EFFECTS OF HOMEOPATHIC DRUGS ON THE ANXIETY-LIKE BEHAVIOUR IN MICE

Magnani P.[1] Conforti A.[2] and Bellavite P.[1]*

[1]Department of Morphological Biomedical Sciences

[2]Department of Medicine and Public Health

University of Verona (I)

*Corresponding Author: paolo.bellavite@univr.it

Abstract

Several models of anxiety-like behaviours have been developed and described in mice, essentially in order to test different anxiolytic drugs, but little is known about the effects of homeopathic medicines. Anxiety-like responses may include physiological parameters as well as behavioural changes (inhibition of ongoing behaviours, scanning, avoidance of light or of the source of danger, time spent in resting state, slow or fast movement, etc.). In our laboratory only "ethological" models are utilized, using exposure of subjects to novel environments (open field test), to unfamiliar aversive places (elevated plus-maze), or to the light/dark choice. We do not expose the animals to pain or other forms of physical stress. Aconitum, Nux vomica, Gelsemium sempervirens, Belladonna, Argentum nitricum, Tabacum, all at 5CH potency in hydroalcoholic (0.3%) solution administerd by i.p. injection, were screened for their potential effects on animal behaviour. Then, the anxiolytic-like effects of Gelsemium sempervirens 5CH were tested in various experimental settings and different mice strains (CD1, C57BL/6J, BALB/c). Control mice were treated with Ethylicum 5CH (vehicle) or with Diazepam (0.5 to 1 mg/kg) in the same hydroalcoholic (0.3%) solution used to dilute the homeopathic medicines. The test was performed with coded drugs/controls in double blind (operations and calculations). We often noted high scattering of data due to inter-individual variability, to change of illumination schedule and of strain. We have observed statistically significant effects of Gelsemium sempervirens in several "symptoms" of anxiety-like behaviour such as the time spent in the illuminated compartment in light/dark test, the decrease of resting time, the distance travelled in the open arms of the plus-maze or in the centre of the open field. The extent of responses to this medicine were in a number of cases comparable to the extent of the responses to Diazepam. Paradoxical "anxiogenic-like" (proving?) effects of Gelsemium were observed in an experiment where also Diazepam inverted its conventional action. A few significant effects of Argentum Nitricum 5CH were also observed and replications studies are now in progress.

Introduction

Among the multiple possibilities to study human pathologies and drug effects, animal models remain one of the most used pathways. Traditional difficulties in accepting these models stem from the argument that there is no concluding evidence that what occurs in animals is equivalent to what occurs in humans. On the other hand, most conventional drugs and, recently, several homeopathic medicines have been tested in animal models, whose main advantages of animal models is multiple testing under controlled conditions and easier access to the study of action mechanism of drugs. In the field of psychopathology, animal models have become an invaluable tool in the analysis of the mechanisms of various disorders and have aided in developing and predicting therapeutic responses to pharmacological agents such as benzodiazepines.

Animal models of anxiety can be grouped into two main subclasses [1]: the first involves the animals conditioned responses to stressful and often painful events (e.g. exposure to electric shock, forceful containment in small space), the second includes the study of unconditioned responses using ethologically-based paradigms and involves the spontaneous reactions to non-painful stimuli (e.g. exposure to a novel highly illuminated test chamber or unfamiliar open field). We decided to utilize ethological models both for ethical reasons and because our aim is to approximate the natural conditions under which the behaviour is influenced by emotional states of fear, curiosity and anxiety, thus allowing for a comprehensive "behavioural profiling". This approach is noteworthy for homeopathic research, where individual and "strange" symptoms may be as significant as classic responses to pain or inflammatory tissue changes. Ethological models, however, present individual differences and variable behavioural baseline levels [1] and this requires strong care for variable parameters linked to environment, handling and testing.

Classification of anxiety disorders is based on symptom clusters and therapeutic response. [2] Anxiety is not a unitary disease but a complex phenomenon that probably involves different neurochemical systems with varied aetiological origins and may be divided in various forms including "state" anxiety (excess anxiety that a subject experiences at a particular moment in presence of an stimulus) and "trait" anxiety (does not vary from moment to moment). It has been suggested that light-dark test and elevated plus-maze device are the most appropriate for assessing state anxiety, while the free-exploratory paradigm can be used for "trait anxiety" [3, 4]. However, also open-field test (see below) is used as a model of state anxiety [1] and few true trait anxiety animal models are used, they rather concern genetic models or chronic exposure to fear-provoking stimuli.

Here we report our preliminary studies investigating the effects of homeopathic medicines on a battery of different tests, measuring mice behaviours in three genetically distinct inbred strains. In this series of experiments we tested Aconitum, Belladonna, Gelsemium, Nux vomica, Argentum nitricum and Tabacum. The choice was obviously suggested by homeopathic Material Medica, reporting a number of neurological and anxiety-related symptoms for those medicines, and in part by pre-existing literature. There were early reports in homeopathic journals showing that in mice, tested using the hole-board test, Gelsemium 3CH and at a lesser extent Gelsemium 5CH reduced the number of exploration attempts, suggesting anxiolytic-like activity because the effect was in the same direction of the effect, which was much more marked, induced by chlordiazepoxide (Librium)[5]. Guillemain et al. [6] showed that Gelsemium 5CH, Sempervirine nitrate 5CH (one of the active principles of Gelsemium) and Argentum nitricum 9CH contrasted the effects of the anxiogenic compound RO 15-3505 (inverse agonist of benzodiazepines) in the labyrinth (plus-maze) test. The same authors reported that RO 15-3505 decreased the affinity of the benzodiazepine receptors in mouse cortex and that this effect was contrasted and reversed by Sempervirine 5CH. This suggests a possible action of Gelsemium on the benzodiazepine receptors and GABAergic inhibitory transmission. More recently, Bousta et al. reported that in some but not all the experimental conditions Belladonna, Gelsemium and Poumon histamine (5CH, 9CH, 15CH)

reduced the stress-induced behavioural alterations of mice in staircase test and light-dark test [7]. However, those results were obtained as reversal of the effects of severe stress (conditioned paradigm) and the findings were highly variable according to the potency used and test performed. As an example, Gelsemium caused a significant increase in step climbed in staircase test in stressed mice and a decrease in normal, unstressed mice. In light-dark box test, Gelsemium caused a decrease of the time spent in the lit compartment, compared to stressed-saline group, simulating an anxiogenic-like effect in this model. Therefore, further studies exploring the neurotropic and behavioural effects of Gelsemium, especially in non-conditioned paradigm, are necessary. That Gelsemium may have neurotropic effects is reinforced by recent data from Mensah laboratory [8] showing that in the rat brain homogenate extremely low doses of this compound (10^{-10} M) and of its active principle gelsemine enhance the production of the neurosteroid allopregnanolone (5a,3a-tetrahydroprogesterone), a highly active stimulator of GABAa receptors and of inhibitory signalling in the central nervous system.

Clearly, anxiety-related symptoms are variable and complex, so that not a single test may reflect the rich pattern of modifications that are present in human pathology. In this first screening study we tried to use the maximum number of test and of parameters compatible with the technical constraints. Our hypothesis was that by testing different homeopathic medicines we could obtain various symptoms different in extent and in quality. Moreover, in accord with the homeopathic rules, we are well aware that possible changes observed in these experimental settings may be interpreted either as “therapeutic” (anxiolytic-like effects) and as “pathogenetic” (anxiogenic-like effects). This may be the case in experimentation like this, where apparently healthy animals are used and their behaviour is observed without pre-conditioning stress procedures. We used different mice strains that have been described as characterized by intermediate (CD-1), high (BALB/c), and low (C57BL/6J) anxiety profiles [9], with the aim to detect different responses to homeopathic medicines which are expected to act differently according to the basic health status of the treated organism [10].Therefore, our experimentation is of exploratory nature and its main goal is to screen several compounds and, once found some active compound, finding the most suitable conditions for assessing its activity on multiple parameters and in different experimental conditions. This is the reason why we changed the animal strain, the housing (e.g. number of animal per cage), the illumination schedule of the room, in the attempt to improve the sensitivity and possibly to find out the best approach to reduce the inter-individual variation coefficients.

Models

We choose the most used and validated models in mice for the study of state anxiety, namely Light-dark exploration test, Open field, and Plus(+) maze. All these models permit to collect several behavioural parameters which have been widely utilized for screening anxiety-modulating drugs anxiety [1]. All the animals were tested individually in 4 separate devices, so that a complete set of up to 64 mice could be tested during a 2-4 hours experimental session. The experiments were done in blind conditions, until completing the calculation phase of the experimental results. A video-tracking camera and software (“Smart” VTS system from PanLab, Barcelona, E) was used to records the sessions and automatically track the position, speed and other indicated parameters of animals in the arenas.

Light-dark exploration test (LD)

The light/dark (LD) exploration test (Figure 1) is based on the innate aversion of rodents to brightly illuminated areas and on their spontaneous exploratory behaviour in response to mild stressors, i.e. novel environment and light. The device permits mice to freely explore two interconnected

departments that vary in size an colour: a white open square (30cm x 30 cm) and a black covered compartment (30 cm x 15 cm). Both departments have 25-cm high walls. A small opening (4x4 cm) allows mice to freely move from the illuminated to the dark chamber and vice versa. The open field is brightly illuminated with 200 lx and the mice are left to explore the space for a 5-min period of testing. Control mice placed into the large, bright, section will rapidly move into the dark chamber and an anxiolytic effect will appear as increase of the time spent in light area[11]. Additional indices of anxiolytic activity have been also proposed such as the distance travelled, the speed, the resting time, the latency time for the first passage, number of transition from one area to the other, an so on. Here the results of the most consistent parameters in our experimental conditions are reported.

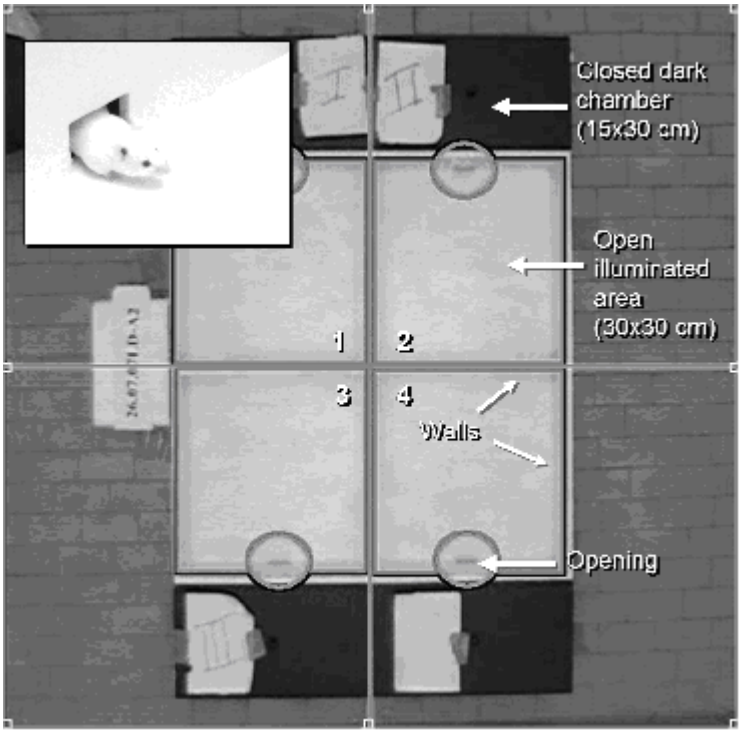

Figure 1. The Light-dark test for simultaneous assessment of behaviour of 4 mice.

Open field (OF)

This test (Figure 2) consists on placing an animal in a unknown environment made by a black-painted wood 50x50 cm platform with 25 cm high surrounding walls, as to observe a number of behaviour patterns, including the tendency to stay on the centre instead of periphery of the field (the latter tendency is called thigmotaxis and often interpreted as anxious behaviour), the number of entrances into a central area, the number of freezing (immobilization) episodes, the instantaneous speed and the parallelism index (tendency to run straight-line instead of wandering or changing direction). White light (100 lx) was present in the room. Placing the subject into the OF arena started the 10-min test. Using the video-tracking system, the arena was virtually divided in two parts, with a square central area having a surface corresponding to 25% of the total surface. Anxiety-like behaviour in the OF is triggered by two factors: individual testing (being the mouse a social animal) and agoraphobia (since the usual cage is much smaller). The main criticism which

one can formulate against this model relates to the implication of anxiety in the results obtained, since it has been observed that anxiolytic treatments do not by themselves increase exploration in the central area but they decrease the stress-induced inhibition of exploration behaviour [1].

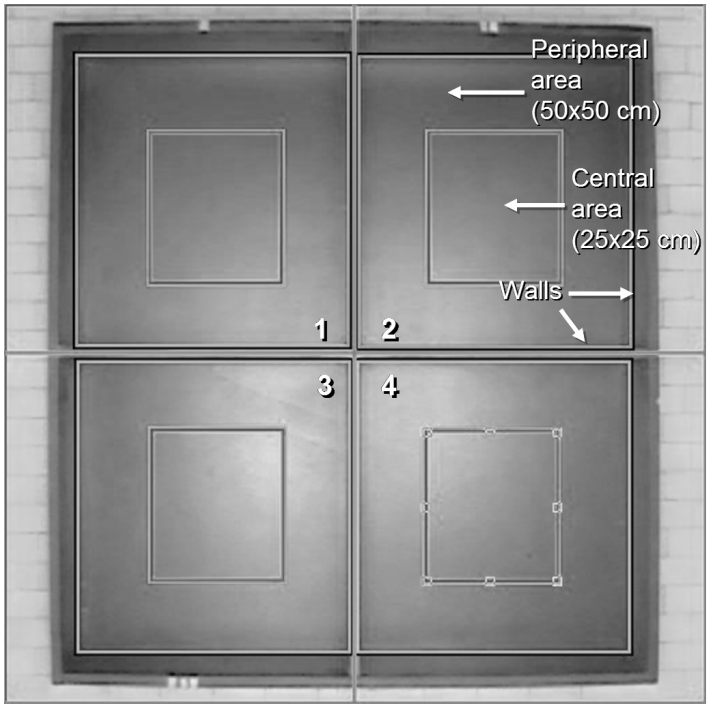

Figure 2. The Open Field test for simultaneous testing of 4 separate mice

Elevated plus-maze (EPM)

The elevated plus maze (EPM) is a widely used behavioural assay (Figure 3). The EPM is in the form of a "plus", made of four 25 cm x 5 cm arms, connected by a 5 cm x 5 cm cross area. Two open arms are facing opposite to each other and the other two orthogonal arms, of the same dimensions, are enclosed by 25 cm x 25 cm vertical walls. Open arms have a 2 mm border to help mice grasping without falling when turning on the open path. The maze is raised 50 cm off the ground so that the open arms combine elements of unfamiliarity, openness and elevation. The system is based on the innate aversion of rodents for open spaces and uses the conflict between exploration and aversion to elevated open spaces. Mice generally taken from their home cages will show a pattern of behaviour characterized by open-arm avoidance with a consistent preference for the closed arms. Anxiolytic-like effects are expected to encourage mice to adventure onto the arms of the maze and make more visits to the open arms, while the inverse tendency is potentiated by anxiogenic agents. Behaviour profiles in the EPM include elements of neophobia, exploration and approach/avoidance conflict; thus the apparatus is referred as an unconditioned spontaneous behavioural conflict model. Briefly, the mice are placed in the closed arms, facing the cross area, and duration/entries in each arm are recorded by the video-tracking system. The main measure of anxiety is the time spent in the open arms (both as absolute values and as percentage of total). Other parameters are the distance travelled and the number of open arm entries as compared with the number of closed arm entries.

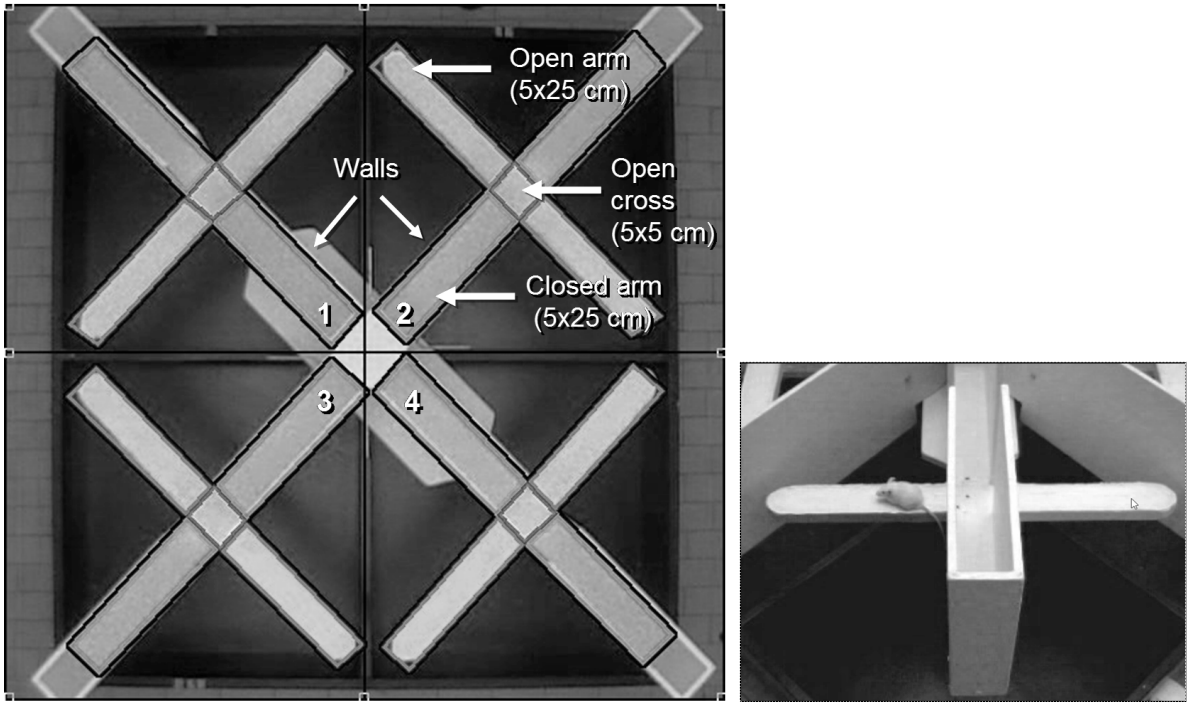

Figure 3. Elevated Plus-Maze test for 4 mice

Homeopathic medicines

The medicines were produced for this experimentation and provided free by Boiron Laboratoires, Lyon (F). All the tested medicines, including Control (placebo) ethylic alcohol were provided at the 4CH potency in 30% Ethanol/distilled water, in 50-ml brown glass ampoules. They were stored at room temperature in a metal cupboard. The first day of each series of treatments, the indicated medicines were diluted 1:100 in sterile, apyrogenic distilled water and strongly succussed with 20 strokes by hand. This step was done by diluting/dynamizing 0.4 ml aliquots of the indicated medicine into a final volume of 40 ml of water in 50-ml plastic tubes (Falcon). The 5CH solutions were then divided in 7.5 ml aliquots in 15-ml plastic tubes (Falcon), wrapped with aluminium foil and stored at +4 °C for the 9 days necessary for the medicine administration. All the procedures were done in sterile conditions and using sterile disposable plasticware. The medicines and the control solutions were administered in the morning for 9 consecutive days (including the last two days, dedicated to the testing procedures) by intraperitoneal (i.p.) injection (0.3 ml for CD1 mice, 0.25 ml for C57BL/6J mice or BALB/c mice, due to their different size, namely a mean of 30 g and 25 g respectively). I.p. injection was chosen in this series of experiments because it is much easier to control the dose/volume and is by far the most used in pharmacological studies in mice. Diazepam (Valium, Roche) (5 mg/ml) were diluted in distilled water and administered as 0.3 ml or 0.25 ml volumes i.p. at the indicated final dose (usually 1 mg/kg) only the days of the experiment, due to their well known pharmacokinetic properties.

Subjects and maintenance

All the experiments were performed at the Faculty of Medicine, Verona University, I. 4-5 weeks old mice of the indicated inbred strains were purchased from Harlan Laboratories (Udine, I) and allowed to acclimate for two weeks before testing. Mice were socially housed (4 or 2 per cage) in plastic cages with water and food available ad libitum. Cages were cleaned and bottles were filled with fresh tap water twice a week. Lights were on between 7 a.m. and 7 p.m. (indicated as day-light

cycle) or between 7 p.m. and 7 a.m. (indicated as inverted-light cycle). The animals belonging to a particular experimental group were randomly distributed in 3 to 8 different cages, according to the different protocols, and the order in which cages were settled in the rack and the mice were injected and tested was balanced between all the cages and all the experimental groups (Figure 4). Homeopathic medicines and control Ethylicum were administered each day at 9 a.m. for 9 consecutive days, including the two last days necessary for the behavioural testing. Mice belonging to the benzodiazepine standard treatment were also injected with control Ethylicum for 9 days, but in the days of testing the control Ethylicum solution was supplemented with Diazepam at the indicated concentrations. All the medicine preparations were independently coded by researchers of the Pharmacology department, so that the operators were unaware of the assignment of the experimental groups. The codes were disclosed only after the completion of the calculation phase. The day of the experiment mice were injected 70 min prior the beginning of tests, which were performed between 10 a.m. and 17 p.m. The testing procedures were done in two subsequent days: the first day (8th day of drug administration) LD and EPM were performed, the second day (9th day of drug administration) OF test was performed. After the tests, mice were put back into social housing in their original home cages. The animals were used only once with the same test in order to avoid the confounding effect of learning and habituation. All studies were conducted in accordance with Italian NIH policies on use of animals in research and the testing procedures were independently approved by the Animal Ethical Committee of the Interdipartimental Centre for Animal Research (CIRSAL) of Verona university and by the Italian Health Ministery.

Figure 4. Position of cages in the rack for a typical experiment with 64 mice distributed in 32 cages (2 animals x cage, 16 animals in 8 cages for each tested medicine and control).

Statistics

The present study primarily focuses on the setting-up of a behavioural laboratory and suitable anxiety-related paradigms for assessing in a sensitive way the responses of mice to homeopathic medicines. Since high variability of individual responses was expected, we used a sufficiently high number of mice for each experimental condition, namely 12 mice in the screening experiments and 16 mice in the confirmatory ones. The two extreme responses were discarded so that the mean values of the other 10 or 14 values were analysed and reported. The means of treated groups were

compared with the means of control group by analysis of variance (ANOVA SPSS, version 11 for Windows, Chicago,IL). Post-hoc t-tests were performed assuming equal variances with LSD corrections to adjust for repeated comparisons. Statistically significant data ($p<0.05$) are reported in tables in bold and p values data showing near-significativity are also reported. Where not indicated, the p values are considered as not significant.

Results

We started by performing two separate experiments in order to test six different homeopathic medicines, besides controls and standard benzodiazepine (Diazepam) drugs at conventional dosages. For each medicine tested, 12 animals of the CD1 strain, one of the most commonly used in behavioural studies, were used. The most interesting findings obtained are reported in tables 1 and 2, where the essential experimental variables are also given as notes.

Table 1. Behavioural parameters of CD1 mice treated with different medicines or equivalent control

			Tested medicines				
Test	Parameter	Value	Control (Ethylicum)	Gelsemium	Nux Vomica	Belladonna	Diazepam 0,5 mg/kg
Light-Dark	% time in light/total time of the test	Mean	22,64	28,76	20,54	24,56	**32,73**
		SD	9,65	5,47	8,92	6,92	6.67
		P(Anova)	-	ns (0,08)	ns	Ns	**0,007**
	Distance travelled in light	Mean	922,6	1113,8	825,7	999,8	**1347,5**
		SD	379,5	249,2	403,2	348,0	350,1
		P(Anova)		ns	ns	ns	**0,01**
	% resting time in light/total time in light	Mean	13,00	12,71	12,87	12,18	13,04
		SD	2,85	1,77	2,23	1,89	2,44
		P(Anova)		ns	ns	ns	ns
Open-field	% time in centre/total time of the test	Mean	6,55	**10,49**	8,20	5,73	**10,42**
		SD	3,50	3,00	4,88	2,98	4,02
		P(Anova)	-	**0,02**	ns	ns	**0,03**
	Distance travelled in centre	Mean	385,7	467,9	248,4	375,8	389,8
		SD	187,8	150,3	118,9	187,7	116,4
		P(Anova)	-	ns	(0,06)	ns	ns
	% resting time in centre/total time in centre	Mean	28,44	**38,59**	30,80	30,12	37,80
		SD	10,92	9,70	13,50	10,99	10,86
		P(Anova)	-	**0,04**	ns	ns	(0,07)

Notes. For each drug 12 animals were housed in three cages (4xcage), daylight cycle.

The time spent in the open, illuminated (white), compartment of LD test is the first measure for anxiety and this was confirmed in the experiment, reported in Table 1, by the effect of the benzodiazepine standard drug Diazepam, which increased the permanence time from 22,64 % to 32,73 % of the total time of the test (+44.6%). Of the three homeopathic medicines tested, only Gelsemium showed a 27% stimulating effect (from 22,64 to 28,76), that was near to the statistical significance. Also the distance travelled in illuminated compartment was increased by Diazepam but not by homeopathic medicines. A useful parameter reflecting the movement behaviour of the mice is the % time spent in resting position (related to the absence of exploratory attitude, or "freezing", or rearing), and this was not modified by any treatment condition. The other indicator is the OF test and here a clear and significant anxiolytic-like effect of Gelsemium appeared at the same extent of the standard conventional drug. The effect regarded the time spent in centre, indicating a tendency to explore the environment and a decrease of thigmotaxis. This was accompanied by an increase of the resting time more than a increase of distance travelled, suggesting that the mice treated with Gelsemium and Diazepam were more relaxed and familiar with the anxiogenic central area as compared with control, untreated, mice.

Table 2. Behavioural parameters of CD1 mice treated with different medicines or equivalent control

			Tested medicines				
Test	Parameter	Value	Control (Ethylicum)	Aconitum	Argentum nitricum	Tabacum	Diazepam 0,5 mg/kg
Light-Dark	% time in light/total time of the test	Mean	13,21	**25,32**	17,16	15,28	16,16
		SD	11,77	10,13	13,90	11,89	9,81
		P(Anova)	-	**0,02**	ns	ns	ns
	Distance travelled in light	Mean	537,3	867,9	476,5	671,3	527,7
		SD	435,1	442,5	367,6	529,2	333,2
		P(Anova)	-	(0,09)	ns	ns	ns
	% resting time in light/total time in light	Mean	12,84	22,76	**24,45**	14,88	19,99
		SD	2,97	10,70	17,42	8,10	10,51
		P(Anova)		(0,051)	**0,023**	ns	ns
Open-field	% time in centre/total time of the test	Mean	6,91	6,84	8,05	9,04	5,88
		SD	3,17	3,17	2,91	4,16	1,83
		P(Anova)	-	ns	ns	ns (0,14)	ns
	Distance travelled in centre	Mean	716,3	744,1	621,9	840,1	701,7
		SD	324,3	285,5	233,0	372,6	212,6
		P(Anova)	-	ns	ns	ns	ns
	% resting time in centre/total time in centre	Mean	13,24	12,40	**18,60**	15,15	13,01
		SD	3,59	5,30	7,55	4,35	5,01
		P(Anova)	-	ns	**0,03**	ns	ns

Notes. For each drug 12 animals were housed in three cages (4xcage), daylight cycle.

In the second LD screening experiment (table 2) we found a significant increase of % time in light/total time after administration of Aconitum, while its anxiolytic-like effect was not reproduced in the OF test. Argentum nitricum showed a peculiar effect, since it consistently increased the % resting time both in LD and in OF arenas. This effect was not accompanied by significant increase of permanence time, suggesting that was not a anxiolytic-like symptom. The major problem of this experiment was the lack of effect of Diazepam, possibly due to the dose or the solubilization, or both. As a matter of facts, we noted that dissolving Diazepam powder in 100% ethylic alcohol for the stock solution was very difficult and then, during dilution in water for preparing the working solutions, there was often a cloud of precipitate that was apparently dissolved by shaking. This prompted us to introduce, in the subsequent experiments, the use of commercial injectable Diazepam (Valium), that was already solubilized and provided in vials (5 mg/ml), and to increase the dose to 1 mg/kg body weight.

A further attempt to test our models and drugs was the use of the genetically inbred BALB/c mouse, which is characterized by a trait of "pathologic" anxiety, probably due to a chronically unbalanced corticosterone response to stress and formation of fear memories[12]. These animals exhibited a behaviour extremely peculiar: once put in the LD arena, they stayed in resting position or moved very slowly. As a consequence, they took much more time in order to enter into the dark chamber (lag time) as compared with other strains. Mice moved more quickly to the dark chamber, thus reducing the time spent in light and this in contrast with the principle according to which the model is working. Therefore, the % time spent in light, as performed in our laboratory, is not a suitable anxiety index for this type of mice. Some significant effect of Diazepam and of Gelsemium in LD test appeared only as a decrease of % resting time in light; this may indicate a positive effect on the exploration attitude of the mice and a decrease of fear-induced immobilization. Similar considerations hold for OF test, where many, but not all, mice put in the centre of arena stayed in that position for long time, without moving to the periphery and this problem caused high inter-individual variability and difficult interpretation of data. In order to avoid such interference, in subsequent experiments with this strain, mice should be always put in the periphery of the arena, so that only the movement from periphery to the centre will become a more reliable parameter of anxiolytic-like behaviour. On EPM test high variability of responses was noted, but a clear result was obtained: Diazepam markedly increased the time and distance in the open arm, while Gelsemium was not effective.

Table 6. Behavioural parameters of BALB/c mice treated with Diazepam and Gelsemium							
Test	Parameter	Value	Control (Ethylicum)	Diazepam 1mg/kg	% effect	Gelsemium	% effect
Light-dark	% time in light/total time of the test	Mean	41,50	32,55	-21.5	46,94	+13.1
		SD	37,21	27,45		40,10	
		P(Anova)		ns		Ns	
	Distance travelled in light	Mean	609,7	718,7	+17.8	862,0	+41.3
		SD	366,2	467,8		471,8	
		P(Anova)		ns		Ns	
	% resting time in light/total time in light	Mean	39,31	**25,23**	**-35.8**	**31,18**	**-20.7**
		SD	12,41	10,12		12,78	
		P(Anova)		**0,001**		**0,037**	
Open-field	% time in centre	Mean	7,94	1,60	-79.8	26,09	+228.5
		SD	13,98	3,28		40,38	
		P(Anova)		ns		(0,068)	
	Distance travelled in centre	Mean	178,9	34,56	-80.8	341,8	+91.0
		SD	294,2	64,1		459,7	
		P(Anova)		ns		Ns	
	% distance travelled in centre/total	Mean	5,93	2,13	-64.1	**26,04**	**+339.1**
		SD	9,19	4,04		38,68	
		P(Anova)		ns		**0,039**	
	% resting time in centre/total time in centre	Mean	46,82	41,71	-12.2	50,13	+7.1
		SD	14,75	23,34		19,71	
		P(Anova)		ns		Ns	
Plus-Maze	% time in open arms	Mean	11,99	**28,54**	**+138.0**	10,07	-16.0
		SD	12,84	15,11		13,81	
		P(Anova)		**0,04**		Ns	
	Distance travelled in open arms	Mean	267,6	**828,8**	**+209.7**	226,3	-15.4
		SD	345,3	480,6		291,7	
		P(Anova)		**<0,001**		Ns	
Notes. For each drug and control 16 animals were housed in 8 cages (2xcage), inverted light cycle							

In the subsequent experiment (table 7), we decided to go back to the use of CD1 mice, which appeared the most suitable and reliable, having intermediate levels of trait anxiety. In this experiment, neither Diazepam nor Gelsemium worked in the LD test. In OF test a significant change in the direction of anxiolytic-like effect was obtained only with Gelsemium. This is not surprising because similar trends in favour of a better OF response to Gelsemium were noted in experiments reported in tables 3,4,5,6. Finally EPM paradigm showed concordance between the effects of the two drugs.

Finding qualitatively different results in different experiments and high variability forced us to continue testing the same paradigm in the same animal strain. Table 8 reports the results of a experiment similar to the previous one, the only difference being a one-week longer housing of animals before starting the treatments (due to Christmas holidays). Quite surprisingly, the reference drug Diazepam caused in all the test a paradoxical anxiogenic effect, which was highly significant in the EPM test. Careful revision of all the procedure excluded any possible error in the drug administration, in the experimental procedure or in the calculation phase. Gelsemium showed no anxiogenic properties in LD nor in OF, where only a significant increase of resting time was caused: the mice moved more slowly in both fields, an effect that at present is difficult to interpret in behavioural terms. The more striking evidence is the net decrease in the EPM parameters, which parallels the effect of Diazepam. Very interestingly, also Gelsemium exhibited the same trend of effects in EPM and in this case we could speculate that the homeopathic medicine induced a "pathogenetic" anxiogenic effect (symptoms "proving", in homeopathic terminology).

Discussion

The goals of these experiments were to obtain behavioural profiles after conventional and homeopathic treatment and to evaluate the limitations and strengths of these models in mice. We explored and changed different experimental settings in order to find the optimal conditions where statistically significant differences in specific behavioural parameters/symptoms emerged. Conflict tests have become widely used for assaying anti-anxiety agents, either for screening purposes or for studying their mechanisms of action [2]. This series of preliminary experiments permit to draw a series of provisional indications.

Gelsemium sempervirens, the homeopathic medicine that preliminary screening test revealed as the most active in reducing anxiety, regulated the behaviour of mice of CD1 and C57BL/6J strains n a number of models. The measurements found to be most consistent and useful for assessing anxiety-related activity are the time spent in the lit compartment of the Light-dark model or the time spent in the central area of the Open field model, expressed as percent of total time of the test. Also the EPM test proved to be a sensitive marker of drug-induced changes of behaviour, possibly unrelated to the other parameters (see the data with BALB/C mice), but further experiments are necessary in order to test its validity in our models.

Table 8. Behavioural parameters of CD1 mice treated with Diazepam and Gelsemium

Test	Parameter	Value	Control (Ethylicum)	Diazepam 1mg/kg	% effect	Gelsemium	% effect
Light-dark	% time in light/total time of the test	Mean	20,85	13,19	-36.7	21,90	+5.0
		SD	7,45	8,74		12,70	
		P(Anova)		(0,053)		ns	
	Distance travelled in light	Mean	712,9	535,4	-24.8	627,9	-11.9
		SD	287,2	343,0		458,6	
		P(Anova)		ns		ns	
	% resting time in light/total time in light	Mean	21,33	18,23	-14.5	**27,23**	**+27.6**
		SD	5,77	2,63		9,42	
		P(Anova)		ns		**0,012**	
Open-field	% time in centre	Mean	7,52	**4,68**	**-37.7**	8,39	+11.6
		SD	2,58	3,09		3,16	
		P(Anova)		**0,015**		ns	
	Distance travelled in centre	Mean	804,2	497,8	**-38.0**	677,5	-15.7
		SD	250,9	287,0		263,1	
		P(Anova)		**0,005**		ns	
	% resting time in centre/total time in centre	Mean	13,03	14,47	+11.0	**21,30**	**+63.4**
		SD	4,14	7,06		10,86	
		P(Anova)		ns		**0,004**	
Plus-Maze	% time in open arms	Mean	17,97	**9,42**	**-47.6**	**10,61**	**-40.9**
		SD	8,04	6,89		7,53	
		P(Anova)		**0,005**		**0,015**	
	Distance travelled in open arms	Mean	683,7	**324,2**	**-52.5**	**401,5**	**-41.2**
		SD	354,8	215,4		306,9	
		P(Anova)		**0,002**		**0,013**	

Notes. For each drug and control 16 animals were housed in 8 cages (2xcage), inverted light cycle

Using the OF test, Gelsemium increased, often in a statistically significant fashion, the time and movement of mice in the centre of arena, with the exception of the last experiment, where the mice exhibited atypical responses even to Diazepam. The changes caused by Gelsemium treatment in OF were much more reproducible and consistent than those caused by Diazepam. This may suggest that the homeopathic medicine does not act simply by antagonizing anxiety but also by stimulating the unconditioned exploratory attitude of the animals, a parameter that is better exploited by OF test. The extent of the Gelsemium effects often approximated the response to 1 mg/kg of the standard

drug Diazepam. Assuming that active principles of Gelsemium, namely Gelsemine and Sempervirine may represent approximately 1% of the weight in the plant mother tincture (in the leaves and in the flowers the dose is about 0,1 mg/g [13]) the dose of those principles in the 5CH potency administered to animals may be estimated in the order of 0,000000001 mg/kg. Subsequent studies are planned in order to test higher potencies (lower doses) of the same medicine.

In several models and in several experiments the stimulating or inhibiting effects of Diazepam and Gelsemium were similar and parallel. However, very often, the effects of Diazepam and of Gelsemium were different in the different test of the same experiment, indicating that the various models explore different neurobiological and behavioural schemes. We also noted high interindividual variability, also in inbred animals. Others have reported that in the same inbred C57BL/6J male mice population, two groups having low and high "trait" anxiety and different neuroendocrine responses to stress could be selected for their latency to freely enter from their home cages into an unfamiliar arena [14], indicating that expression of trait anxiety displays a high interindividual variability in inbred mice. Many of the inconsistencies found with drugs that alter central nervous system may be explained by the fact that these are mixed models, in the sense that the mouse displays different strategies of defence while exploring the available space: in EPM avoidance of open arms when the mouse is in the closed arm and escape from open arm to enter a safe closed arm; in LD paradigm tendency to explore open arena and escape from bright light. These conflicting choices could be influenced in opposite direction by neurotropic drugs.

The lack of reproducibility of the anxiolytic effect of the standard drug Diazepam, and even its inverted paradoxical action, indicates that these models are very sensitive to the experimental conditions like dosages, administration schedules, animal housing, light cycle, temperature, food, manual handling and possibly other elusive factors. In general, we noted that the anxiolytic effect of Diazepam, starting from LD test, decreased when we changed the illumination schedule. Another important variable factor may be the age of animals, that seem to markedly affect the response the anxiolytics and anaesthetics in animal models.[15, 16] The discovery of benzodiazepines in the early 1960s and their considerable commercial success in the treatment of anxiety fuelled the development of numerous animal models of anxiety; conversely, the predictive validity of these models was based on their ability to detect the pharmacological action of BDZs. However, subsequent studies showed that non-BDZ anxiolytics (e.g. Buspirone, a 5-HT1A partial agonist) were found to be inactive in some anxiety tests [3], while traditional BDZs have been found to be inactive in some models or even produce paradoxical anxiogenic effects [9]. These findings raise some concerns about the methodological foundations of the current paradigm of benzodiazepines as the reference anxiolytic compounds both in animal and human studies.

Particularly noteworthy was the finding that the inversion of effects (paradoxical anxiogenic effects) have been obtained in the same experiment and in the same experimental model. It is also evident that when the basal activity of untreated animals was low (e.g. the time spent in open arm in experiment of table 7) the Diazepam and Gelsemium effect was to increase it, as expected, while when the basal activity was already high and mice were basically less anxious (e.g. the time spent in open arm in experiment of table 8), the Diazepam and Gelsemium effect was to decrease it (paradoxical anxiogenic effect). This is recalling the similia rule according to which the direction of the drug effect is opposite in healthy versus ill or stressed individuals (Wilder rule) [10, 17].

Acknowledgments

This work was supported by a grant from Laboratoires Boiron s.r.l. (Milano, I) to Verona University. The authors wish to thank Dr. Naoual Boujedaini for her advice and cooperation.

References

(1) Bourin M, Petit-Demouliere B, Dhonnchadha BN, Hascoet M. Animal models of anxiety in mice. *Fundam Clin Pharmacol* 2007 December;21(6):567-574.

(2) Pinheiro SH, Zangrossi H, Jr., Del-Ben CM, Graeff FG. Elevated mazes as animal models of anxiety: effects of serotonergic agents. *An Acad Bras Cienc* 2007 March;79(1):71-85.

(3) Belzung C, Griebel G. Measuring normal and pathological anxiety-like behaviour in mice: a review. *Behav Brain Res* 2001;125:141-149.

(4) Clement Y, Joubert C, Kopp C, Lepicard EM, Venault P, Misslin R, Cadot M, Chapouthier G. Anxiety in mice: a principal component analysis study. *Neural Plast* 2007;2007:35457.

(5) Binsard AM, Guillemain J, Platel A, Savini EC, Tetau M. Etude psycho-pharmacologique de dilutions homéopathiques de Gelsemium et d'Ignatia. *Ann Homeop Fr* 1980;22(3):35-50.

(6) Guillemain J, Rousseau A, Dorfman P, Tetau M. Recherche en psychopharmacologie. *Cah Biother* 1989;103:53-66.

(7) Bousta D, Soulimani R, Jarmouni I, Belon P, Falla J, Foment N, Younos C. Neurotropic, immunological and gastric effects of low doses of Atropa belladonna L., Gelsemium sempervirens L. and Poumon histamine in stressed mice. *J Ethnopharmacol* 2001;74(3): 205-215.

(8) Venard C, Boujedaini N, Belon P, Mensah-Nyagan AG, Patte-Mensah C. Pharmacological modulators of the glycinergis sstem regulate allopregnanolone biosynthesis in the rat spinal cord. *Trabajos del Instituto Cajal*; 2007 Feb 17; Madrid, p. 262.

(9) Ennaceur A, Michalikova S, van RR, Chazot PL. Are benzodiazepines really anxiolytic? Evidence from a 3D maze spatial navigation task. *Behav Brain Res* 2008 March 17;188(1): 136-153.

(10) Bellavite P, Ortolani R, Pontarollo F, Pitari G, Conforti A. Immunology and Homeopathy. 5. The Rationale of the 'Simile'. *Evid Based Complement Alternat Med* 2007 June;4(2): 149-163.

(11) Bourin M, Hascoet M. The mouse light/dark box test. *Eur J Pharmacol* 2003 February 28;463(1-3):55-65.

(12) Brinks V, de Kloet ER, Oitzl MS. Strain specific fear behaviour and glucocorticoid response to aversive events: modelling PTSD in mice. *Prog Brain Res* 2008;167:257-261.

(13) Irwin RE, Adler LS. Correlations among traits associated with herbivore resistance and pollination: implications for pollination and nectar robbing in a distylous plant. *Am J Botany* 2006;93:64-72.

(14) Jakovcevski M, Schachner M, Morellini F. Individual variability in the stress response of C57BL/6J male mice correlates with trait anxiety. *Genes Brain Behav* 2007 August 3 (e-pub).

(15) Bessa JM, Oliveira M, Cerqueira JJ, Almeida OF, Sousa N. Age-related qualitative shift in emotional behaviour: paradoxical findings after re-exposure of rats in the elevated-plus maze. *Behav Brain Res* 2005 July 1;162(1):135-142.

(16) Tobin JR. Paradoxical effects of midazolam in the very young. *Anesthesiology* 2008 January;108(1):6-7.

(17) Bellavite P. Complexity science and homeopathy. A synthetic overview. *Homeopathy* 2003;92:203-212.