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RESEARCH ARTICLE

Transcranial light affects plasma monoamine levels and expression of brain encephalopsin in the mouse

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ABSTRACT

Encephalopsin (OPN3) belongs to the light-sensitive transmembrane receptor family mainly expressed in the brain and retina. It is believed that light affects mammalian circadian rhythmicity only through the retinohypothalamic tract, which transmits light information to the suprachiasmatic nucleus in the hypothalamus. However, it has been shown that light penetrates the skull. Here, we present the effect of transcranial light treatment on OPN3 expression and monoamine concentrations in mouse brain and other tissues. Mice were randomly assigned to control group, morning-light group and evening-light group, and animals were illuminated transcranially five times a week for 8 min for a total of 4 weeks. The concentrations of OPN3 and monoamines were analysed using western blotting and HPLC, respectively. We report that transcranial light treatment affects OPN3 expression in different brain areas and plasma/adrenal gland monoamine concentrations. In addition, when light was administered at a different time of the day, the response varied in different tissues. These results provide new information on the effects of light on transmitters mediating mammalian rhythmicity.

KEY WORDS: OPN3, Circadian rhythm, Signal transmitter, Hypothalamus, Cerebellum

INTRODUCTION

Encephalopsin (OPN3) is a part of the opsin superfamily. Opsins are light-sensitive heptahelical G-protein-coupled transmembrane receptors, which are mostly expressed in the retina. Opsin function can generally be divided into two parts: light absorption and G-protein activation (Terakita, 2005). Absorption of photons by the retinal-binding pocket of opsin protein causes the photoisomerisation from 11-*cis* to all-*trans* conformation. This change allows G-protein binding and activation of the phototransduction cascade (Bellingham and Foster, 2002). Different wavelengths stimulate different opsins (Kumbalasiri and Provencio, 2005).What is more, in the retina, opsins are located in various tissues and in a variety of cell types (Porter et al., 2012). These proteins are stimulated by light in both visual and non-visual systems, regulating events such as circadian rhythms (Kumbalasiri and Provencio, 2005).

Rhodopsin has been identified in the avian brain (Wada et al., 1998). OPN3 was the first opsin found to be expressed in mammalian brain; OPN3 was highly expressed in the paraventricular nucleus (PVN) of the hypothalamus and in the medial preoptic area, both of which involve encephalic phototransduction in nonmammalian vertebrates (Blackshaw and Snyder, 1999). Apart from these mRNA findings by Blackshaw and Snyder (1999), we have recently found OPN3 existing at the protein level in the paraventricular area (hypothalamus), cerebral cortex and cerebellum in the adult mouse brain (Nissilä et al., 2012).

Light is the key factor in mammalian rhythmicity, because it entrains the circadian system according to the solar day (Bellingham and Foster, 2002). Based on a generally accepted paradigm, in mammals a light signal reaches the hypothalamus via the retinohypothalamic tract (RHT); more specifically, until now it had been thought that non-visual phototransduction would occur only in intrinsically photosensitive ganglion cells (ipRGCs) in the retina (Bailes and Lucas, 2010; Provencio et al., 1998). The suprachiasmatic nucleus (SCN), as a target for the RHT is located in the anterior part of the hypothalamus. The SCN is the circadian pacemaker in higher vertebrates and controls molecular and physiological rhythms, including sleep and alertness, physical activity, hormone levels and body temperature (Ralph et al., 1990; Toh, 2008).

Monoamines are a group of neurotransmitters that are secreted mainly in the brain and adrenal gland. The catecholamines adrenaline, noradrenaline (NA) and dopamine (DA) are synthesized from tyrosine to form DOPA. Serotonin (5-HT) is synthesized from tryptophan to form 5-hydroxytryptophan (5-HTP). After these first steps, both catecholamines and 5-HT are hydroxylated and decarboxylated to form functional molecules (Folk and Long, 1988; Fuller, 1980; Iverson, 1967; Siegel et al., 1999). After their synthesis, monoamines are stored in vesicles, whereas DA is hydroxylated into NA when necessary, and released to the synaptic cleft with calcium-dependent exocytosis when action potential reaches monoamine nerve terminals (Siegel et al., 1999). In the rodent brain, monoamines mediate various central nervous system functions and dysfunctions, including disturbances of the circadian rhythm and several other neurological disorders (Libersat and Pflueger, 2004; Penev et al., 1993). Monoamines are also released from the adrenal gland during stress (Sanchez et al., 2003).

Albeit largely forgotten in the scientific community, it has long been known that a significant amount of light penetrates the skull and reaches the brain in mammals (Brunt et al., 1964; Campbell et al., 2001; Persinger et al., 2013). Studies in rodents show that hooded or enucleated animals conduct light information through extraretinal routes (Wetterberg et al., 1970; Zweig et al., 1966). Similar characteristics of extraretinal photoreception are found in humans. When light is administered transcranially, the functional connectivity in the lateral visual network of the brain increases (Starck et al., 2012). What is more, the circadian system has been shown to be functional, even without external light cues (Rao and Diwan, 1995). We have recently shown that OPN3 protein is widely distributed in the mouse brain (Nissilä et al., 2012) and it has also been shown that brain monoamine production can be enhanced by external stimulus (Hampp et al., 2008; Sudo and Miki, 1995).

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Consequently, in this study, we investigate whether transcranial light affects the expression of brain OPN3 and plasma monoamines in mice.

RESULTS

We illuminated mice under anaesthesia, either just after the beginning of the light period (morning-light group, ML) or just after the beginning of the dark period (evening-light group, EL) for 4 weeks, five times a week and for 8 min per mouse per treatment. Controls were also anaesthesized and light rods were inserted in their ears, but no light was given. The mice were weighed three times a week. After 4 weeks of light treatment, mice were killed and samples of hypothalamus, cerebellum, retina, adrenal glands, plasma and liver were collected. Expression of OPN3 in the hypothalamus and cerebellum was analysed by western blot and the concentrations of monoamines in adrenal gland and plasma were analysed by HPLC.

Body mass and length

The body masses of the animals increased significantly during the study within the groups; no significant differences between groups were seen at the beginning of the study (Table 1). At the end of the study, the mass of mice in the EL group was significantly lower (P<0.05) compared with animals in the control group. The length of the EL group mice was also significantly lower (P<0.05). No changes were seen in BMI between groups at the end of the study.

OPN3 protein abundance in the mouse hypothalamus and cerebellum

Western blotting was used to analyse the abundance of OPN3 protein in the mouse hypothalamus. The antibody detected a single band of approximately 45 kDa, consistent with the predicted protein size for OPN3. In general, significant differences were observed between the control and experimental groups in the OPN3 content of the hypothalamus samples; compared with the control group, the amount of OPN3 was 2.70 times higher in the ML group (P<0.001) and 0.52 times lower in the EL group (P<0.05) (Fig. 1A). In the cerebellum, OPN3 content was 0.14 times (P<0.001) and 0.63 times (P<0.05) lower in the ML and EL groups, respectively (Fig. 1B). There were no differences in OPN3 expression between groups in the retina, although the molecular mass of retinal OPN3 appeared to be larger than in brain samples, which implies that the molecular mass of retinal OPN3 is slightly larger than brain OPN3.

Monoamines in plasma and adrenal gland

The amount of adrenaline, NA, DA and 5-HT was analysed by HPLC in plasma and the adrenal gland of the mice. Cortisol concentrations were measured from serum. Plasma DA concentration was significantly higher in the ML group than in the control group (P<0.01), but no difference was found between the EL group and the control group. There was no detectable DA in the control group plasma. Adrenaline concentration in EL group plasma was 0.44 times lower (P<0.05), but no difference was seen in the ML group compared with controls (Fig. 2A). 5-HT and NA showed no significant differences in either of the test groups compared with controls in plasma samples. In the adrenal gland, NA and DA concentrations were 3.70 times and 4.29 times higher, respectively, in the ML group (P<0.001) and 3.05-fold and 3.48-fold higher, respectively, in the EL group compared with controls. Adrenaline concentration in the ML and in the EL groups was 0.39 times and 0.35 times lower (P<0.001), respectively, compared with controls (Fig. 2B). Cortisol concentrations were similar in all of the experimental groups. To control the effects of anaesthesia, we used mice housed under similar conditions, which were unanaesthesized. As a result, we found that anaesthesia does not have an effect on OPN3 expression, but monoamine concentrations were altered (data not shown). Anaesthesia affected the results, especially in adrenaline samples, where it increased the adrenaline concentration compared with unanaesthesized controls. We believe that this difference is caused by the stress the animals suffered during the treatment.

Table 1. Mouse body mass, length and BMI

	Control	ML	EL
Body mass, start (g)	28.21±0.41	28.25±0.41	27.39±0.42
Body mass, end (g)	31.03±0.29***	30.52±0.37***	29.48±0.54**,#
Length (cm)	9.76±0.08	9.69±0.04	9.49±0.06#
BMI	3.26±0.03	3.25±0.03	3.27±0.04

BMI values are calculated from the mass at the end of the experiment. **P<0.01, ***P<0.001, compared with values before treatment; #P<0.05, compared with control group. Values are means±s.e.

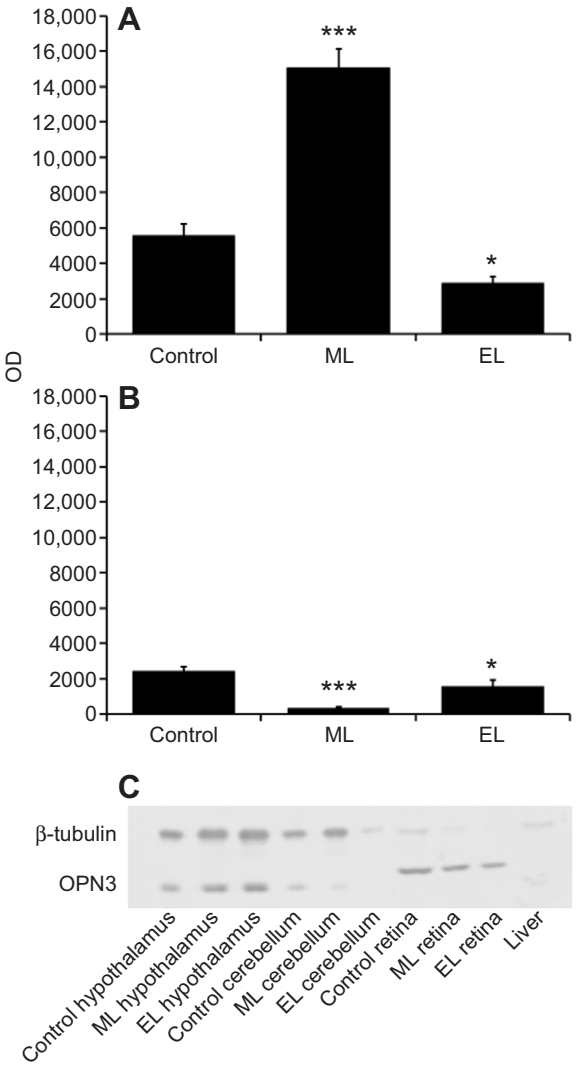

Fig. 1. Expression of OPN3 in the hypothalamus and cerebellum of mouse brain. (A) A bar graph summarizing results from western blot analysis showing mean optical density (OD) in hypothalamus. (N=9–10 samples per group). (B) Bar graph summarizing results from western blot analysis showing mean optical density in cerebellum (N=10 samples per group). ML, morning-light group; EL, evening-light group. Error bars indicate s.e. *P<0.05, ***P<0.001. (C) Representative western blot membrane showing expression of OPN3 with molecular mass of 45 kDa. β-tubulin was used as a loading control.

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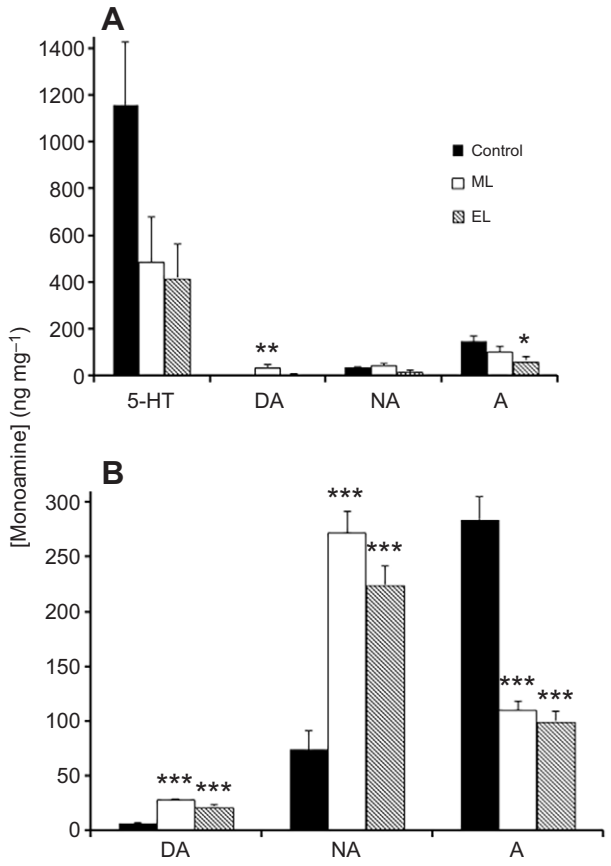

Fig. 2. Monoamine concentrations in mouse plasma and adrenal gland. (A) Bar graph summarizing results from HPLC analysis showing mean concentration of monoamine in plasma (*N*=4–10 samples per group). (B) Bar graph summarizing results from HPLC analysis showing mean concentration of monoamine in adrenal gland (*N*=10 samples per group). ML, morning-light group; EL, evening-light group; 5-HT, serotonin; DA, dopamine; NA, noradrenaline; A, adrenaline. Error bars indicate s.e. **P*<0.05, ***P*<0.05, ****P*<0.001.

DISCUSSION

To the best of our knowledge, our study was the first to investigate whether transcranial light affects the protein expression of brain OPN3. Our data showed that, when compared with controls, the amount of OPN3 differed significantly in transcranially illuminated mice. Moreover, the results were significant, even if the absorption maximum of our light source (approximately 450 nm) and the absorption maximum of OPN3 (approximately 485 or 500 nm depending on the lighting conditions) differed considerably (Koyanagi et al., 2013). Furthermore, as seen in Fig. 3, the absorption maximum of the light source used declines rapidly towards 500 nm. Nevertheless, the production of OPN3 was regulated by transcranial light illumination.

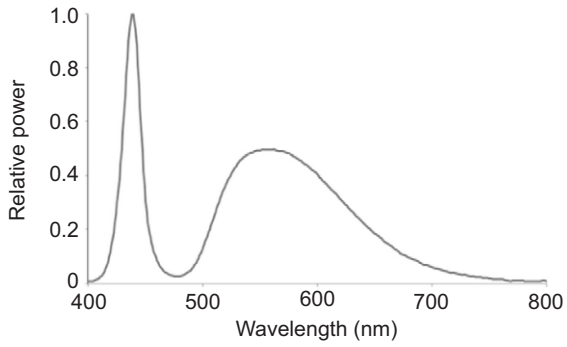

Fig. 3. Spectrum of the light source used for transcranial illumination. Light spectrum of the Valkee NPT 1000 device.

In general, it is well known that opsins are photosensitive compounds (Max et al., 1995; Panda et al., 2003; Ruby et al., 2002) acting as brain photoreceptors in vertebrates (Nakane et al., 2010; Wada et al., 1998). Furthermore, OPN3 is found in the mouse brain both at the mRNA and protein level (Blackshaw and Snyder, 1999; Nissilä et al., 2012), and it could act as signal transmitter for light (Fischer et al., 2013; Koyanagi et al., 2013). We do not know of any previous studies investigating whether transcranially administered light has an effect on the amount of OPN3 in the mammalian brain. Interestingly, when light was administered at a different time of day, the response varied in different parts of the brain.

Based on the findings of our study, it is reasonable to hypothesize that light-activated molecules can also be stimulated transcranially, not only through the retina. Until now, the best known route for light-mediated information is thought to be through the RHT (Bailes and Lucas, 2010; Provencio et al., 1998), where melatonin is the key molecule responsible for entraining circadian rhythms (Cassone, 1990). However, it is possible that there is an alternative target for regulating circadian rhythm, which is, as yet, unknown. The target could be opsins, which have been shown to receive light information in the avian brain (Nakane et al., 2010), and here we find that transcranial light has effects on OPN3 expression in the mammalian brain. Moreover, it is documented that light entrains mammalian rhythmicity (Zubidat et al., 2010), partly by affecting the structure of opsins (Blasic et al., 2012). Variation in OPN3 expression is also dependent on the illuminated tissue and on the timing of light exposure. Since opsins have a long evolutionary history and appear in almost all animal groups (Porter et al., 2012), they might play a similar role to that of the third eye of lizards, possibly partly regulating circadian melatonin production (Eakin, 1973).

Interestingly, the amount of OPN3 in the hypothalamus of transcranially illuminated mice was higher when light exposure occurred in the morning. It is already known that opsins act as deep brain photoreceptors in vertebrates (Nakane et al., 2010) and that light penetrates the skull in mammals (Campbell et al., 2001). It has also been shown that light directly stimulates neurons in the rodent hypothalamus (Lisk and Kannwischer, 1964). Therefore, in our opinion, brain OPN3 expression could also be stimulated directly by transcranial light. It seems, however, that the stimulation has variable effects depending on the time of the light treatment, because the amount of OPN3 was reduced in the EL group compared with the controls. Since melatonin levels are high during the night, and given that melatonin changes neurohypophysial hormone release in the hypothalamus (Yasin et al., 1996), OPN3 expression might also be driven by this melatonin rhythmicity. Melatonin may inhibit the effects of transcranial light and OPN3 production when light illumination occurs late. However, there is no evidence that melatonin is the only molecule entraining the circadian system (Bromundt et al., 2014; Jurvelin et al., 2014; Yamazaki et al., 1999) and therefore there could also be other pathways for OPN3 deactivation. One of these may be γ-aminobutyric acid (GABA), which could be a target molecule to inhibit OPN3 action. Leszkiewicz and Aizenman (2003) found that when light was administered to retinal and cortical cells, the activity levels of $GABA_A$ receptors were enhanced. GABA is an inhibiting molecule, so it may also decrease OPN3 expression. If it is true that GABA has a role in OPN3 deactivation, our results would indicate that the activation of GABA by light and deduction of OPN3 production is dependent on the time the light is given, because the amount of OPN3 increased in the ML group. In this context, we must

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remember that mice are a nocturnal species and that their circadian rhythm is reversed in comparison to diurnal species. Therefore, the effects of rhythm-entraining factors might be reversed when compared with diurnal species.

The time of day when light was administrated also affected OPN3 expression in the cerebellum. We found that transcranial light reduced the amount of OPN3 in both the ML group and EL group. OPN3 has been found in the cerebellum in previous studies at the protein level (Nissilä et al., 2012) but, as in the hypothalamus, it has not been known whether light has an effect on its concentration. Again, GABA could be a potential signalling neurotransmitter, which also alters OPN3 levels in the cerebellum. Wade et al. (1988) showed that light causes a response in the cerebral cortical tissue. They found that, when dissected rat brain was exposed to white light, GABA was released from the cerebral cortex. This could inhibit brain OPN3 production. Furthermore, Calver et al. (2003) imply that $GABA_B$ receptors act through a G-protein-coupled system. If this is true, GABA might compete with opsins for the same binding sites in the G-protein-coupled receptor. Furthermore, the cerebellum is considered to be the centre of motor control (D'Angelo et al., 2011), and the reduced amount of OPN3 in the ML group might indicate that the control of motor coordination is also reduced.

DA and NA concentrations increased and adrenaline concentrations decreased significantly in the plasma and adrenal gland of both test groups. Other metabolites (5-HT, cortisol) studied showed no differences when compared with the control group. According to our results and previous studies (Enrico et al., 1998; Feenstra et al., 2000; Nieoullon and Coquerel, 2003; Tanaka et al., 2000), the increased amount of NA and DA in the ML group may be linked to stress caused by the experiment. Transcranial light treatment caused a delay in the beginning of the resting state of the mice, which may have stressed the animals. However, Gordon and Bains (2005) have been able to enhance NA release by stimulating the hypothalamus, so transcranial light could be a similar enhancing factor stimulating NA release at least. Furthermore, both behaviour and monoamine oxidase A activity are altered by disturbing the SCN, which implies that light may have direct effects on the activity of monoamines by altering the circadian rhythm and mood (Hampp et al., 2008). The findings of Sudo and Miki (1995) emphasize the importance of light. They find that levels of adrenaline in urine were significantly higher during the dark period than during the light period. The results of this study support that finding; the levels of adrenaline were significantly higher in the control group than in the transcranially illuminated test groups. Additionally, although anaesthesia itself increased the concentration of adrenaline in control mice, transcranial illumination reduced adrenaline expression in both test groups. Furthermore, DA and NA transporters are inhibited under isoflurane anaesthesia, leading to lower levels of DA and NA (Shahani et al., 2002). Since all groups were anaesthetized, our results show that light possibly restores DA and NA levels.

Because of our study design, we do not know the causal relationship between monoamine and OPN3 expression. However, in the tiger salamander, endogenous dopamine has been shown to boost opsin expression when light is administered during the daily dark phase (Alfinito and Townes-Anderson, 2001). Furthermore, in the zebrafish, dopamine increased opsin production just after the beginning of the light period (Li et al., 2005). According to the results of this study, light might increase the OPN3 production through dopamine stimulation also in mammals, in addition to the direct effect of transcranial light on OPN3 expression. Similarly, lower adrenaline concentration may reduce the OPN3 production also. However, the causal relationships between catecholamine and OPN3 expression in the context of light exposure remain to be investigated further.

In conclusion, our study is the first to show that transcranial light has a significant effect on OPN3 expression in the mouse brain. We also were able to show that OPN3 expression varies depending on the time of day when the transcranial light was administered. In future, it will be important to study whether different wavelengths and intensities of light, or the length of light treatment, cause different responses in opsin expression. In addition, it would be interesting to investigate whether the increase in the concentration of OPN3 results from increased levels of OPN3 production by OPN3-expressing cells or from an increase in the number of OPN3-expressing cells in the brain. Furthermore, as we investigated the effects of transcranial light in OPN3 expression only at the protein level, it would be interesting to also study changes in the expression of OPN3 mRNA.

MATERIALS AND METHODS

Animals

Thirty adult C3H/HeNHsd *rd/rd* male mice (*Mus musculus*; Harlan Laboratories, Venray, The Netherlands) were used in this study, which was approved by the Finnish national committee for animal experimentation (licence number ESAVI/567/04.10.03/2012). The animals were 8–10 weeks old at the beginning of the study. The mice used in the study were blind. Their rods had degenerated in the 5th week after birth and their cones secondarily, which leads to image-forming blindness as an adult (Cavusoglu et al., 2003).

Experimental procedure

All the mice were kept under a 12 h:12 h light:dark cycle. Mice were randomly assigned to three different groups: a control group, a morning-light group (ML) and an evening-light group (EL). The animals were weighed three times a week. Transcranial light was given via ear canals for 4 weeks, five times a week. Light treatment was given under anaesthesia (isofluran, anaesthesia 3%, maintenance 1.5%) for 8 min per mouse. The ML group was exposed to light just after the beginning of the light period (treatment time 7:30 h–9:30 h) simulating a time when mice are typically inactive, and the EL group was exposed just after the beginning of the dark period (treatment time 19:00 h–21:00 h), simulating a time when mice are typically active (Chang et al., 2002). The EL group was illuminated in a lightless room without disturbing the circadian rhythm. The intensity of the light in the headphones of the light source (Valkee NPT 1000, Valkee Inc., Oulu, Finland) was 2.00×10^{15} photons cm^{-2} s^{-1}. Light spectrum is shown in Fig. 3. A nonmeasurable small fraction of the mechanical heat produced by the light source (totally less than 14.7 mW) conducts into the ear canal and there is no significant heat effect caused by the device. The control group was anaesthetized and headphones were inserted, but no light was given. After four weeks of light treatment, the mice were killed by cervical dislocation, the animals were measured and samples of hypothalamus, cerebellum, liver, retina and plasma were collected between 8:00 h and 14:00 h and stored at −80°C. Body mass index (BMI) was calculated using the general BMI formula: $BMI=mass/length^2$. Masses are reported in kilograms and length in metres.

SDS-PAGE and western blotting

Total protein concentrations of the brain tissue samples were measured according to Bradford (1976). Homogenization buffer (62.5 mmol l^{-1} Tris-HCl, pH 6.8), containing leupeptin (1 µg ml^{-1}), pepstatin A (1 µg ml^{-1}) and PMSF (1 mmol l^{-1}) was added to the samples and homogenized (Qiagen TissueLyser, Retsch, Haan, Germany).

Samples with equal amounts of protein (12.06 µg per lane) were loaded in a separating gel (4–12%, Amersham ECL Gel, GE Healthcare Bio-Sciences, Uppsala, Sweden) and electrophoretically separated at 160 V for 60 min. The separated proteins were transferred to nitrocellulose membrane (0.45 µm, Bio-Rad Laboratories, Hercules, CA, USA) according to the method of Towbin et al. (1979).

After electroblotting, the membranes were blocked with 5% non-fat milk powder in TBS for 1 h at room temperature. After washing (3×5 min) in

TBST (TBS+0.05% Tween 20), the membranes were incubated in primary antibody for OPN3 (45 kDa, anti-encephalopsin antibody ab75285, Abcam, Cambridge, UK). OPN3 antibody was diluted in TBST (1:500) and incubated for 2 h at room temperature. Then the membranes were washed (3×5 min) and incubated with secondary antibody [1:3000, goat anti-rabbit IgG (H+L)-AP-conjugate, Bio-Rad Laboratories] in TBST for 2 h at room temperature. Antibody detection was performed with bromo-4-chloro-3-indolyl phosphate mono-(toluidinium) salt/nitro blue tetrazolium (BCIP/NBT) substrate. β-tubulin (molecular mass 50 kDa) was used as a loading control (anti-β-tubulin antibody ab6046, Abcam). β-tubulin was diluted in TBST-antibody solution (1:1500) and incubated for 2 hours with the primary antibody. Immunoreactive band intensities compared with background were analysed using the VersaDoc imaging system (Bio-Rad). Liver was used as a negative control and retina as a positive control.

Monoamine concentration analysis by HPLC

Monoamine concentrations were determined by high-performance liquid chromatography (HPLC) in plasma and adrenal gland samples, as described by Nieminen et al. (2004). 5-HT samples were analysed using the same technique. Briefly, plasma samples were deproteinized before injection into the HPLC autosampler. The running buffer contained 50 mmol l^{-1} NaH_2PO_4, methanol and aluminium chlorohydrate in a 77:15:8 ratio, respectively (pH 3.22). The running buffer was filtered and 200 mg Na-dodecylsulphate and 77.4 mg Na-EDTA were added to buffer. The total concentration of base solution was 5 µg ml^{-1} in 0.1 $HClO_4$.

Cortisol assay

Cortisol concentration was assayed by ELISA in blood serum samples with a DIAsource kit for human cortisol according to the instructions of the commercial kit (KAPDB270, DIAsource ImmunoAssays SA, Louvain-La-Neuve, Belgium). Calibrators, controls and samples were made as duplicates, and absorbances were read at 450 nm using a microplate reader (SPECTROstar Nano, BMG LAbtech, Ortenberg, Germany). The sensitivity for cortisol was 0.4 µg dl^{-1}, and calibrators were used to validate the method.

Statistics

In this study, all values are expressed as means±s.e. One-way ANOVA was used for comparisons between groups for the amounts of OPN3, cortisol and monoamines. In the case of significant changes, pair-wise comparisons between the test group and control group were made using the Tukey post-test. The length, mass and BMI comparisons between groups were also made with one-way ANOVA and Tukey post-test. Mass changes within groups were determined using the paired *t*-test.

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Competing interests

S.M. and S.S. have no conflicts of interest to declare. J.N. is the founder and a shareholder and M.T. is a minor shareholder of Valkee Ltd. A.F. received a grant from Valkee Ltd for this study.

Author contributions

All authors were responsible for designing the study. A.F. was responsible for conducting the study and for the western blot and HPLC results. A.F., S.M. and S.S. were responsible for analysing the results. All authors were responsible for drafting and revising the article.

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