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ORIGINAL PAPER

Effects of a dragonfly (*Anax i.*) homeopathic remedy on learning, memory and cell morphology in mice

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Background: **Homeopathy is a form of alternative medicine in which uses highly diluted preparations that are believed to cause healthy people to exhibit symptoms similar to those exhibited by patients. The aim of this study was to investigate the effects of dragonfly (*Anax imperator, Anax i.*) on learning and memory in naive mice using the Morris water maze (MWM) test; moreover, the effects of dragonfly on MK-801-induced cognitive dysfunction were evaluated.**

Methods: **Male balb-c mice were treated with dragonfly (30C and 200C) or MK-801 (0.2 mg/kg) alone or concurrently (n = 10). Dragonfly (D) and MK-801 were administered subchronically for 6 days intraperitoneally 60 min and 30 min, respectively, before the daily performance of the MWM test.**

Results: **This study revealed that in the familiarization session and first session of the MWM test, *Anax i.* D30 significantly decreased escape latency compared to the control group, although MK-801, D30 and D200 significantly increased escape latency at the end of five acquisition sessions. *Anax i.* combined with dizocilpine maleate (MK-801) also significantly decreased escape latency in the familiarization session and first session of the MWM test, although this combination increased escape latency compared to the MK-801 alone group at the end of the test. Time spent in escape platform's quadrant in the probe trial significantly decreased while mean distance to platform significantly increased in MK-801, D30 and D200 groups. In the MWM test, *Anax i.* combined with MK-801 significantly decreased speed of the animals compared to the MK-801 alone group. General cell morphology was disturbed in the MK-801 group while D30 and D200 seemed to improve cell damage in the MK-801 group.**

Conclusions: **These results suggest that the homeopathic *Anax i.* can impair learning acquisition and reference memory, and it has beneficial effects on disturbed cell morphology.** *Homeopathy* (2016) **105**, 96–101.

Keywords: Homeopathy; Dragonfly (*Anax imperator*); Adipokinetic hormone; Mice

Introduction

Anax imperator, belonging to the order Odonata and the family Aeshnidaean, is an insect commonly known as the Emperor Dragonfly or Blue Emperor. It is a large (averaging 78 mm in length) and powerful species of hawker dragonfly.[1]

The neurosecretory cells in the corpus cardiacum of insects synthesize a set of peptide hormones that are called

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adipokinetic, hypertrehalosaemic or hyperprolinaemic, depending on the type of insect. These neuropeptides act as hormones (neurohormones) and are especially necessary when the oxidative metabolism of insects is high; for example, when flight muscles contract maximally or over long periods. Hence, insects need large amounts of energy that must be mobilized from fat stores in their body.[2–4] Adipokinetic hormones (AKHs) are metabolic neuropeptides mediating mobilization of energy substrates from body fat in many insects. In addition, AKH peptides have excitatory effects on motor neurons, and evidence supports a central role for AKH in locomotion in some insects.[5]

Insect AKHs are a large family of peptide hormones involved in the mobilization of sugar and lipids from the fat body during energy-requiring activities, such as flight and locomotion, but they also contribute to the homeostasis of hemolymph sugar. Interestingly, insect AKH receptors are structurally and evolutionarily related to gonadotropin-releasing hormone receptors in vertebrates.[6]

Using a heterologous (locusts and cockroaches) and a homologous bioassay, the neuropeptide pGlu-Val-Asn-Phe-Ser-Pro-Ser-Trp-NH_2 was isolated from extracts of the corpora cardiaca of the Emperor dragonfly, *A. imperator*. Therefore, it was named Ani-AKH, to denote *A. imperator* AKH.[7] Results from a previous study suggested that AKH may contribute to neuronal function in the human central nervous system.[8]

In this paper, we aimed to study the effect of homeopathic dilutions of *A. imperator (Anax i.)* on learning and memory in naive mice. Homeopathy is a medical theory and practice asserting that disease can be cured by remedies that produce symptoms in a healthy person similar to those suffered by a patient with the malady; the basic principle of homeopathy, known as the law of similars, is "let like be cured by like." The remedies are usually administered in low doses.[9] Dragonfly (*A. imperator*) is a homeopathic remedy that was proposed to be used in attention deficit hyperactivity disorder (ADHD). This condition might be reproduced in rodents by using MK-801 (dizocilpine maleate) a non-competitive NMDA receptor antagonist that causes memory deterioration and hyperlocomotion. The aim of this study was to investigate the effects of dragonfly on learning and memory in naive mice, using the Morris water maze (MWM) test; moreover the effects of dragonfly on MK-801 induced cognitive dysfunction and hyperlocomotion was evaluated. Sections through the hippocampus of the mouse brain were probed with antibodies to evaluate general cell morphology.

Material and methods

Animals

Inbred male BALB/c ByJ mice (Uludag University, Bursa, Turkey) were 7−8 weeks old when they arrived at the laboratory. The mice were kept in the laboratory for two weeks before the onset of the experiments. Mice were maintained under standard laboratory conditions (12-h light: 12-h dark cycle, lights on at 07:00 h, 21 ± 1°C). All animals received food and water ad libitum. All procedures described in this paper were conducted in accordance with the European Community Council's directive for the ethical treatment of animals (86/609/EEC) and with the approval of the Kocaeli University Medical Faculty (7/3/2013).

Experimental groups and drug administration

We directly used dragonfly (*A. imperator*) 30C and 200C liquid homeopathic remedy (25% alcohol) purchased from Helios Homoeopathy, UK, in test groups. We used vehicle (saline with 25% alcohol) in control groups. D30C was the 30 times diluted form of the remedy while D200C was diluted 200 times. Male balb-c mice weighing 30−40 g were treated with dragonfly (30C and 200C) or MK-801 (0.2 mg/kg) alone or concurrently (n = 10). The experimental division of the groups was: 1. Vehicle; 2. D30C; 3. D200C; 4. MK-801 + vehicle; 5. MK-801 + D30C; 6. MK-801 + D200C. *Anax i.* (D) and MK-801 were administered subchronically for 6 days intraperitoneally 60 min and 30 min, respectively, before the daily performance of the MWM test in a volume of 0.05 ml/10 g body weight.

Morris water maze test (MWM)

The MWM was a circular pool (90 cm diameter and 30 cm height) filled with water (22°C) to a depth of 14 cm and rendered opaque by the addition of small black balls. The pool was located in a dimly lit, soundproof test room with a various visual cues, including a white-black colored poster on the wall, a halogen lamp, a camera and the experimenter. The maze was divided into four quadrants, and three equally spaced points served as starting positions around the edge of the pool. The order of the release positions varied systematically throughout the experiment. A circular escape platform (6 cm diameter and 12 cm high) was located in one quadrant 1 cm above the water surface during the familiarization session and 1 cm below the water surface during the other sessions.

Video tracking was conducted with a video camera focused on the full diameter of the pool. Navigation parameters were analyzed by the Ethovision 3.1 video analysis system (Noldus, The Netherlands). The following parameters were evaluated: escape latency (s), which was defined as the time required to find the hidden platform in the acquisition sessions; time spent in the quadrant that included the escape platform (s) in the probe trial; and the mean distance to the platform (cm) in the probe trial. The mice were trained in the MWM for five days (Familiarization session, S1, S2, S3, S4). During the familiarization session and acquisition phase of the experiment, each mouse was given three trials. The delay between the trials was 60 s, and a 1-day interval was used between each session. For each trial, the mouse was taken from the home cage and placed into the water maze at one of three randomly determined locations with its head facing the center of the water maze. After the mouse had found and climbed on to the platform, the trial was stopped, and the

escape latency was recorded. If the mouse did not climb onto the platform in 60 s, the trial was stopped, and the experimenter guided the mouse to the platform; an escape latency of 60 s was then recorded.

Twenty-four hours after the last acquisition session, a 'probe trial' was used to assess the spatial memory retention of the location of the hidden platform. During this trial, the platform was removed from the maze and the mouse was allowed to search the pool for 60 s. The percent of time spent in each quadrant was recorded.

Nissl staining

After 3 weeks, all experimental groups were perfused under ether anesthesia first with phosphate-buffered saline and then with buffered 4% paraformaldehyde. Brains were dissected and postfixed in the same fixative. Following fixation, brain tissues were embedded in paraffin and sectioned at 5 μm. The paraffin sections were assessed *via* Nissl staining.

The paraffin-embedded tissue slices were deparaffinized with xylene. Nissl staining was performed with 0.1% cresyl violet (Sigma) using a standard procedure.[10] Finally, the sections were mounted for analysis. Images of the stained sections were captured with a Leica DFC290 HD color digital camera mounted on a Leica DM1000 microscope with a 40× objective and stored in the Tagged Image File format.[11]

Semi-quantitative analysis of Nissl staining

Staining patterns were examined in three sections of brain tissue from each mouse. Light microscopic analyses were performed by a researcher blinded to the sample grouping.[12,13] The signal strength of the images was graded on a 5-point scale: 0 (no staining), 1 (weak staining, light-blue staining in scattered cells observable using the light microscope), 2 (moderate staining, regionally defined light-blue staining observable with the microscope), 3 (strong staining, regionally defined medium-blue staining, with intensity gradients visible by eye), 4 (very strong staining, dense blue color and intensity gradients, easily visible by eye).[12,13]

Statistics

The results of the MWM test were evaluated by one-way ANOVA followed by Tukey's post-hoc test when significant differences were detected. The data are expressed as the mean values ± SEM. The differences were considered statistically significant when the p value was less than 0.05.

Results

Effects of a dragonfly (*A. imperator*) on learning and memory in naive mice in the MWM test

In the familiarization and first sessions of the MWM test, D30 (115.3 ± 11.36; 102.3 ± 16.08; respectively) ($p < 0.01$; $p < 0.05$) and D200 (120 ± 11.75; 100.8 ± 10.82; respectively) ($p < 0.05$; $p < 0.05$) significantly decreased escape latency compared to the control group (162.7 ± 7.26; 144.9 ± 9.53) [$F(2,29) = 6.39$, $p = 0.005$; $F(2,29) = 4.02$; $p = 0.02$; respectively; Figure 1a] although in the third and fourth sessions D30 (123 ± 12.19; 118.4 ± 16.04) ($p < 0.05$; $p < 0.01$) and D200 (129.6 ± 14.08; 110.5 ± 13.79) ($p < 0.05$; $p < 0.01$) significantly increased escape latency compared to the control group (70.3 ± 13.18; 48.2 ± 8.48; respectively) [$F(2,29) = 6.08$, $p = 0.006$; $F(2,29) = 8.53$; $p = 0.0013$; respectively; Figure 1a]. D30 (15.63 ± 2.03) ($p < 0.001$) and D200 (15.52 ± 1.86) ($p < 0.001$) significantly decreased time spent in the quadrant that included

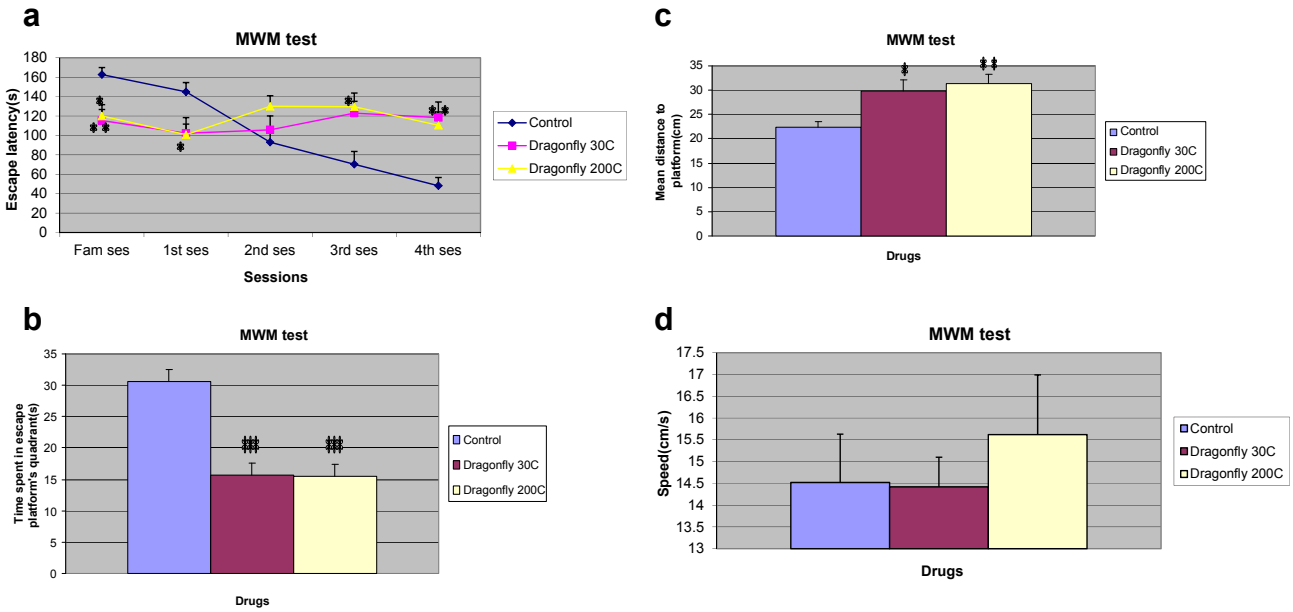

Figure 1 Effects of *Anax i.* 30C and 200C given intraperitoneally for 6 days in the Morris water maze (MWM) test: **(a)** on escape latency. **(b)** On time spent in the quadrant that included the escape platform. **(c)** Mean distance to platform. **(d)** Speed. Data are means ± SEM (n = 10 for each group). *p < 0.05, **p < 0.01, ***p < 0.001 *vs* control group.

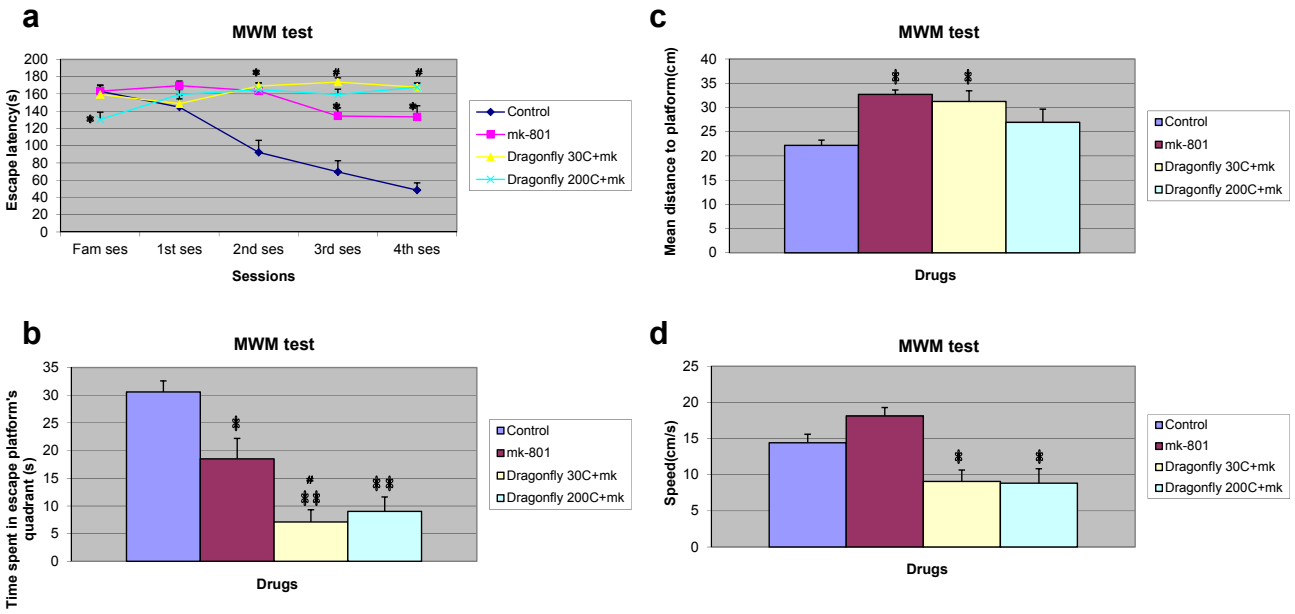

Figure 2 Effects of MK-801 and MK-801 + *Anax i.* 30C and MK-801 + *Anax i.* 200C given intraperitoneally for 6 days in the Morris water maze (MWM) test: **(a)** on escape latency. **(b)** On time spent in the quadrant that included the escape platform. **(c)** Mean distance to platform. **(d)** Speed. Data are means ± SEM (n = 10 for each group). *p < 0.05, **p < 0.01, *vs* control group.

the escape platform compared to controls (30.51 ± 2.02) in the probe trial of MWM test [F(2,29) = 19.01; p < 0.0001; Figure 1b.] while D30 (29.82 ± 2.25) (p < 0.05) and D200 (31.33 ± 1.97) (p < 0.01) significantly increased mean distance to platform compared to control (22.38 ± 1.06) [F(2,29) = 6.81; p = 0.004; Figure 1c]. There was no significant difference between control (14.52 ± 1.11), D30 (14.42 ± 0.68) and D200 (15.62 ± 1.37) groups when the effect of *Anax i.* on speed of animals in naive mice was evaluated in the MWM test [F(2,29) = 0.36; p = 0.69; Figure 1d].

Effects of a dragonfly (*A. imperator*) on learning and memory in MK-801 administered mice in the MWM test

There was a significant difference between groups when escape latency during familiarization, 2nd, 3rd and 4th sessions was evaluated [F(3,39) = 4.63, p = 0.0077; F(3,39) = 18.95, p < 0.0001; F(3,39) = 26.82, p < 0.0001; F(3,39) = 45.28, p < 0.0001; respectively; Figure 2a]. MK-801 (163.4 ± 4.58, 134.4 ± 9.41, 133.4 ± 12.87; respectively) (p < 0.001), MK-801 + D30 (169.1 ± 3.71, 173.7 ± 4.86, 167.8 ± 4.36; respectively) (p < 0.001) and MK-801 + D200 (165 ± 7.72; 159.3 ± 6.15; 167.7 ± 4.95; respectively) (p < 0.001) significantly increased escape latency in the 2nd, 3rd and 4th sessions of the MWM test compared to the control group (92.3 ± 13.85; 69.7 ± 12.86; 48.5 ± 8.38; respectively) (Figure 2a). Escape latency significantly decreased in the MK-801 + D200 (130.3 ± 8.47) (p < 0.05) group compared to the MK-801 alone group (163.2 ± 6.43) in the familiarization session and significantly increased in the MK-801 + D30 (167.8 ± 4.36) (p < 0.05) and MK-801 + D200 (167.7 ± 4.95) (p < 0.05) groups compared to the MK-801 alone group (133.4 ± 12.87) in the 4th session of MWM test (Figure 2a). There was a significant difference between groups when time spent in the quadrant that included the escape platform was evaluated in the probe trial of the MWM test [F(3,39) = 15.67; p < 0.0001, Figure 2b]. MK-801 (18.5 ± 3.69) (p < 0.05), MK-801 + D30 (7.1 ± 2.22) (p < 0.001) and MK-801 + D200 (9 ± 2.62) (p < 0.001) significantly decreased time spent in the quadrant that included escape platform compared to controls (30.59 ± 2) while it significantly decreased in the MK-801 + D30 (p < 0.05) group compared to the MK-801 alone group (Figure 2b). Mean distance to platform significantly increased in the MK-801 (32.71 ± 0.9) (p < 0.01) and MK-801 + D30 (31.24 ± 2.19) (p < 0.01) groups compared to control (22.17 ± 1.08) in the probe trial of the MWM test [F(3,39) = 6.31; p = 0.0015, Figure 2c]. In the probe trial of the MWM test, the speed of the animals significantly diminished in the MK-801 + D30 (9.07 ± 1.57) (p < 0.001) and MK-801 + D200 (8.82 ± 2) (p < 0.001) groups compared to the MK-801 alone group (18.11 ± 1.18) [F(3,39) = 8.66; p = 0.0002, Figure 2d]. The use of dragonfly impaired memory performances both when used alone or in MK-801-treated animals.

Effects of a dragonfly (*A. imperator*) remedy on general cell morphology in the hippocampus of mice

Neuronal injury was quantified by loss of Nissl substance in the CA1 region as described previously. In the control and D30 groups, CA-1 cells showed prominent Nissl substance (4, very strong staining) whereas the CA-1 cells of the MK-801 group showed a lack of Nissl substance (1, weak staining). The CA-1 cells of the D200 and D200 + MK-801 groups showed mild decreases in

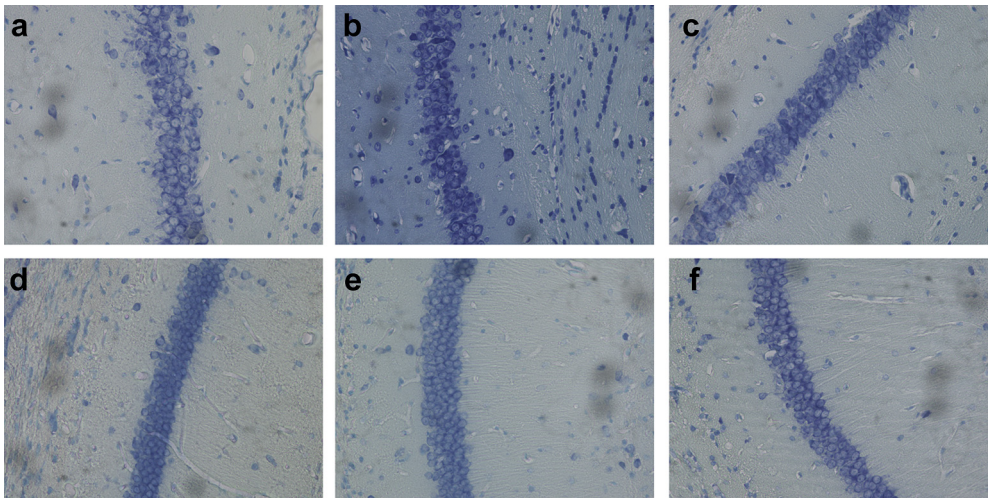

Figure 3 Sections through the hippocampus of the mouse brain were immunohistochemically examined for general cell morphology. Immunoreactivity is detected within the CA1 field of hippocampus. CA1, hippocampal field: pcl, pyramidal cell layer; so, stratum oriens; sr, stratum radiatum. Bar, 25 μm. **(a)** Control. **(b)** *Anax-i* 30C. **(c)** *Anax-i* 200C. **(d)** MK-801. **(e)** *Anax-i* 30C + MK-801. **(f)** *Anax-i* 200C + MK-801.

neuronal Nissl substance (3, strong staining). The CA-1 cells of the D30 + MK-801 group showed severe decreases in neuronal Nissl substance (2, moderate staining) (Figure 3).

Discussion

The effects of a dragonfly (*A. imperator*) remedy on learning and memory in naive mice and MK-801 treated mice were evaluated in this study. In the familiarization and first session of the MWM test, D30 significantly decreased escape latency compared to the control group although MK-801, D30 and D200 significantly increased escape latency at the end of five acquisition sessions. Our results showed that a homeopathic dragonfly remedy increased learning acutely but disturbed learning and memory when administered chronically in the MWM test. General cell morphology was disturbed in the MK-801 group while D30 and D200 seemed to improve cell damage in the MK-801 group. Other studies with different strain of animals, with different behavioral methods and tasks are needed for better understanding the difference between acute and chronic administration. Also different molecular studies are needed to understand the impairing mechanism of dragonfly 30C and 200C on learning and memory and for the results obtained with Nissl.

The order Odonata includes some of the most ancient and beautiful insects ever to roam the Earth, as well as some of the largest flying invertebrates ever to live. Odonata consists of three groups: Anisoptera (which includes dragonflies), Zygoptera (which includes damselflies), and Anisozygoptera (a relict group represented by only two living species). The dragonfly (*A. imperator*) homeopathic remedy is used for ADHD, obsessive-compulsive disorder, headache, sinusitis and influenza.

Energy homeostasis is a fundamental property of animal life, providing a genetically fixed balance between fat storage and mobilization. The importance of body fat regulation is emphasized by dysfunctions resulting in obesity and lipodystrophy in humans. AKHs mobilize lipids, carbohydrates and/or proline from insect fat body stores. In addition, AKHs inhibit lipid and protein synthesis in the fat body. AKH inhibits egg production indirectly by interfering with the formation of energy stores in the fat body that are mobilized to fuel egg production.[14] In a recent study, adipokinetic neuropeptides were identified in the corpora cardiaca of the major families of all three suborders of the Odonata. A sequence assignment revealed that the investigated Odonata species always contains only one adipokinetic peptide, and this is always an octapeptide.[15] Results from a previous study suggested that AKH may contribute to neuronal function in the human central nervous system,[8] which is corroborated in our study. In addition, AKH peptides have excitatory effects on motor neurons, and evidence supports a central role for AKH in locomotion in some insects.[5]

Homeopathy is a practice guided by the theory that disease is cured by remedies that produce, in a healthy person, effects similar to the symptoms that a patient suffers. Homeopathic remedies are usually administered in minute doses.[9] The basic principle of homeopathy, known as the "law of similars", is "let like be cured by like". *Anax i.* (*A. imperator*) has been used in ADHD, obsessive-compulsive disorder, headache, sinusitis and influenza.

In a recent study, Magnani et al.[16] investigated the anxiolytic-like activity of Gelsemium sempervirens in the open field and light−dark tests. The results provided evidence that G. sempervirens acts on the emotional reactivity of mice, and anxiolytic-like effects were apparent. Also, Venard et al.[17] examined brain transmitters with homeopathic treatment. This study was the first basic demonstration of cellular effects of *G. Sempervirens*. In our previous study, we found that *Anax i.* had antidepressant, anxiolytic, and analgesic effects in naive mice and also caused hyperlocomotion and weight loss.[18]

Conclusion

According to our results, we have demonstrated that a chronic treatment with dragonfly impaired learning acquisition and reference memory and, surely, further studies are needed to better understand the differences between an acute or chronic administration of this remedy. In conclusion, the results of our study suggest that there may be potential for further research in the above-mentioned areas for homeopathic dragonfly remedy. Future studies are needed to elucidate the mechanisms of the homeopathic dragonfly remedy.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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