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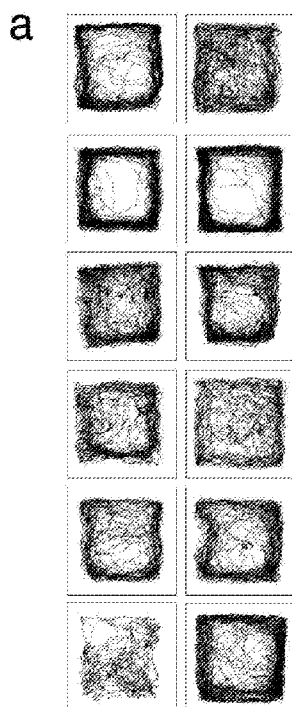


Figure 1

(57) Abstract: The present invention relates to a method for screening for compounds having anxiolytic or anti-depressant effect. In particular, the invention relates to a method of screening for test agents having anxiolytic or antidepressant effect on a population of insects (e.g. flies of the family *Drosophila*) which involves correlating the distribution of the insects in a test area with the effect of the test agent on the population of insects.



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- 1 -

SCREENING FOR ANXIOLYTIC DRUGS BY TESTING DROSOPHILA IN AN OPEN-FIELD TEST

5 The present invention relates to a method for screening for compounds having anxiolytic or anti-depressant effect. In particular, the invention relates to a method of screening for test agents having anxiolytic or antidepressant effect on a population of insects (e.g. flies of the family *Drosophila*) which involves correlating the distribution of the insects in a test area with the effect of the test agent on the population of insects.

10 There is a need to be able to model psychiatric phenotypes in invertebrates. This would provide new and - compared to vertebrate models - faster approaches to understanding the neuronal and molecular bases of these common human disorders. However, very few validated models exist.

15 It has now been found that pharmacological, genetic and environmental interventions alter behaviour in flies of the family *Drosophila*. In particular, it has been found that the anxiolytic drug diazepam decreases *Drosophila* wall-following (also called 'thigmotaxis' or 'centrophobism'), while the anxiogenic drug sodium lactate increases wall-following. Disruptions of the serotonergic system, lesions in two stress-related neuropeptide pathways, pain and immobilization have all now been found to affect wall-following behaviour in *Drosophila*. The data presented herein indicate that human anxiety is an ancient emotion whose underlying mechanisms are shared across a wider range of metazoans than previously appreciated. Hence the findings disclosed herein can be used to screen for test agents having an anxiolytic or antidepressant effect in humans.

20 In one embodiment, therefore, the invention provides a method of screening for the effect of a test agent on a population of insects, comprising the steps:

- 25 (a) providing a population of insects in a test area or test areas, wherein the test agent has been administered to said population or is being administered to said population;
- (b) determining the distribution of the insects in the test area(s);
- (c) correlating the distribution of the insects in the test area(s) with the effect of the test agent on the population of insects.
- 30

 In another embodiment, the invention provides a method for screening for an agent having anxiolytic or antidepressant effect, the method comprising the steps:

- (a) providing a population of insects in a test area or test areas, wherein a test agent has been administered to said population or is being administered to said population;

- 2 -

(b) determining the distribution of the insects in the test area(s);

(c) correlating the distribution of the insects in the test area(s) with the effect of the test agent on the said population of insects,

5 wherein a test agent whose administration results in an increased proportion of the said population being located in open or central regions of the test area(s) or away from the walls of the test area(s) during the test period or at the end of the test period compared to a control population to whom the test agent has not been administered is indicative of the test compound having an anxiolytic or antidepressant effect.

10 As used herein, the term "anxiolytic effect" refers to a substance or compound which is capable of relieving or reducing a state of anxiety or neurosis.

As used herein, the term "anti-depressant effect" refers to a substance or compound which is capable of reducing or relieving one or more symptoms of depression.

15 Preferably, the substance, agent or compound is one which is capable of having an anxiolytic or anti-depressant effect in mammals, most preferably in humans.

The number of insects in the population may be any suitable number. Preferably, the number is large enough in order to obtain a statistically significant result. The number may be, for example, 1-200, 1-150, 1-100, 1-50, 1-20, 1-10, 5-50, preferably 10-40, more preferably 20-30 insects per test area or areas.

20 The test area(s) may be any suitable size and is preferably enclosed to prevent loss of the insects. The area(s) may have walls, open areas and/or central areas. It may, for example, be 1-20cm², more preferably 1-10cm² and most preferably about 1-5cm² in floor area.

A number of test areas may be tested concurrently. In one embodiment, the invention relates to a method as disclosed herein wherein a plurality of screening assays are performed, 25 using the same or different test agents, concurrently.

The subclass *Pterygota* includes most of the world's insect species. The subclass is further divided into two groups – the *Exopterygota* and the *Endopterygota*. Insects in the Superorder *Exopterygota* undergo a simple or incomplete metamorphosis. The life cycle includes just three stages – egg, nymph, and adult. During the nymph stage, gradual change 30 occurs until the nymph resembles the adult. Only the final molt stage has functional wings.

A large number of familiar insects fall within the superorder *Exopterygota*. There are sixteen orders in this subdivision, including Order *Odonata* - Dragonflies and Damselflies; Order *Orthoptera* - Crickets, Grasshoppers and Locusts; Order *Dictyoptera* - Roaches and Mantids; Order *Hemiptera* - True Bugs; and Order *Thysanoptera* - Thrips.

- 3 -

The majority of the world's insects undergo complete metamorphosis and are included in the superorder *Endopterygota*. The largest of these nine insect orders are Order *Coleoptera* - Beetles; Order *Diptera* - True Flies; Order *Lepidoptera* - Butterflies and Moths; and Order *Hymenoptera* - Ants, Bees, and Wasps.

5 The invention preferably relates to insects of one of the above Orders. Preferably, the insect is of the order *Diptera*. Most preferably, the insect is from one of the following families: *Culicidae* - mosquitoes; *Tipulidae* – crane flies; *Simulidae* – black flies; *Muscidae* – house flies; *Cecidomyiidae* – gall midges; *Calliphoridae* – blow flies; or *Drosophilidae* – pomace flies. In some particularly preferred embodiments, the insect is from the family *Drosophila*, most
10 preferably *Drosophila melanogaster*. In other embodiments, the insect is one of the following: *Drosophila (Sophophora) simulans*; *Drosophila (Sophophora) sechellia*; *Drosophila (Sophophora) yakuba*; *Drosophila (Sophophora) erecta*; *Drosophila (Sophophora) ananassae*; *Drosophila (Sophophora) pseudoobscura*; *Drosophila (Sophophora) persimilis*; *Drosophila (Sophophora) willistoni*; *Drosophila (Drosophila) mojavensis*; *Drosophila (Drosophila) virilis*; or
15 *Drosophila (Drosophila) grimshawi*.

In yet other embodiments, flies from the genera *Calliphora*, *Phormia* and *Musca* may be used.

In yet further embodiments, insects such as mosquitoes (*Culicidae*) may be used, e.g. of the genera *Anopheles*, *Culex* or *Aedes* genera or other insects that might have damaging
20 effects on crops or human health.

In some embodiments of the invention, male insects are preferred; in other embodiments, female insects are preferred.

In general, all of the insects which are used in any one method will be of the same species.

25 In some embodiments, it is desirable to use insects which are modified (genetically, chemically, physically, physiologically or otherwise) to become more susceptible to anxiety or depression and hence such insects can be used to screen for compounds having increased anxiolytic or antidepressant effect.

Preferred modified insects include the following:

30 - insects with reduced or increased serotonin transporter (SERT), e.g. insects expressing RNAi against the *SerT* mRNA;
- insects having loss of function or reduced or enhanced function of 5-HT receptors, e.g. 5HT1A or 5HT-2B-like receptors;

- 4 -

- insects having loss of function or reduced or enhanced function of corticotropin releasing hormone (CRH);

- insects with reduced or enhanced expression of diuretic hormone 44 (DH44-R1).

5 In other embodiments, a (genetically non-uniform) population of insects may be used to screen for mutations in that population that affect anxiety-like behaviour (ALB).

Hence the invention also provides a method of screening for genetic mutations in a population of insects which affect anxiety-like behaviour, the method comprising the steps:

(a) providing a genetically non-uniform population of insects in a test area;

(b) determining the physical distribution of the insects in the test area;

10 (c) correlating the distribution of genetic mutations in the insects in the test area with the physical distribution of the insects.

The invention further provides a method of screening for genetic mutations which reduce anxiety or depression in a population of insects, the method comprising the steps:

(a) providing a genetically non-uniform population of insects in a test area;

15 (b) determining the physical distribution of the insects in the test area;

(c) correlating the distribution of genetic mutations in the insects in the test area with the physical distribution of the insects,

wherein a genetic mutation which:

20 (i) is present in a sub-population of the insects which are predominantly located in open or central regions of the test areas or away from the walls of the test area during the test period or at the end of the test period, and which

(ii) is not present in a sub-population of the insects which are predominantly located away from open or central regions of the test areas or near from the walls of the test area during the test period or at the end of the test period

25 is indicative of the genetic mutation having an anxiety-reducing or depression-reducing effect.

The invention also provides a method of screening for genetic mutations in a population of insects which increase anxiety or depression, the method comprising the steps:

(a) providing a genetically non-uniform population of insects in a test area;

(b) determining the physical distribution of the insects in the test area;

30 (c) correlating the distribution of genetic mutations in the insects in the test area with the physical distribution of the insects,

wherein a genetic mutation which:

- 5 -

(i) is present in a sub-population of the insects which are predominantly located away from open or central regions of the test areas or near from the walls of the test area during the test period or at the end of the test period, and which

(ii) is not present in a sub-population of the insects which are predominantly located in open or central regions of the test areas or away from the walls of the test area during the test period or at the end of the test period,

is indicative of the genetic mutation having an anxiety-increasing or depression-increasing effect.

Once mutations are found which affect (either increase or reduce, preferably reduce) anxiety or depression, insects having such mutations may be used to screen for agents which counter that affect.

In other embodiments, the insects referred to herein may be replaced by another motile animal (excluding rodents), for example fish (e.g. zebrafish, *Danio rerio*) or amphibians (e.g. frogs or toads, or their tadpoles). Hence the invention relates to all methods and all agents as described herein wherein the references to an insect or insects are replaced with motile animal, fish or amphibian, *mutatis mutandis*.

The term "administering" includes, *inter alia*, all means which result in the test agent being capable of exerting its effect on the population of insects. For example, the insects may be allowed to feed on the test agent, the insects may be physically contacted with the test agent or the insects may be exposed to a fluid (liquid or gas) which comprises the test agent. In some embodiments, the insects are exposed to a mist or vapour comprising the test agent.

The invention involves the step of determining the distribution of the insects in the test area. This may be done by any suitable means. In a preferred embodiment, the distribution of the insects is determined using a camera and appropriate computer software. In a particularly preferred embodiment, the distribution of the insects is tracked using videography. Preferably, the computer software is LabVIEW tracking software.

The distribution of the insects may be determined over a set time period. Examples of suitable time periods include 1-200 minutes, preferably 1-100 minutes, more preferably 1-50 minutes and most preferably about 10 minutes.

At the end of the test period, the accumulated distributions or final distribution of the insects in the test area are analysed and correlated with the effect of the test substance. For example, the accumulated distributions of the insects may refer to information on the total paths taken by the insects or physical locations of the insects during the test period. Such information may be taken and stored at defined intervals. This information may take the form of x-y plots of the paths of the insects or x-y plots of the positions of the insects. For example, the overall time

- 6 -

spent by the insects in the centre of the test area(s) or the time spent by the insects in open regions or overall mean distances from the centre of the test area(s) may be correlated with effect of the test agent. In other embodiments, the final positions of the insects are correlated with the effect of the test substance.

5 In some embodiments, the overall time spent by the population near or away from the walls or near or away from the central region of the test area(s) is determined and compared to a control population to whom the test compound has not been administered.

 In other embodiments, the mean distances of each member of the population from the centre of the test area(s) or a point in the test area(s) away from one of the walls is determined.

10 In the context of testing of agents having an anxiolytic or antidepressant effect, the proportion of the population located in open or central areas of the test area(s) during or at the end of test period compared to the proportion of the population located near the walls or sides is determined; and these proportions may be compared to control populations to whom the test agent has not been administered.

15 In particular, a test agent whose administration results in an increased proportion of the said population being located in open or central regions of the test area(s) during the test period or at the end of the test period compared to a control population to whom the test agent has not been administered is indicative of the test compound having an anxiolytic or antidepressant effect.

20 Furthermore, a test agent whose administration results in a proportion of the said population being located in open or central regions of the test area(s) for a greater amount of time compared to a control population to whom the test agent has not been administered is indicative of the test compound having an anxiolytic or antidepressant effect.

25 In some embodiments, the information is pre-filtered in order to eliminate tracking failures or dead insects.

 Preferably, the increase in the proportion of the population or increased time is a significant increase ($p > 0.05$).

30 The distribution of the insects may be determined using the apparatus described in Cell 139, 405-415 (October 2009), the contents of which are specifically incorporated herein by reference.

 As used herein, the term "open regions" of the test area(s) refers to areas which are away from any of the sides or walls of the test area(s) or away from other physical objects which are present in the test area. In other embodiments, the term "open areas" refers to areas which are not in proximity with the walls or sides of the test area(s).

- 7 -

The methods of the invention may be carried out under conditions of increased stress. In order to increase the stress on the insects, the method may be carried out at an increased temperature, e.g. 30-40°C, preferably at about 37°C. Alternatively, the insects may be
5 restrained or immobilised prior to the method, e.g. so that movement is restricted or prevented. For example, the insects may be restrained for about 1 hour prior to the method. Alternatively, the insects may be subjected to pain before or during the method.

The invention also provides an anxiolytic or antidepressant agent which has been found using a screening method of the invention.

10 The invention further provides a method for preparing a pharmaceutical composition, comprising the steps:

(a) screening for a test agent having an anxiolytic or antidepressant effect in accordance with the invention, and

(b) admixing a test agent which has been found to have an anxiolytic or antidepressant
15 effect with one or more pharmaceutically acceptable carriers, diluents and/or excipients.

FIGURE LEGENDS

Figure 1: Pharmacological effects on *Drosophila* wall following.

20 a, Representative locomotion paths from 12 flies placed in 1 cm arenas collected during a 10-minute experiment show a diversity of locomotor patterns, including prominent wall following in most flies.

b, Diazepam reduces wall following behaviour. Wild type Canton-S flies maintained an average distance of 3.1 mm from the centre of arena when fed food containing
25 0.5% dimethyl sulfoxide (DMSO), but walk closer to the centre when fed food also containing 3 mM diazepam ($p = 0.02$, $R^2 = 0.049$, $N = 60, 40$). The location maps show the distribution of flies in the asymmetrical space of the square arena (see Methods).

c, Sodium lactate increases centre-avoiding, wall following locomotion. Flies increase
30 the mean distance they keep from the centre, from 3.1 mm to 3.7 mm ($p = 5.7 \times 10^{-7}$, $R^2 = 0.191$, $N = 57, 59$). An identical amount of sodium chloride does not have such an effect ($p = 0.45$, $R^2 = -5.478 \times 10^{-3}$, $N = 40, 37$).

- 8 -

Figure 2: The serotonergic system affects fly wall following.

- a, Induction of a *SerT* RNAi transgene *SerT^{GD3824}* for 3 nights produces an increase in distance from the centre of the chamber ($p = 8 \times 10^{-4}$, $R^2 = 0.106$, $N = 59, 40$).
- b, Subjecting progeny of driver strain *n-syb-GAL4*, *Tub-GAL80^{ts}* and Canton-S to 3 nights of 31 °C treatment has no significant effect ($p = 0.816$, $R^2 = -0.009$, $N = 64, 44$).
- c, Overnight over-expression from a *SerT* cDNA transgene (*SerT^{Scer\UAS.cPa}*) results in a decrease in centrophobism ($p = 0.0027$, $R^2 = 0.038$, $N = 93, 116$).
- d, RNAi knockdown of the 5-HT1A-like *Drosophila* 5-HT1B receptor (*5-HT1B^{JF01851}*) produces an increase in centre-avoidance, as does ($p = 9.2 \times 10^{-4}$, $R^2 = 0.044$, $N = 125, 98$)
- e, knockdown of the 5-HT2B-like *Drosophila* 5-HT2 receptor ($p = 0.02$, $R^2 = 0.035$, $N = 59, 70$).
- f, Selective acute inactivation of neurotransmission from serotonergic neurons with SHI^{ts} results in an increase in thigmotaxis ($p = 6.986 \times 10^{-5}$, $R^2 = 0.132$, $N = 48, 59$).

Figure 3: Stress signalling systems and stress influence fly wall following.

- a, Knockdown of fly tachykinin in *n-syb-GAL4*, *Tub-GAL80^{ts}*/*Tk^{JF01818}* flies produces a decrease in wall following as measured by mean distance from the arena centre ($p = 0.01$, $R^2 = 0.031$, $N = 90, 91$).
- b, Knockdown of a CRH-like peptide receptor with *Dh44-R1^{JF03208}* results in decreased wall following ($p = 2.4 \times 10^{-4}$, $R^2 = 0.069$, $N = 97, 95$).
- c, A transition from 25 °C to 37 °C results in Canton S increases flies proximity to the arena's corner ($p = 2.6 \times 10^{-9}$, $R^2 = 0.256$, $N = 60, 59$).
- d, One hour immobilization increases wall following ($p = 0.021$, $R^2 = 0.035$, $N = 59, 37$).

Figure 4: Overnight exposure to 31 °C has no effect on fly wall following.

- a, F1 progeny of Canton S flies (*w*; *n-syb-GAL4*, *Tub-GAL80^{ts/+}*) were subjected to a single night at 31 °C, resulting in no change in centre avoidance ($p = 0.64$, $R^2 = -0.007$, $N = 60, 60$).
- b, F1 progeny of *w¹¹¹⁸* flies (*w*; *n-syb-GAL4*, *Tub-GAL80^{ts/+}*) were subjected to a single night at 31 °C, resulting in no change in centre avoidance ($p = 0.10$, $R^2 = 0.013$, $N = 60, 60$).

Figure 5: Genes not expected to influence thigmotaxis have no effect on wall following.

a, Mutations in *rutabaga* do not affect wall following. There is no difference in centrophobism between wild type flies and those that carry the *rut¹* lesion ($p = 0.97$, $R^2 = -0.009$, $N = 56, 51$).

b, The thigmotaxis of *rut²⁰⁸⁰* flies is statistically indistinguishable from near-isogenic wild type flies ($p = 0.37$, $R^2 = -0.001$, $N = 80, 60$).

Induction of RNAi targeting c, *CtBP* ($w+; n-syb-GAL4, Tub-GAL80^{ts} / + CtBP^{GD4268}$) ($p = 0.75$, $R^2 = -0.012$, $N = 38, 40$) and d, PDK have no effect on centre avoidance,

two genes chosen with no expectation to have an effect on wall following ($p = 0.11$, $R^2 = 0.008$, $N = 99, 97$).

EXAMPLES

The present invention is further defined in the following Examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. The disclosure of each reference set forth herein is incorporated herein by reference in its entirety.

Example 1: Summary of methods

Locomotion assays were performed on male flies in a 20-plex array square arenas. Arenas were 1cm square, with a height of 1.6mm and a transparent plastic lid. The array was lit from above with microscope lamps. Individual cold anaesthetized flies were introduced into each arena. After recovery from anaesthesia, flies' movements were tracked via videography and LabVIEW tracking software. Tracking location data for each fly was recorded as x and y coordinates and analyzed with MATLAB and R. Flies were raised and maintained at 24°C on a standard cornmeal/molasses medium on a 12:12 hrs light/dark cycle at 60% humidity, unless

- 10 -

they carried *Tub-Gal80^{ts}* or *UAS-shi^{ts}* in which case they were raised at 18°C. Lines carrying UASdsRNA transgenes were obtained from the Vienna Drosophila Research Centre or the Bloomington Drosophila Stock Center.

5 Example 2: Full methods

Fly strains Flies were cultured and maintained on fly medium at 24 °C and 60% humidity, on a 12 hrs light: 12 hrs dark cycle unless otherwise mentioned. Wild-type stocks were *w¹¹¹⁸* and Canton-S (*CS*). Strains carrying double-stranded RNAi transgenes were obtained from stock centers. The Vienna Drosophila Research Centre (VDRC) *UAS-dsRNA* lines used were: 5-*HT2^{KK110704}* (v102105), *SerT^{GD3824}* (v11346), *CtBP^{GD4268}* (v37609) and *Pdk^{GD5280}* (v37966). Other lines, including RNAi lines made by the Transgenic RNAi Project, were obtained from the Bloomington Drosophila Stock Center (BDSC): *5-HT1B^{JF01851}* (25833), *Tk^{JF01818}* (25800), *Dh44-R1^{JF03208}* (28780), *SerT^{Scer\UAS.cPa}* (24464), and *rut¹* (9404). The pan-neural driver strain *n-syb-GAL4*, *Tub-GAL80^{ts}/TM* was generated by standard methods from the constituent transgenics: *n-syb-GAL4* was a gift from Bassem Hassan and *Tub-GAL80^{ts}* was acquired from BDSC (14657498). The *rut²⁰⁸⁰* stock was a gift from Scott Waddell; the *Trh-GAL4* stock was a gift from Edward Kravitz.

Transgenic animal preparation For temperature-controlled transgenic expression *nsyb-GAL4*; *Tub-Gal80^{ts}/TM3* virgin females were crossed with males carrying the responder transgene (i.e. *UAS-RNAi* or *UAS-cDNA*). The progeny of these crosses were raised at 18°C to maintain repression of the responder gene. Newly eclosed non-*TM3* males were isolated and maintained as a group of 24 flies in vials (2.4 cm diameter × 9.4 cm height) for 2–3 days before the behavioural assays. Vials of these animals were either maintained at 18°C until assaying, or treated to ~16 hr long overnight interval(s) at 31 °C. A single night of induction was initially used for each responder; if no behavioural effect was seen the experiment was repeated with 3 consecutive nights at 31 °C, days at 18. Prior to the behavioural assay, flies were held for a 2-3 hr recovery period at 25 °C. In addition to the non-induced flies carrying *UAS* transgenes, further control animals were generated by crossing the driver line with wild type virgins (either Canton-S or a *w¹¹¹⁸* strain received from VDRC) and raising the progeny under identical regimes as the flies carrying the responder transgenes. Since *rutabaga* is on the X chromosome *rut* mutant females were crossed with male Canton-S flies to yield mutant F1 males and *rut* males were crossed with Canton-S females to yield near-isogenic wild type F1 males.

- 11 -

Drug treatments Diazepam was dissolved in 10% v/v dimethyl sulfoxide solution; sodium lactate (Sigma) solution was made with distilled water. Drug solutions were then diluted 20-fold into molten fly food to a final concentration of 3mM for each (and 0.5% v/v dimethyl sulfoxide in the case of diazepam). For controls, equivalent volumes of solvent were added. Flies were food
5 deprived in empty vials for 6 hrs before transfer to the drugged vials for ~18 hrs at 24°C prior to behavioural testing.

Stressors Pain was induced by heating the flies to 37 °C while in the arena. For restraint stress, each individual fly was immobilized in a small space formed between two nested 20 µl pipette
10 tips (Eppendorf). This arrangement allowed ventilation through the tips, but prevented any substantial locomotion. Flies were restrained for a period of one hour prior to behavioural testing.

Behavioural assay Batches of 20-30 male flies were isolated under carbon dioxide anaesthesia and placed in food vials on days prior to the behavioural assay. Just before assaying, a batch
15 was knocked into an ice-chilled vial for 1-2 min cold anaesthesia to subdue them before they were placed individually to an arena with forceps. Each arena was a 1 cm square with a height of 1.6 mm; twenty arenas were laser cut from a sheet of black acrylic to allow video analysis of 20 flies simultaneously. The array was covered with a transparent plastic sheet and was placed in an incubator, lit from the sides by two white LED microscope lamps (Gain Express Holdings).
20 To image the flies an AVT Guppy F-046B CCD camera (Stemmer Imaging) equipped with a 12 mm CCTV-type lens was positioned above the behavioural arenas and connected to a computer via a IEEE 1394 cable. Flies were allowed to freely explore the arena during a 10-minute test session. The live video feed was analyzed for the flies' positions by custom virtual instruments written in LabVIEW (National Instruments), extracting centroid x-y position data and
25 recording it to a text file as previously described²⁸.

Data analysis Fly centroid x-y location data were filtered to eliminate tracking failures and dead or missing flies before being used to calculate distances from the arena's center with the Pythagorean equation. Mean distance was preferred over proportion of time spent in centre for
30 its independence from an arbitrary central area and its normal distribution; however time spent in the centre gave identical changes in wall following (data not shown). Linear regressions were performed with the R statistical package and plots were drawn using the ggplot2 library. Coefficients of determination are reported as adjusted R^2 . Location maps were generated with

- 12 -

MATLAB using centroid x-y data. Data were binned into a square matrix with a similar number (156) of cells as pixels in a typical arena-sized portion of the video. This matrix was smoothed with a Gaussian (full width at half maximum = 9 cells) and symmetrized by averaging all four quadrants with each other and their own inverses, to yield a single average quadrant matrix.

- 5 Averaged histogram maps were then normalized such that the sum of all elements equals 1; maps to be compared directly within an experiment were placed on a shared colour scale.

Results

- Placed in an arena, flies stay close to the walls during much of their spontaneous locomotion in a way that is reminiscent of rodent behaviour in open fields¹ (Fig. 1a). We tested pharmacological agents, behavioural interventions and genetic alterations. We fed wild type flies food containing 3 mM diazepam, an anxiolytic GABAA receptor modulator and allowed them to explore a 1 cm square arena. Diazepam, but not solvent, induced flies to decrease their distance from the centre of the arena (Fig. 1b). By contrast, adding 3 mM of the anxiogenic agent sodium lactate to the food increased the time spent edge walking (Fig. 1c). This was a specific effect of lactate as sodium chloride had no effect on thigmotaxis.

- Genetic manipulations of the serotonergic system have profound effects on mammalian anxiety, so we asked whether similar interventions might influence fly wall following. To reduce fly SERT function an RNA interference (RNAi) transgene was targeted at the SerT messenger RNA⁵, in combination with two other transgenes that allow temporal control of neural expression using overnight-elevated temperature⁶. This approach was used to control for strain genetic background and to exclude developmental effects. While subjecting flies to a single night of SerT RNAi induction produced no effect on wall following, three nights at 31 °C significantly increased wall following (Fig. 2a). We controlled for possible effects of warm treatment by testing strains carrying the driver transgenes only, comparing their behaviour with and without 31 °C exposure: this pretreatment alone did not produce an effect on wall following (Fig. 2b). We noted a significant difference between the RNAi carriers and Canton-S F1 non-carriers without any treatment (Fig. 2a, b). Examination of F1 progeny of w¹¹¹⁸ flies from the RNAi stock center and other RNAi F1 animals described below confirmed that wall following varied with the specific genetic background being tested, making it important to compare isogenic (or near-isogenic) strains⁷ (Fig. 4). In contrast to the knockouts, mice expressing elevated levels of SERT display a low anxiety phenotype⁸. Flies subjected to overnight induction of a SerT over-expression transgene⁹ similarly showed a marked decrease in wall following (Fig. 2c).

Drosophila has four serotonin receptors, including several that are similar to mammalian 5-

- 13 -

HT1A¹⁰ and 5-HT2B receptors¹¹. Loss of function in 5-HT1A produces increased anxiety-related behaviour in rodents¹². Targeting a fly 5-HT1A-like gene with RNAi increased wall following behaviour after one night of induction in adult flies (Fig. 2d). While this result is consistent with an overall anxiogenic effect in the loss-of-function mouse, it is distinct in that mouse 5-HT1A must be reduced during development to affect anxiety-like behaviours, although targeting 5-HT1A in adult mice with drugs does impact anxious behaviours¹². Mammals have three distinct 5HT2-type receptors, while fly has a single one that is most similar to 5-HT2B¹¹.

While testing behaviour in 5-HT2B knockout mice has not been possible due to developmental defects, increasing 5-HT2B signalling with the selective agonist BW 723C86 produces anxiolytic effects¹³. Thus reduction of 5-HT2B signalling is anticipated to have an opposite, anxiogenic effect. Indeed, targeting a 5-HT2B-like gene with RNAi produced a significant increase in avoidance of the arena's centre (Fig. 2e). To further assess the role of serotonin in wall following, we selectively inactivated serotonergic transmission with temperature-sensitive dynamin targeted to tryptophan hydroxylase-bearing cells; this produced a marked increase in wall following (Fig. 2f).

Stress in various forms can drive anxiety and several stress-signalling neuropeptide systems influence mammalian anxiety-related behaviours; we speculated whether manipulation of their insect counterparts would have effects on wall following. The tachykinins and their receptors have long been connected to mammalian stress responses and anxiety-like behaviours¹⁴. An RNAi transgene was targeted against *Drosophila* tachykinin¹⁵ expression and produced a significant reduction in wall following (Fig. 3a). Increased corticotropin releasing hormone (CRH) signalling is associated with anxiety disorders¹⁶ and CRH receptor knockout and antagonists have anxiolytic effects¹⁷. The CRH-like peptide in fly is diuretic hormone 44 (DH44)¹⁸, which binds to several receptors including DH44-R1¹⁹. Interestingly, DH44-R1-bearing cells in the central brain have been linked to sexually dimorphic responses to chronic physiological stress²⁰. Overnight expression of an RNAi transgene to target DH44-R1 produced a reduction in wall following (Fig. 3b). Since mammalian tachykinins mediate pain signalling and pain itself is a powerful driver of anxiety and comorbid disorders²¹, we reasoned that pain might also influence fly wall following. Indeed, subjecting flies to painful heat produced a dramatic increase in the distance flies kept from the arena's centre (Fig. 3c). We next attempted to stress the flies with a behavioural intervention. Some stressors, such as separation from parents, cannot be modeled in flies, but we can model restraint stress, where the animal is contained so that movement is restricted or impossible. A large literature attests to the behavioural consequences of physical restraint in rodents^{23,24}. We restrained flies by placing

- 14 -

them in a small space formed between two nested pipette tips for a period of one hour prior to behavioural testing. This arrangement allowed ventilation through the tips, but prevented any substantial locomotion. Flies immobilized in this way showed a significant increase in thigmotaxis in our assay (Fig. 3d).

While the manipulations described above influenced fly wall following in the same direction as anxiety-like behaviour in mammals, we aimed to establish whether this was a false positive result that might have arisen if thigmotaxis-modulating genes constituted a large proportion of the genome. A literature search found that loss-of-function in adenylate cyclase 1 gene has no anxiety-related effects in mammals²⁵. Similarly, loss of function alleles in the adenylate cyclase 1 homologue²⁶ *rutabaga* produced wall following that was indistinguishable from wild type flies (Fig. 5a, b). Additionally, two further RNAi lines chosen *ad hoc* had no impact on thigmotaxis (Fig. 5c, d).

The findings disclosed herein indicate that a process homologous to anxiety exists in flies and presumably therefore in other invertebrates. In three processes known to be crucial to anxiety – the serotonergic, GABAergic and stress signalling systems - every manipulation that we tested the direction the of effect is consistent with results from rodent models of anxiety. We also show that not all manipulations alter fly thigmotaxis – the effects are specific to those predicted from the rodent models.

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CLAIMS

1. A method for screening for an agent having anxiolytic effect, the method comprising the steps:

5 (a) providing a population of insects of the family *Drosophila* in a test area, wherein a test agent has been administered to said population or is being administered to said population;

(b) determining the distribution of the insects in the test area; and

(c) correlating the distribution of the insects in the test area with the effect of the test

10 agent on the said population of insects,

wherein a test agent whose administration results in an increased proportion of the said population being located in open or central regions of the test areas or away from the walls of the test area during the test period or at the end of the test period compared to a control population to whom the test agent has not been administered is indicative of the test compound
15 having an anxiolytic effect.

2. A method of screening for the effect of a test agent on a population of insects, comprising the steps:

20 (a) providing a population of insects in a test area or areas, wherein the test agent has been administered to said population or is being administered to said population;

(b) determining the distribution of the insects in the test area(s); and

(c) correlating the distribution of the insects in the test area(s) with the effect of the test agent on the said population of insects.

25 3. A method for screening for an agent having anxiolytic effect, the method comprising the steps:

(a) providing a population of insects in a test area, wherein a test agent has been administered to said population or is being administered to said population;

(b) determining the distribution of the insects in the test area; and

30 (c) correlating the distribution of the insects in the test area with the effect of the test agent on the said population of insects,

wherein a test agent whose administration results in an increased proportion of the said population being located in open or central regions of the test areas or away from the walls of the test area during the test period or at the end of the test period compared to a control

- 17 -

population to whom the test agent has not been administered is indicative of the test agent having an anxiolytic effect.

4. A method for screening for an agent having antidepressant effect, the method comprising the steps:

- (a) providing a population of insects in a test area or areas, wherein a test agent has been administered to said population or is being administered to said population;
- (b) determining the distribution of the insects in the test area(s); and
- (c) correlating the distribution of the insects in the test area(s) with the effect of the test agent on the said population of insects,

wherein a test agent whose administration results in an increased proportion of the said population being located in open or central regions of the test area(s) or away from the walls of the test area(s) during the test period or at the end of the test period compared to a control population to whom the test agent has not been administered is indicative of the test agent having an antidepressant effect.

5. A method of screening for genetic mutations in a population of insects which affect anxiety-like behaviour, the method comprising the steps:

- (a) providing a genetically non-uniform population of insects in a test area;
- (b) determining the physical distribution of the insects in the test area; and
- (c) correlating the distribution of genetic mutations in the insects in the test area with the physical distribution of the insects.

6. A method of screening for genetic mutations which reduce anxiety or depression in a population of insects, the method comprising the steps:

- (a) providing a genetically non-uniform population of insects in a test area;
- (b) determining the physical distribution of the insects in the test area; and
- (c) correlating the distribution of genetic mutations in the insects in the test area with the physical distribution of the insects,

wherein a genetic mutation which:

- (i) is present in a sub-population of the insects which are predominantly located in open or central regions of the test areas or away from the walls of the test area during the test period or at the end of the test period, and which

- 18 -

(ii) is not present in a sub-population of the insects which are predominantly located away from open or central regions of the test areas or near from the walls of the test area during the test period or at the end of the test period

is indicative of the genetic mutation having an anxiety-reducing or depression-reducing effect.

5

7. A method of screening for genetic mutations in a population of insects which increase anxiety or depression, the method comprising the steps:

(a) providing a genetically non-uniform population of insects in a test area;

(b) determining the physical distribution of the insects in the test area; and

10 (c) correlating the distribution of genetic mutations in the insects in the test area with the physical distribution of the insects,

wherein a genetic mutation which:

(i) is present in a sub-population of the insects which are predominantly located away from open or central regions of the test areas or near from the walls of the test area during the test period or at the end of the test period, and which

15

(ii) is not present in a sub-population of the insects which are predominantly located in open or central regions of the test areas or away from the walls of the test area during the test period or at the end of the test period,

is indicative of the genetic mutation having an anxiety-increasing or depression-increasing effect.

20

8. A method as claimed in any one of claims 2 to 7, wherein the insect is from *Exopterygota* or *Endopterygota*.

9. A method as claimed in any one of claims 2 to 7, wherein the insect is of the order *Diptera*.

25

10. A method as claimed in claim 9, wherein the insect is of the family *Drosophilidae*.

11. A method as claimed in claim 10, wherein the insect is *Drosophila melanogaster*.

30

12. A method as claimed in any one of the preceding claims, wherein the distribution of the insects is determined using a camera and appropriate computer software.

- 19 -

13. A method as claimed in any one of the preceding claims, wherein the distribution of the insects is determined over a set time period.

14. A method as claimed in any one of the preceding claims, wherein the accumulated
5 distributions and/or final distribution of the insects in the test area are analysed at the end of the test period and correlated with the effect of the test substance.

15. A method as claimed in any one of the preceding claims, wherein the overall time spent
10 by the insects in the centre of the test area(s) or the time spent by the insects in open regions or overall mean distances from the centre of the test area(s) is correlated with the effect of the test agent.

16. A method as claimed in any one of the preceding claims, wherein the overall time spent
15 by the population near or away from the walls or near or away from the central region of the test area(s) is determined and compared to a control population to whom the test compound has not been administered.

17. A method as claimed in any one of the preceding claims, wherein the distribution
20 information is pre-filtered in order to eliminate tracking failures or dead insects.

18. A method as claimed in any one of the preceding claims, wherein the method is carried
out at an increased temperature, e.g. 30-40°C, preferably at about 37°C.

19. A method as claimed in any one of the preceding claims, wherein the insects are
25 restrained or immobilised prior to the method.

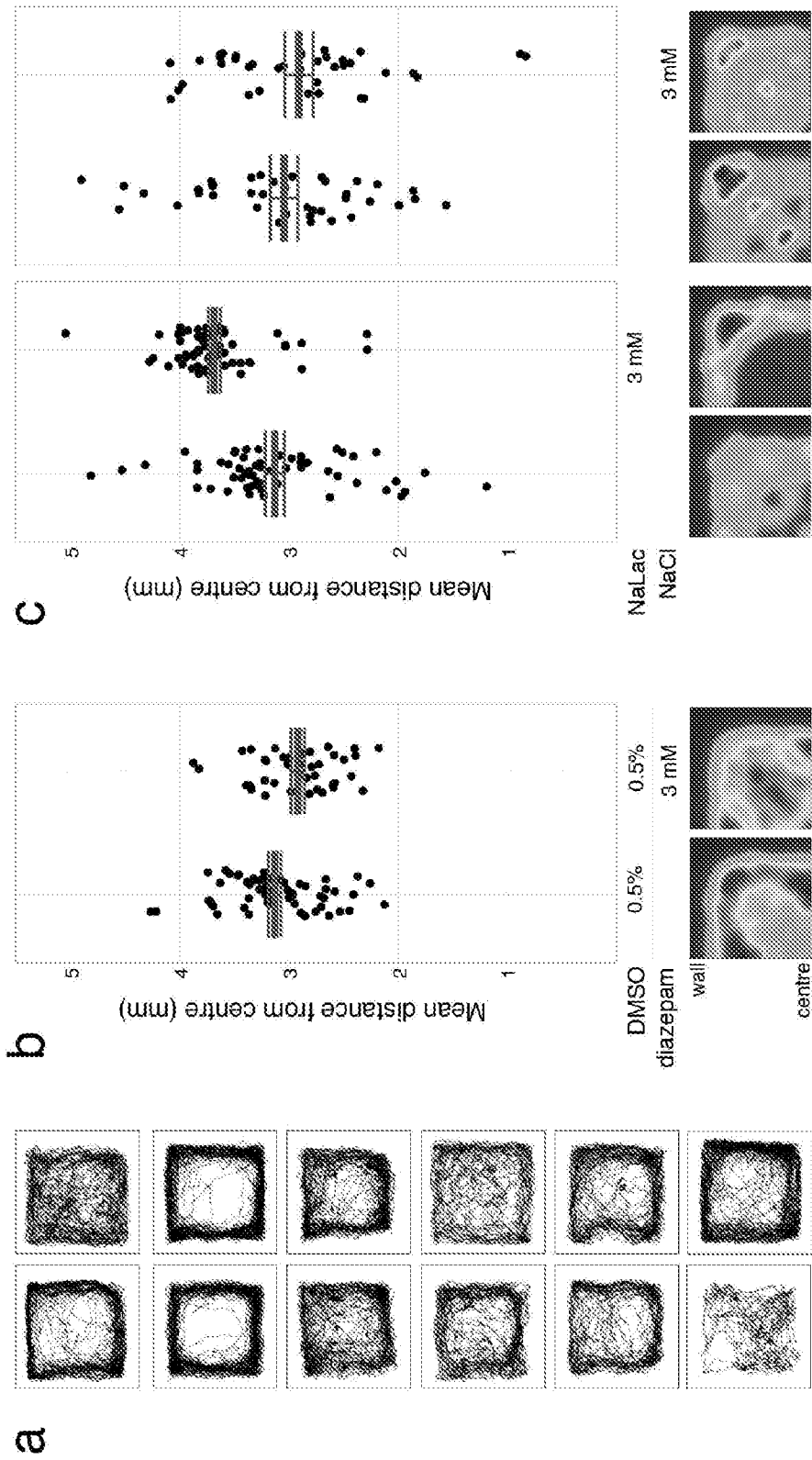
20. A method as claimed in any one of the preceding claims, wherein the insects are
subjected to pain before or during the method.

21. An anxiolytic agent or antidepressant agent which has been found using a screening
30 method of any one of the preceding claims.

22. A method for preparing a pharmaceutical composition, comprising the steps:

- 20 -

- (a) screening for a test agent having an anxiolytic or antidepressant effect using a method as defined in any one of claims 1 to 20, and
- (b) admixing a test agent which has been found to have an anxiolytic or antidepressant effect by such a method with one or more pharmaceutically acceptable carriers, diluents or excipients.



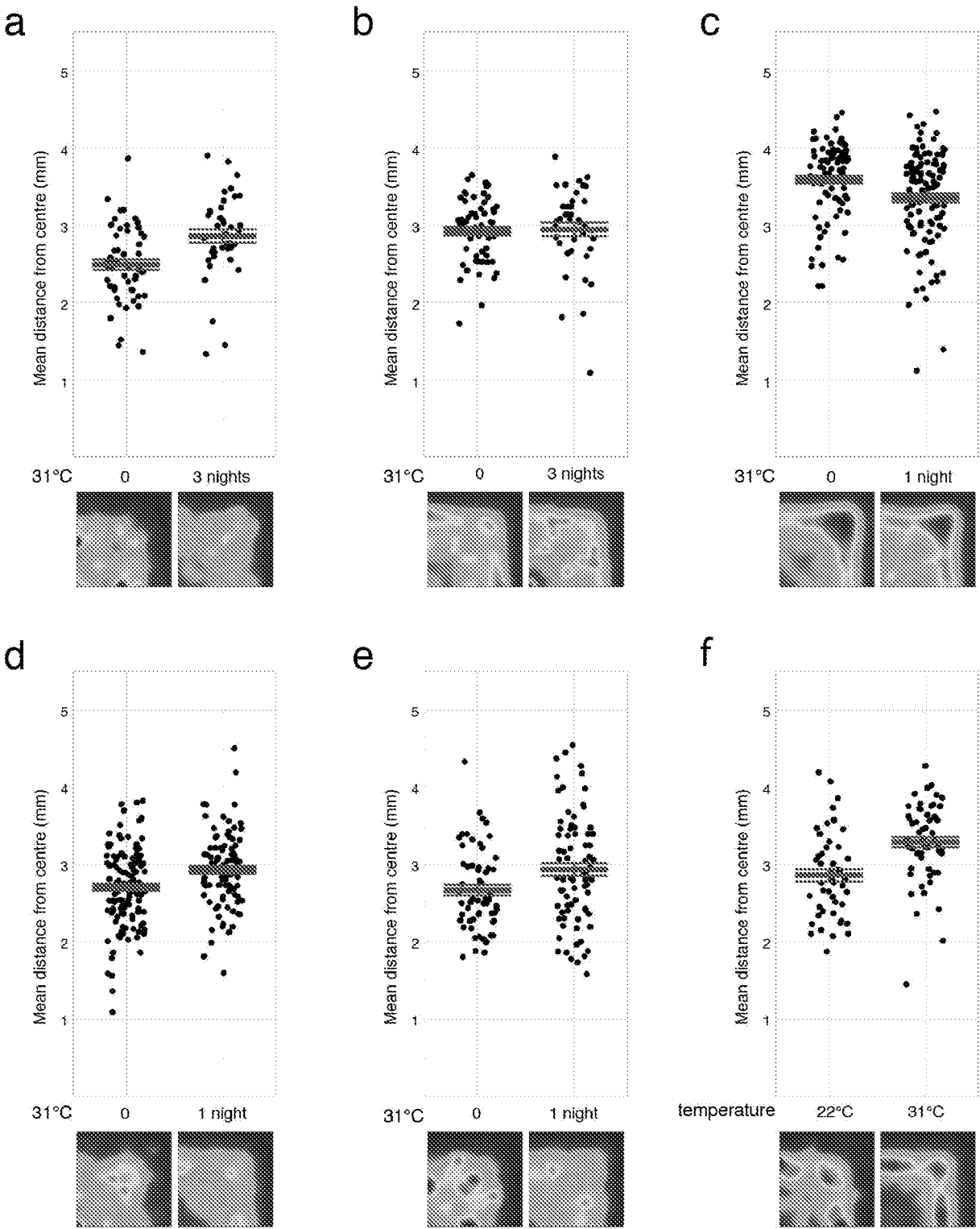


Figure 2

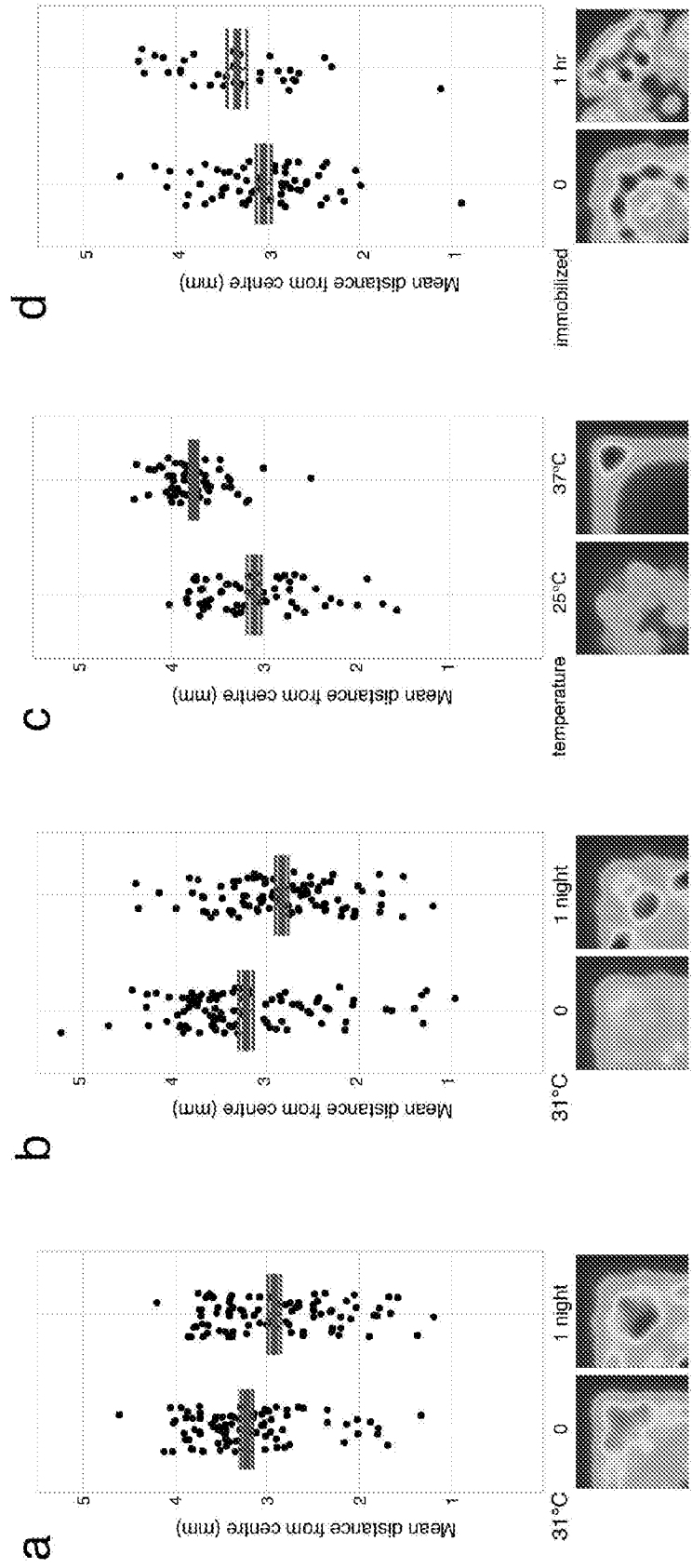


Figure 3

Figure 4

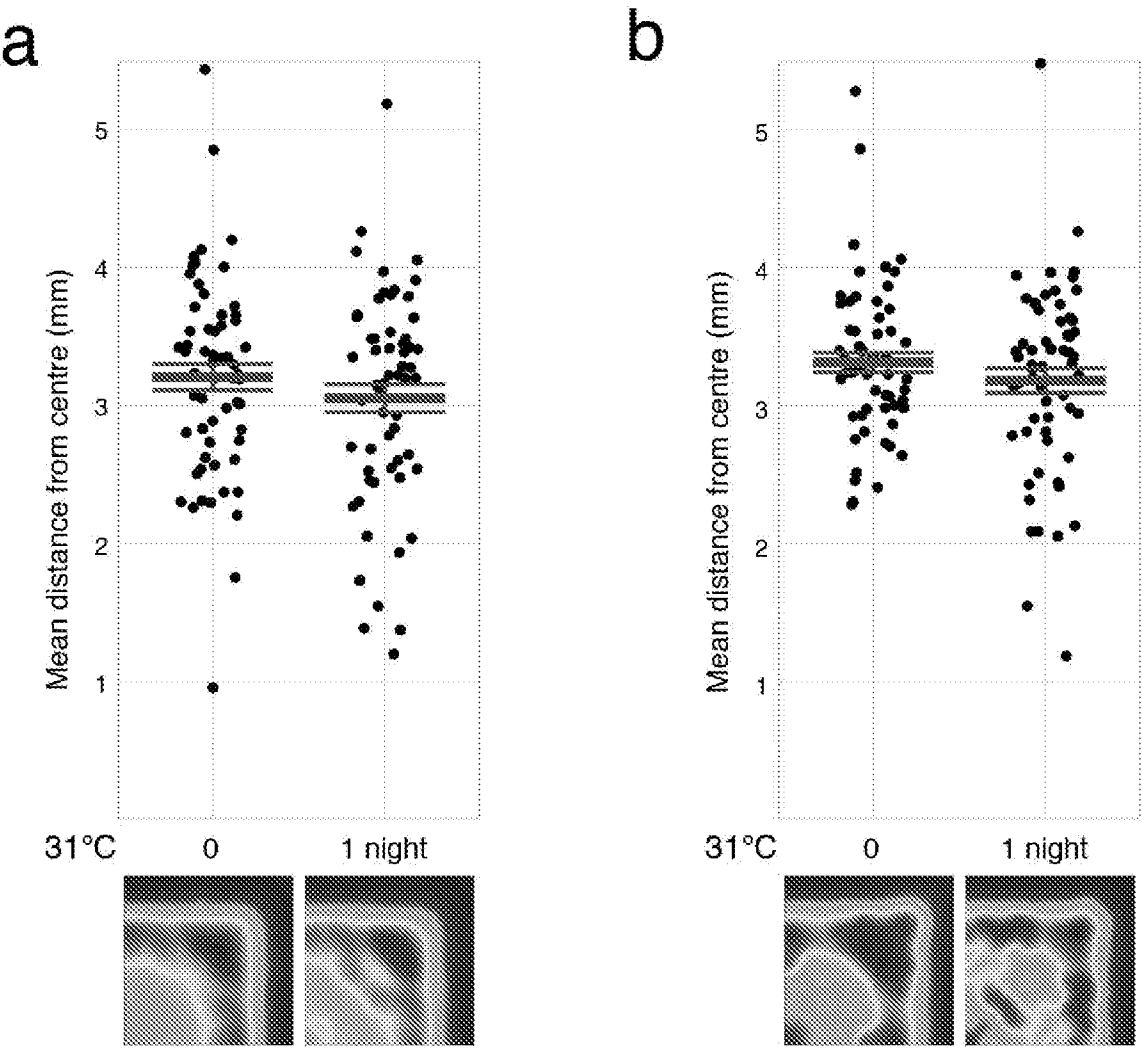
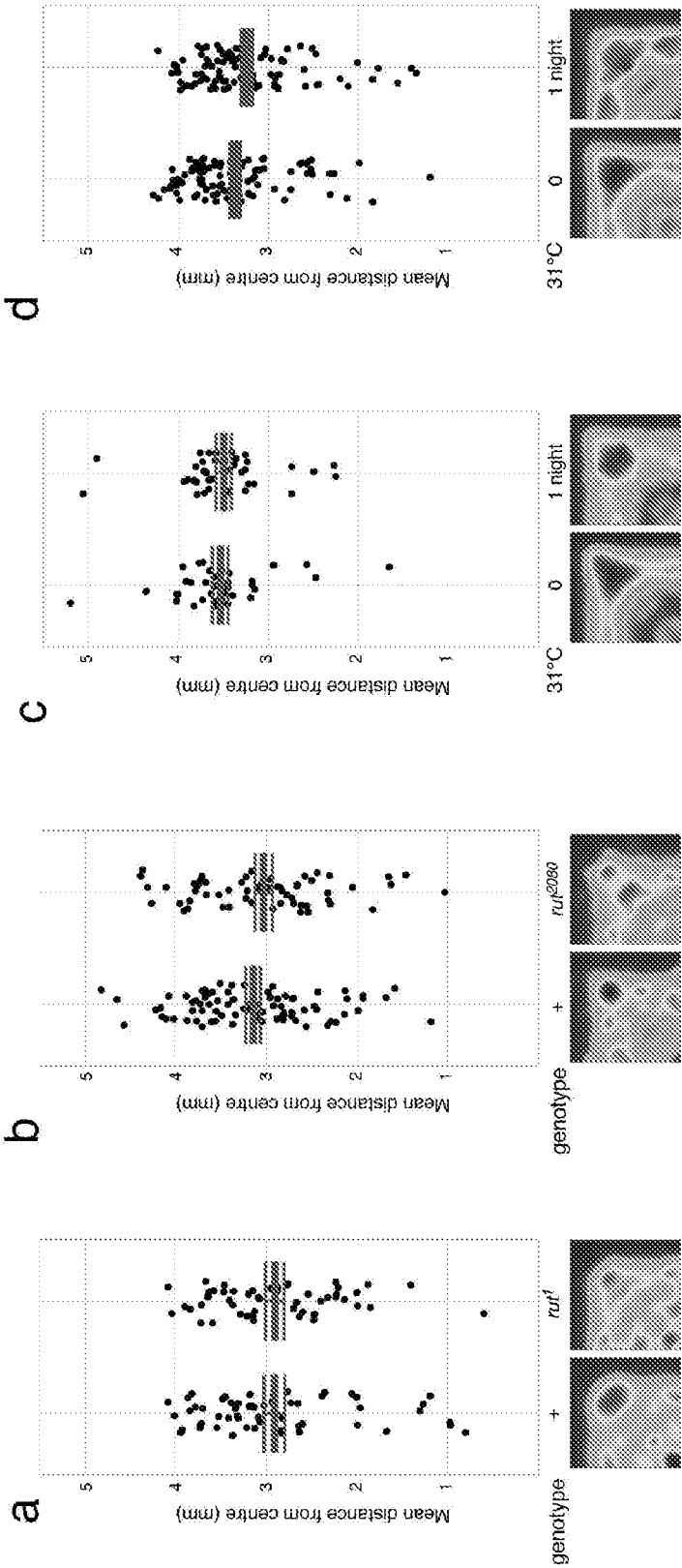


Figure 5



INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2012/051009

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/50

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	L. KAHSAL ET AL: "Neuropeptides in the Drosophila central complex in modulation of locomotor behavior", JOURNAL OF EXPERIMENTAL BIOLOGY, vol. 213, no. 13, 1 July 2010 (2010-07-01) , pages 2256-2265, XP55031901, ISSN: 0022-0949, DOI: 10.1242/jeb.043190	2,8-20
Y	abstract, p. 2257, fig. 2 ----- -/--	1,3,4,22

X

Further documents are listed in the continuation of Box C.

1

See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

9 July 2012

Date of mailing of the international search report

02/08/2012

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INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2012/051009

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEBRETON S ET AL: "Mutations affecting the cAMP transduction pathway disrupt the centrophobism behavior", JOURNAL OF NEUROGENETICS, ELSEVIER, AMSTERDAM, NL, vol. 23, no. 1-2, 1 January 2009 (2009-01-01), pages 225-234, XP009160610, ISSN: 0167-7063	5,8-20
Y	abstract, p. 226-229	6,7,22
X	----- KHAWAJA A M ET AL: "TACHYKININS: RECEPTOR TO EFFECTOR", INTERNATIONAL JOURNAL OF BIOCHEMISTRY AND CELL BIOLOGY, EXETER, GB, vol. 28, no. 7, 1 January 1996 (1996-01-01), pages 721-738, XP000956480, ISSN: 1357-2725, DOI: 10.1016/1357-2725(96)00017-9	21
Y	abstract, p. 732	1,3,4,22
X	----- RONALD S. DUMAN ET AL: "A Molecular and Cellular Theory of Depression", ARCH GEN PSYCHIATRY, vol. 54, 1 July 1997 (1997-07-01), pages 597-606, XP55031905,	21
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Y	----- KERRY J. RESSLER ET AL: "Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor", NATURE, vol. 470, no. 7335, 24 February 2011 (2011-02-24), pages 492-497, XP55031906, ISSN: 0028-0836, DOI: 10.1038/nature09856	6,7,22
	abstract	
