Figure 5 also shows that the 9CH exhibited a much higher inter-assay repeatability than the 7CH since the latter potency was active in some experiments (e.g., 4th and 5th) and not in others.

In summary, *Gelsemium* exhibited a highly statistically significant effect in two main and validated behavioural test in mice; however, these effects appeared as highly dependent on the experimental conditions and did not concern all the tested potencies. Anxious behaviour of mice is caused by various factors that operate on the emotional level: the fact of being tested individually (the animals are used to living in groups), of being placed into a new and unfamiliar space, and agoraphobia (the arena is very large compared to the cages in which the animals are customarily housed). Therefore, what is measured is the remedy's effect both in augmenting the tendency and interest to explore, and in diminishing the anxiety provoked by the test itself (which tends to inhibit exploratory behaviour).

Discussion

Anxiety is a characteristically human experience, and is in a certain sense inescapable for human beings endowed with free will and conscious of their precarious nature. Anxiety within certain limits is inevitable, innocuous and even useful, since it makes it possible to confront exogenous stressors with enhanced vigilance. Yet, if it occurs to an excessive degree, or inappropriately to the context, it can become

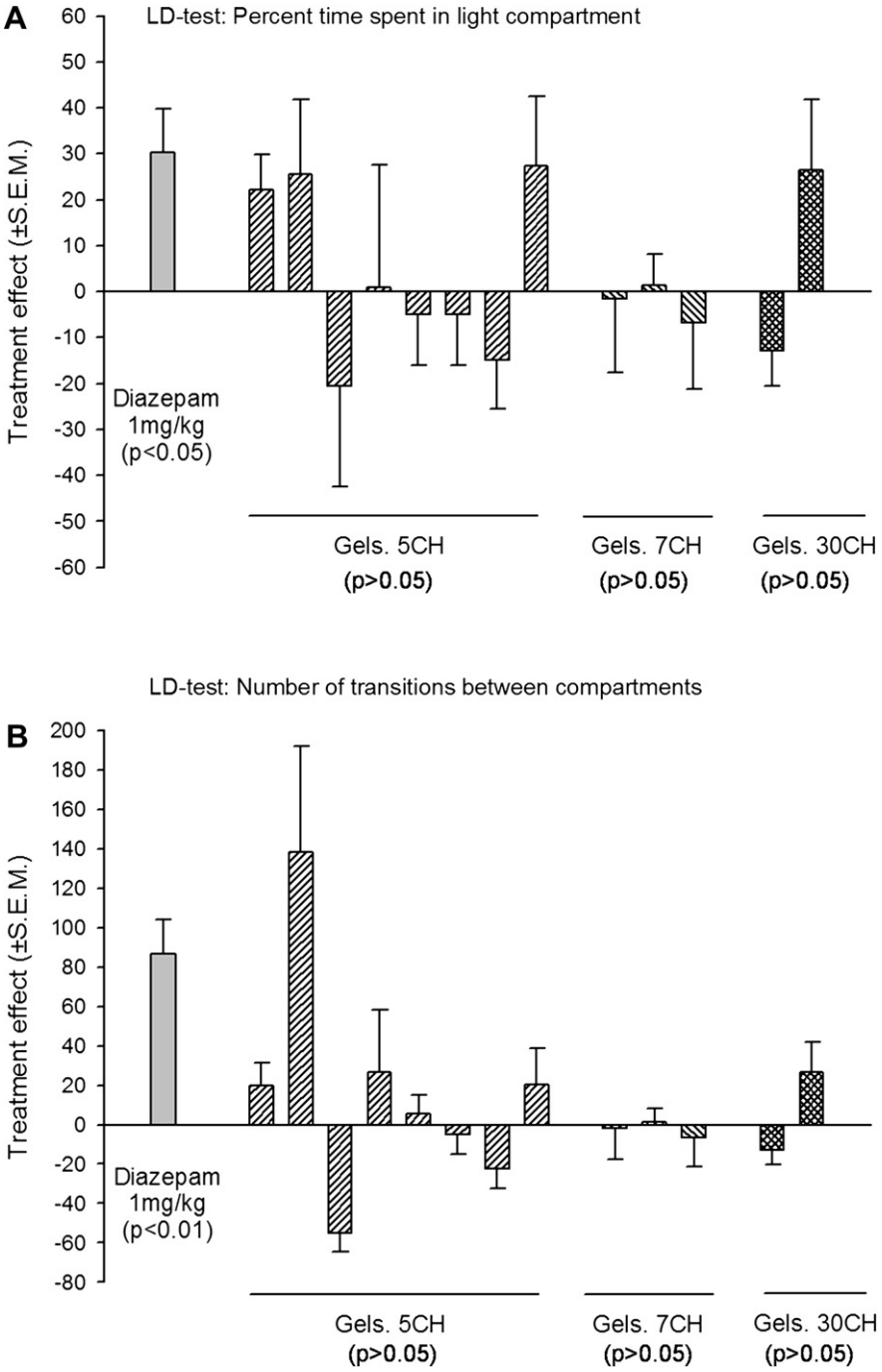

Figure 3 Effects of *Gelsemium* 5CH, 7CH and 30CH on the two main behavioural parameters in the LD test. Data from 5 experiments with Diazepam ($N = 69$ mice) are pooled and mean values ± S.E.M. from each experiment are reported of 8 experiments with Gels 5CH (total $N = 117$), 3 experiments with Gels 7CH ($N = 47$) and 2 experiments with Gels 30CH ($N = 31$). In each experiment the values for each treated mouse are calculated as the % effect as compared with the mean values of control mice of the same experiment. *p* Values reported in figure are post-hoc analysis of ANOVA comparing Diazepam or *Gelsemium* potencies with control group as described in Methods.

a source of suffering, dangerous enough to even compromise the ability to cope with day-to-day life. It provokes various types of disturbances in both the psychic (insecurity, indecision, fear to the point of panic attacks, sleep disorders) and physical (palpitations, muscle tension, nausea, vomiting) spheres. Often, the physical disturbances themselves become new sources of anxiety for the patient. There are various forms and various classifications of anxiety, including anxious states, phobic disturbances, post-traumatic stress disorders, and atypical disturbances. Decades after their introduction into therapeutic use, benzodiazepines continue to be the most commonly prescribed anxiolytic drugs, because of their effectiveness in controlling anxiety symptoms, panic syndrome and sleep disturbances. However they pose the dangerous problem of dependency and of adverse or side effects (daytime drowsiness, dulling of cognition). Such problems have not been entirely resolved through the recent introduction of other classes of anxiolytic drugs, such as HT receptor agonists, which are better suited to chronic conditions but not always effective.

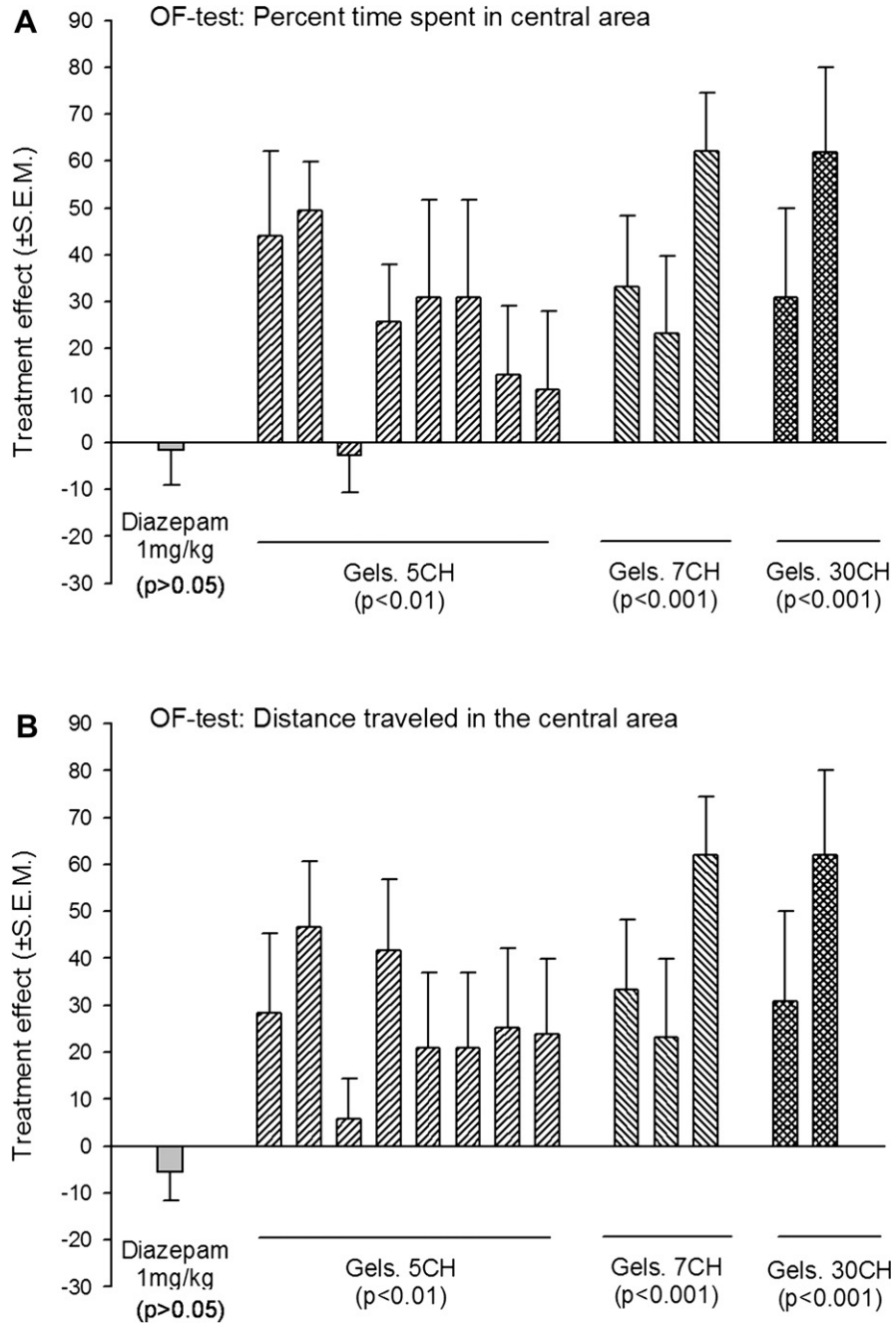

Figure 4 Effects of *Gelsemium* 5CH, 7CH and 30CH on the two main behavioural parameters in the OF. Data from 5 experiments with Diazepam ($N = 69$ mice) are pooled and mean values ± S.E.M. from each experiment are reported of 8 experiments with Gels 5CH (total $N = 118$), 3 experiments with Gels 7CH ($N = 47$) and 2 experiments with Gels 30CH ($N = 32$). In each experiment the values for each treated mouse are calculated as the % effect as compared with the mean values of control mice of the same experiment. *p* Values are post-hoc analysis of ANOVA comparing Diazepam or *Gelsemium* potencies with control group as described in Methods.

Consequently, many patients turn to complementary therapies, and to homeopathy in particular. This treatment alternative merits careful consideration, but to date there are obstacles to the general acceptance of homeopathy – as also of other fields of medicine – due to the scarcity of scientific studies elucidating its indications, limitations and mechanisms of action.

Effects in animal models

Of the many available methods for studying human pathologies and drug effects, animal models continue to be one of the most commonly used. In the field of psychopathology, animal models have become an invaluable tool for analysing the mechanisms of various disorders, and have aided in developing and predicting therapeutic responses to pharmacological agents such as benzodiazepines.

As emerges from this review, in homeopathy relatively few experimental studies have been carried out on animal models in the field of anxiety and psychopathological disorders. The scarcity of basic research in homeopathy, and the frequent presence of methodological flaws, is a problem that also affects other lines of research.[40–43] Though there

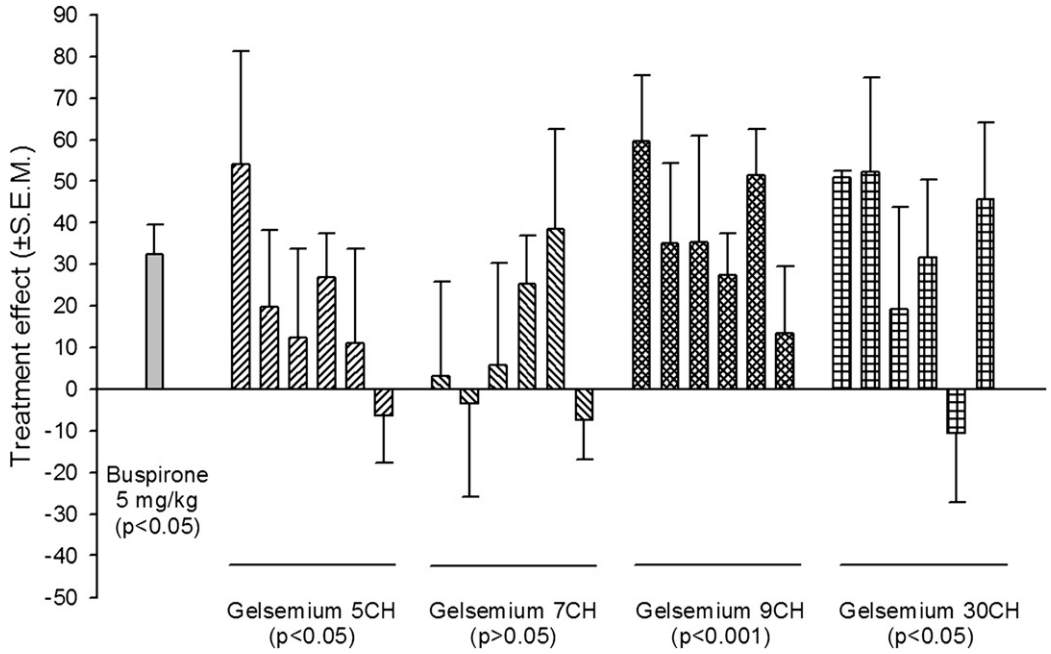

Figure 5 Effects of *Gelsemium* 5CH, 7CH, 9CH and 30CH on the percent time spent in the illuminated area in the LD test of the second series of experiments (2 mice per cage, LD performed in the 9th day). Data from 5 experiments with Buspirone ($N = 40$ mice) are pooled and mean values ± S.E.M. from 6 experiments ($N = 8$ mice per group per experiment) are reported with *Gelsemium* potencies. In each experiment the values for each treated mouse are calculated as the % effect as compared with the mean values of control mice of the same experiment. *p* Values are post-hoc analysis of ANOVA comparing Buspirone or *Gelsemium* potencies with control group as described in Methods.

is doubtless some interesting evidence for the efficacy of homeopathic remedies in murine model of behaviour and experimental intoxication, the results reported in the reviewed contributions are not sufficiently consistent to provide a solid base for understanding the phenomenon and its mechanisms of action. Only a few results – for *Ignatia*, *Gelsemium*, *Chamomilla* – have been confirmed by separate laboratories, but there is also interesting evidence for the possible efficacy of other remedies such as *Cicuta virosa*, *Cannabis*, *Coca*, *Argentum nitricum*, *Nux vomica*, *Belladonna*, and confirmation of a statistically proven effect of high dilutions of antibodies against neuronal antigens. Some authors report indirect evidence for interference of the homeopathic remedy (*Ignatia* and *Gelsemium*) with receptors for glycine[18] or neurosteroid enzymes.[44,45]

The case of *Gelsemium sempervirens*

In traditional medicine, and in particular in homeopathy, *Gelsemium* has always been attributed anxiolytic and analgesic properties, but scientific studies supporting these assertions are rare. We can cite some preliminary observations in the homeopathic literature, but these are not entirely clear nor methodologically reliable (see above). There are two studies which show that *Gelsemium* can have a preventive action against experimental stress (electric shock) in mice[23] or against convulsions provoked by lithium and pilocarpine in rats.[46] It should be noted, that the latter protective effect against convulsions was obtained using a pure extract (24 drops/2 l of drinking water), rather than a homeopathic dilution, mixed with Scutellaria lateriflora (Skullcap) and Datura stramonium (Jimson Weed).

Behavioural effects on mice: Our experiments confirm and consolidate the evidence that *Gelsemium*, in homeopathic dilutions, has positive effects on mouse behaviour, when tested in the OF, reducing the symptom of ''thigmotaxis''. In this test, the conventional anxiolytic drug Diazepam did not have any effect, indicating that this behavioural effect of *Gelsemium* is based on a different mechanism and has a broader spectrum of target function. Thigmotaxis is a characteristic behaviour pattern of mice, consisting of a tendency to stay or move close to walls or vertical surfaces in an OF.[47,48] It is generally considered a sign of emotions correlated with anxiety and fear.[9,49,50] When an animal initially explores an enclosed space, it tends to stay in close contact with the perimeter of the space, and this tendency can be quantified by measuring either the amount of time spent in the central/peripheral area, or the distance travelled (path length). This behaviour is also termed 'wall-following' or 'centrophobic'.[51] Thigmotaxis is particularly apparent upon the first exposure to a novel space, and helps the animal to define the boundaries of an unfamiliar environment. However, if relied upon continually, it then prevents the animal from pursuing other spatial strategies for mapping the environment.

Thigmotaxis is a very conspicuous behaviour in anxious animals, and plays a role in the formation of avoidance behaviour and cognition. It is therefore a primordial behaviour, with a genetic basis, that is ecologically important and used by both animals and humans for exploring the environment. In unconditioned behaviour tests in which an animal is placed for the first time in an arena, it also reflects novelty-induced anxiety, general activity, exploratory behaviour and decision making.[3]

As we have shown, *Gelsemium* possesses an anxiolytic-like activity and augments the exploratory tendency in novel environments, without any effects on the motor coordination or sedation of the animals. *Gelsemium* has anti-anxiety effects without any neurotoxic or sedative side effects. Our data of the first series of experiments, showing a greater effect in the OF (where Diazepam is not effective) suggest that the behavioural effect of this remedy is probably mediated by mechanisms that differ, at least in part, from the classical modulation of BZDps on the GABA receptor. These data are in agreement with other reports[2,9,52,53] showing that OF behaviours of mice were reduced by Diazepam at high doses, which may be within the sedative-hypnotic range.[9,52] It is very intriguing that this test was sensitive to the homeopathic medicine only, since this would suggest that the latter has a broader action on emotional symptoms than conventional drugs. Differences between the type and severity of external stressors or in the experimental setup might account for the high variability of results reported in different experimental conditions and by different laboratories. It has been noted that the extent to which an anxiolytic compound can facilitate exploratory activity depends on its baseline level in the control group.[6]

Anxiety is not a unitary symptom and consists of a series of different syndromes, ranging from simple disturbances of normal behaviour to fully fledged pathologies such as unmotivated panic attacks. Different methods may explore different aspects of the anxiety syndromes, such as 'state' and 'trait' anxiety.[4] Belzung and Griebel suggest that the LD test and the elevated plus-maze are the most appropriate devices for assessing 'state anxiety,' whereas the free-exploratory paradigm can be used for 'trait anxiety'.[3,5] However, the OF test is also used as a model of state anxiety,[2] and few true trait anxiety animal models are used, generally involving genetic paradigms or chronic exposure to fear-provoking stimuli.

In the second series of our studies we have found that *Gelsemium* acts more markedly and significantly as anxiolytic in the LD test when it is carried out on the day after the OF test, rather than on the preceding day as was done in the first series of experiments reported here. If confirmed, this result would indicate that the mere fact of being re-subjected to a behavioural tests augments the degree of baseline anxiety, and thus facilitates exertion of the tranquilising effect of the homeopathic remedy compared to the control solution.

Action mechanism: The mechanisms of action of *Gelsemium* at the cellular level could involve the limbic system and the spinal marrow, at the level of the centres which control pain and anxiety. In fact it has been observed that gelsemine, the main component of *Gelsemium*, stimulates biosynthesis of the neurosteroid 3α,5α-tetrahydroprogesterone (3α,5α-THP), also named allopregnanolone in the central nervous system of rats, through activation of receptors for glycine (Gly-R).[45] The 3α,5α-THP is produced by an enzymatic cascade which includes 5alpha-reductase and 3alpha-hydroxysteroid oxidoreductase. 3α,5α-THP acts on allosteric sites situated on the γ-amino butyric acid type A (GABAA) receptor or on the strychnine-sensitive glycine receptor (Gly-R).[54,55] Gabaergic neurosteroids mediate the anxiolytic effects of various drugs[56,57] and of ethanol.[58]

In a preliminary communication, the authors showed that the effect of the entire extract of *Gelsemium* plant is superior to that of the purified gelsemine.[44] More recently, the same group has tested the effects of *Gelsemium* and gelsemine in a range of homeopathic dilutions (5, 9, 15CH) and shown that the 5CH and 9CH dilutions stimulate synthesis of 3α,5α-THP both in the hippocampus and in the spinal marrow *in vitro*.[59] A particularly interesting fact is that the effects of the homeopathic remedy *in vitro* were blocked by strychnine, a well-known selective inhibitor of glycinergic receptors. Based on these considerations and our findings, a hypothetical diagram showing the possible action mechanism (s) of *Gelsemium* is reported in Figure 6.

Because 3α,5α-THP endogenously synthesized in the central nervous system significantly modulates anxiety or nociceptive mechanisms through paracrine and autocrine modes,[60] substances which are capable of stimulating 3α,5α-THP formation in neural networks appear as potentially interesting for the development of effective anxiolytic or analgesic therapies. Glycine is an amino acid that functions as a neurotransmitter with inhibitory effect and stimulates biosynthesis of 3α,5α-THP starting from progesterone (an effect known to be blocked by strychnine by antagonism at the receptor level). The stimulating effects of glycine and gelsemine suggest possible uses of glycinergic agents and gelsemine for stimulating these metabolic pathways involving neurosteroids, in disorders of the nervous system where they are reduced, and which are accompanied by symptoms of anxiety, social phobia, and potentially also psychiatric disorders[61,62] and encephalopathy.[63–66]

Gelsemine was present in the mother tincture of the potencies used in our investigations at a concentration of 6.5×10^{-4} mol/l. Since each dilution step involves a 100 times decrease in concentration, the theoretical concentration of gelsemine in the 5CH, 7CH, 9CH and 30CH solutions was 6.5×10^{-14}, 6.5×10^{-18}, 6.5×10^{-22} and 6.5×10^{-64} mol/l respectively. Since 1 mol/l of any substance contains 6.022×10^{23} molecules at standard conditions (Avogadro constant), our 7CH working solution contained approximately 10^5 molecules/l (10^2 molecules/ml) and an injection of 0.3 ml contained approximately 33 molecules. Of course, the 9CH and 30CH potencies in our conditions were well beyond the Avogadro limit. Thus, our results, obtained in fully randomized, controlled and blind conditions, confirm and reinforce the observations – emerging from several research fields – that biologically active compounds may indeed have high-dilution effects which mimics those of lower dilutions (higher doses): in homeopathy there does not exist linearity or proportionality between molecular concentration of active principles and biological effect.[39,41,67–71] So far there is no uniting theoretical explanation for these observations, but recent hypotheses seem to point to organisation of the solvent water on a mesoscopic scale: the nano-heterogenous structure of water can be determined by interactive phenomena such as coherence, epitaxy, temperature–pressure processes during strong agitation, and formation of colloidal nanobubbles containing gaseous inclusions of oxygen, nitrogen, carbon

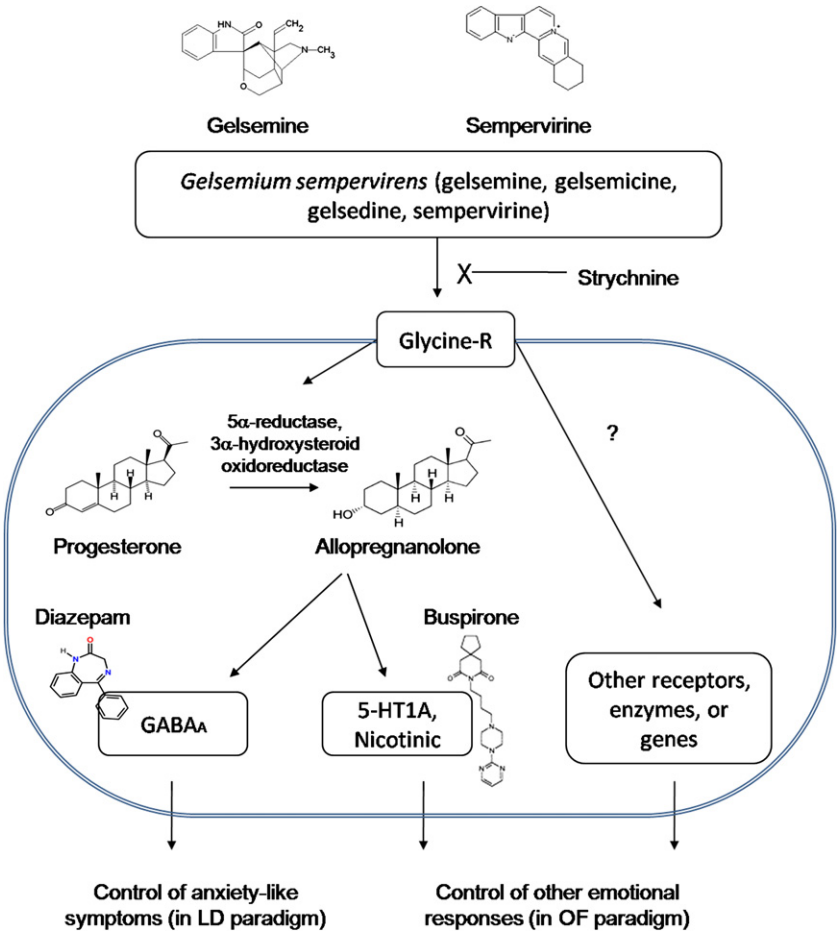

Figure 6 Hypothetical mechanism of action of *Gelsemium*. The homeopathic medicine has been found[59] to act on strychnine-sensitive glycine receptors inducing Cl^- influx and increasing the synthesis of the neurosteroid allopregnanolone, which in turn may act both on the GABA receptor – at a binding site different from benzodiazepines – and on other receptors including the serotonine (5-HT1A) and nicotinic-acetylcholine receptor. Due to the multicomponent nature of *Gelsemium* and to our finding of effects also on the OF paradigm, differently from the conventional anxiolytic drugs, the existence of other neurosteroid-independent mechanisms may be hypothesized.

dioxide, silica, and possibly the remedy source material.[72–79] We have also previously suggested a analogy between this type of non-linearity and a hypothetical fractal-type organisation of water nanoheterogeneities,[80–82] due to the iterative process of dilution/succussion.

Meanwhile, extrapolating these data to the human use of homeopathic medicines, it is quite interesting that qualitatively similar effects are obtained using different potencies, suggesting that the choice of a 'right' potency could not be so determinant for the final outcome. However, or results (see Figure 5) also show that, even under strictly controlled experimental conditions some potencies may be more active than others (e.g., the 9CH exhibited a much higher inter-assay repeatability than the 7CH) and the same potency (e.g., 7CH) may be active in some experiments (e.g., 4th and 5th in Figure 5) and not in others. The magnitude of the effects found in our studies approaches that of conventional anxiolytics (in the models where these latter drugs work), suggesting that the statistically significant effects of *Gelsemium* in mice could also prove to be clinically relevant in humans. These unusual properties of high dilutions, which merit further investigation, are potentially relevant not just to homeopathic pharmaceutical practice, but also to basic research into cell sensitivity to regulation.

Conclusions and prospects

In this work, we have reported the evidence for the effectiveness of some homeopathic remedies in controlling anxiety symptoms in animals. Even though anxiety in experimental animals in a particular setting obviously differs from anxiety in humans, it remains true that some behaviour patterns (fear, flight, neophobia, thigmotaxis, etc.) are ancestral and have a common neurobiological basis in humans and animals. In point of fact, these models have been used from the outset in preclinical studies of all anxiolytic drugs, and proven themselves to be valid and predictive. Especially when using multiple tests simultaneously, in situations that do not involve conditioning with excessive and artificial stress, it is possible to outline a profile of activities that reflect the emotional state of the animal and can be objectively evaluated.

Because we are discussing homeopathy, it is necessary to specify an important methodological point. The fact that *Gelsemium* (or some other remedy such as *Ignatia*

and the other mentioned in this review) has a demonstrable action on some of the neurobiological mechanisms that control anxiety does not mean it is indicated in all the behavioural disorders associated with these types of symptoms. This caveat is grounded in the same experimental findings which show the variability of animal responses depending on the tests employed and other details which distinguish different experimental approaches.

The fact that *Gelsemium* is more active in the OF test under certain conditions, but more active in the LD test under others (possibly dependent on the way the animals are raised, or housed, or their previous experiences with tests that might create habituation or altered expectations) indicates that the effect is highly sensitive to even slight conditioning effects. If these considerations are extrapolated to man, we must conclude that *Gelsemium* – even in very high dilutions – can work, but the 'sensitive type' must be selected on the basis of a very thorough experience and careful discrimination of the different symptoms and the individual's history.

Classical homeopathy, as well as most other alternative approaches, emphasises a holistic view of disease in which individual judgement and treatment are important. This implies that a homeopathic remedy must be chosen by taking into consideration the organism as a whole, including personality and behaviour, and not merely the symptoms in the affected organ system. As a consequence, a reductionist experimental approach can be valid for the scientific advancements that it allows, but cannot furnish a 'proof' or a 'disproof' of homeopathy itself. Therefore, care should be always taken when interpreting the results of animal studies with respect to possible mechanisms of action of homeopathy.

From the above review, it emerges in particular that *Gelsemium* is the remedy that has been most studied in experimental models. Its action, at the level of the living animal and of key neurobiological systems in the control of anxiety, has been proven by multiple studies. Solid statistical evidence demonstrates that the effects of the different potency levels investigated (5CH, 7CH, 30CH) do not follow a classical dose-response relationship regarding their mere dilution level, confirming a phenomenon frequently observed with high homeopathic dilutions in other laboratory models.[66–70] *Gelsemium*'s mechanism of action at the central level is also starting to be elucidated. It is therefore one of the first remedies, already empirically used in human patients with symptoms correlated to anxiety, to have been proven effective in experimental animals. It is to be hoped that a growing number of researchers will carry out equally careful and systematic experimental work on many other remedies, to progressively expand the body of knowledge on their effects and mechanisms of action.

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