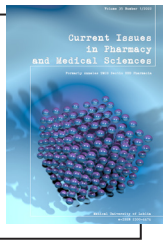
DOI: 10.2478/cipms-2022-0002 Curr. Issues Pharm. Med. Sci., Vol. 35, No. 1, Pages 6-10

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Current Issues in Pharmacy and Medical Sciences

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Effects of linagliptin on morphine dependence in larval zebrafish (*Danio rerio*)

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ARTICLE INFO

Received 7 July 2021
Accepted 30 May 2022

Keywords:
linagliptin,
dipeptydyl peptidase-4 inhibitors,
glucagon-like peptide 1,
addiction, morphine,
zebrafish.

ABSTRACT

Drug addiction is a chronic, recurrent disease of the central nervous system that leads to the development of comorbidities and premature death. Despite extensive scientific research concerning addiction, no effective method of addiction pharmacotherapy has been known so far. Glucagon-like peptide 1 has been suggested to play a role in the rewarding effect of addictive drugs. Linagliptin is a selective dipeptidyl peptidase-4 inhibitor that suppresses the rapid degradation of endogenous glucagon-like peptide-1. In clinical practice, it is used as an antidiabetic drug, but recent studies have confirmed its role in the activity of the central nervous system. This pilot study was conducted to ascertain whether linagliptin might influence morphine dependence – a locomotor activity test was carried out to assess the intensity of morphine withdrawal symptom. The obtained results clearly confirmed that linagliptin (0.01 and 0.1 mM) reduced the locomotor activity in morphine-dependent larval zebrafish. The undertaken experiments clearly indicates that linagliptin is involved in the addictive effects of morphine, thus, further studies on higher organisms should be carried out.

INTRODUCTION

According to the World Health Organization (WHO) guidelines, drug addiction is a chronic, recurrent disease of the central nervous system that leads to the development of comorbidities and premature death. Mental addiction is the compulsion to take the next dose of an addictive substance in order to feel better. Symptoms of mental addiction are long-lasting and persist even several years after the withdrawal of the addictive substance. Physical dependence develops after long-term exposure to addictive substances and is the result of adaptive changes in brain neurons [1-3]. It manifests itself as the withdrawal signs characteristic for a given group of substances, which appear after a person stops taking the addictive substance [2] or after the administration of an opioid receptor antagonist, for example, naloxone. Due to the time of manifestation, morphine withdrawal symptoms can be divided into early, peak and late stages. In humans, the early stage of morphine withdrawal symptoms is characterized by the appearance of lacrimation, rhinitis, sneezing, coughing and yawning. Later on, the symptoms worsen (the peak stage) and are accompanied by pain, chills, gastrointestinal symptoms (diarrhoea, anorexia), photophobia and temperature rise. After a few days, the symptoms of the withdrawal syndrome begin to subside (the late stage). In rodents, withdrawal from the addictive substance manifests itself with jumpings, teeth chattering, wet dog shakes, paw tremors and diarrhoea.

Currently, there is no known ideal animal model that would reflect all aspects of addiction. In behavioural experiments, the state of dependence is often tested by assessing the intensity of withdrawal symptoms or rewarding properties of addictive substances in rodents. Nowadays, there are some new models of addiction. One, namely the zebrafish model, is becoming a popular model species in behavioural neuroscience research. This model allows for drawing quick conclusions with low financial outlays. Furthermore, clear similarities in physiology and metabolism between zebrafish and mammals are observed. However, to date, there has been known no methodology to develop morphine withdrawal symptoms in zebrafish larvae. Nevertheless, the locomotor activity test has already been used to assess the intensity of ethanol- [4] and cocaine-induced [5] withdrawal symptoms in zebrafish. In this paper, the authors present for the first time, a methodology to study the intensity of naloxone-induced withdrawal symptoms in morphine-dependent zebrafish. This has been adapted from the methodology used in rodents. In this experiment, increasing doses of morphine were used to prevent the development of tolerance.

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It has been experimentally confirmed that there are peptides in the digestive tract, called "incretins", which regulate the functioning of the digestive system. Incretins include the glucose-dependent insulinotropic peptide (GIP) and the glucagon-like peptide (GLP-1 peptide). The GLP-1 peptide, formed in the gastrointestinal tract in response to food intake [6], increases the secretion of endogenous insulin, reduces glucagon synthesis and slows down gastric emptying [7]. Currently, drugs that increase the activity of the GLP-1 peptide are used in the treatment of type II diabetes [8]. These are two groups: GLP-1 peptide analogues, and inhibitors of the enzyme dipeptidyl peptidase-4 (DPP-4). The GLP-1 peptide analogues act directly on receptors for the GLP-1 peptide, whereas DPP-4 inhibitors inhibit the activity of the DPP-4 enzyme and, therefore, increase the activity of the endogenous GLP-1 peptide, indirectly. It is now known that receptors for the GLP-1 peptide are located not only in the digestive system, but also in the heart [9], kidneys [10], lungs [11] and the brain [12]. Considering the location of GLP-1 peptide receptors in the mesolimbic system structures, it can be assumed that increasing the activity of these receptors may affect the action of addictive substances. Numerous studies have shown that increasing the GLP-1 peptide activity reduces the rewarding effects of various addictive substances, *i.e.*, cocaine [13,14], amphetamine [13], nicotine [15,16] and alcohol [17,18], in rodents. Taking into account the literature data mentioned above, and being aware that the GLP-1 receptors are expressed in key brain areas controlling the reward and motivated behaviours (including the ventral tegmental area, VTA), innervated by hindbrain GLP-1 neurons [19], it can be assumed that that stimulation of GLP-1 receptors in VTA contributes to the reduction of the rewarding effect of the addictive substance.

Studies have also been carried out concerning the presence of GLP-1 receptors in zebrafish. A receptor with double selectivity for GLP-1 and glucagon [20] was identified and, therefore, the aim of this study was to formulate a withdrawal model based on the locomotor activity of zebrafish and to assess whether GLP-1 receptors are involved in addiction processes.

MATERIALS AND METHODS

Animals and general conditions of experiments

Zebrafish *(Danio rerio)* larvae, obtained from the Centre of Experimental Medicine at the Medical University in Lublin, were used. The fertilized eggs were collected via natural spawning. Embryos were reared under standard light/dark conditions in the embryo medium (the E3 medium) (1.5 mM HEPES, pH 7.1-7.3, 17.4 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$, 0.18 mM $Ca(NO_3)_2$, and 0.6 μM methylene blue) in an incubator at 28.5°C. From the 5th day of their life onwards, the larvae were fed 4 times a day with standard food – rotifers + dry food Gemma Micro 75 (Skretting France, Vervins, France). The research groups consisted of n=20-30 individuals. During the experiments, there were a maximum of 10 animals per well in 6-well plates. The substances were dissolved in the E3 medium and the fish were placed in the solution obtained in this manner for 24 hours (absorbed by the entire body surface). Behavioural experiments were conducted between 8.00 a.m. and 3.00 p.m., maintaining the natural day-night cycle (14h/10h) and at a constant water temperature of 27-28°C, with lighting of 1010±80 lx, oxygenation of 7.20 mg O_2/L and water pH of 7.2. The animals were used only once for the experiments, and after the experiment was completed, the fish were euthanized with a 250 mg/L solution of tricaine mesylate. All experiments were carried out according to the National Institute of Health Guidelines for the care and use of laboratory animals and the European Council Directive of 24 November 1986 regulating the Care and Use of Laboratory Animals (86/609/EEC), and were approved by the Local Ethics Committee (61/2019).

Substances and doses used in the experiment

The following substances were used in the experiments: morphine hydrochloride trihydrate (Cosmetic Pharma, Poland), naloxone hydrochloride dihydrate (Sigma-Aldrich, St. Louis, MO, USA) and linagliptin (Medchem Express, USA). The first two substances were dissolved in the E3 solution, whereas linagliptin was initially dissolved in dimethylsulfoxide (DMSO) and then suspended in the E3 solution. These solutions were freshly prepared on each day of the experiments.

Locomotor activity test

The locomotor activity test is used to assess the development and intensity of physical dependence on various substances, including morphine [21]. The test was performed with the aid of the automatic tracking device (ZebraBox system; Viewpoint, Lyon, France) and total locomotor activity was quantified using ZebraLab software (Viewpoint, Lyon, France). The total distance moved was defined as the distance travelled (in cm) by the fish during a single 10-minute session.

The locomotor activity test was applied for the assessment of morphine withdrawal in zebrafish. A 1-day-old *Danio rerio* larvae were used for this purpose. From day 1 to day 14 of the experiment, the fish were placed in the E3 medium or a morphine solution. The concentration of the morphine solution was 0.01 mM for the first 7 days, and 0.1 mM for the next 7 days. On day 15 of the experiment, the fish were placed in the E3 medium or the 10^{-4}M morphine solution, and then transferred to 24-well plates with a 1 ml of 0.01 mM naloxone solution. Thereafter, the plates were immediately placed in the chamber of an automatic tracking device, and after 5 minutes of habituation, the locomotor activity of the zebrafish was measured for 10 minutes. To investigate the effect of linagliptin (0.01 and 0.1 mM) on the expression of morphine withdrawal symptoms, the animals were placed in the linagliptin solution on day 15 of the experiment for 30 minutes before placing the fish in the naloxone solution. The control animals were placed in the E3 medium.

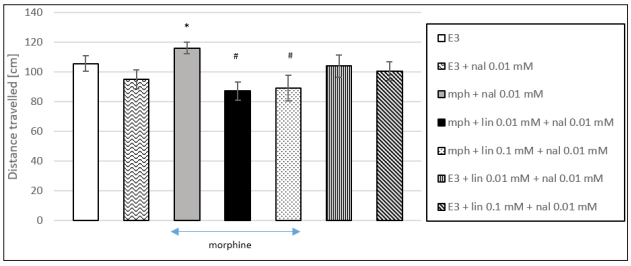
Statistical analysis of the results

The results are presented as the mean ± standard error (± SEM). Statistical analysis was conducted and the figure generated with the aid of GraphPad Prism 8.0.2. One-way analysis of variance (ANOVA) was used to analyze

the results. *Post-hoc* comparisons between the tested groups were made using Tukey's test. The differences were considered statistically significant if p was less than 0.05 ($p < 0.05$).

RESULTS

The conducted experiments showed that a single dose of linagliptin had a statistically significant effect on the locomotor activity of the zebrafish, which were placed under chronic exposure to increasing doses of morphine ($F_{(3,88)} = 4.230$, $p = 0.0077$). Statistical analysis using Tukey's test showed that placing the fish in a naloxone solution induced a statistically significantly increased locomotor activity in animals that had previously been immersed in morphine for a long time, as compared to the fish that had been immersed for a long time in the E3 solution, and then transferred to the naloxone solution ($p < 0.05$). The animals exposed to the linagliptin solution (0.01 and 0.1 mM) before being placed in the naloxone solution showed statistically significantly lower locomotor activity ($p < 0.05$) compared to the group of fish receiving only morphine. Statistical analysis also showed that naloxone alone and linagliptin alone, both at the dose of 0.01 and 0.1 mM, did not statistically significantly affect the mobility of *Danio rerio* compared to the control group (Figure 1).



Experimental 1-day-old *Danio rerio* larvae were immersed in a morphine (mph) solution for 14 days (days 1-7 of the experiment – 0.01 mM solution, days 8-14 of the experiment – 0.1 mM solution), and then transferred to the naloxone (nal) solution (0.01 mM) (mph + nal 0.01 mM group). Subsequently, the locomotor activity was measured for 10 min. Some animals were exposed to the linagliptin (lin) solution (0.01 and 0.1 mM) before being placed in the naloxone solution (mph + lin 0.01 mM + nal 0,01 mM group; mph + lin 0.1 mM + nal 0.01 mM group). The locomotor activity was then measured for 10 min. Data are shown as the means ± SEM and are expressed as the distance travelled [centimetres] by the zebrafish, $n = 20$-30 larvae per group; $*p < 0.05$ *vs.* E3+nal 0,01 mM group; #$p < 0.05$ *vs.* mph + nal 0.01mM group) (Tukey's test)

Figure 1. The effect of linagliptin (lin) (0.01 and 0.1 mM) on the locomotor activity of morphine(mph)-treated zebrafish

DISCUSSION

The role of the GLP-1 peptide in the effect of morphine was investigated by the use of a selective, reversible DPP-4 inhibitor – linagliptin, which increases the level of the endogenous GLP-1 peptide.

While following animal experimental guidelines to limit rodent studies, pilot experiments were conducted using *Danio rerio* to assess morphine withdrawal symptoms. Due to the limited literature data [22,23] concerning behavioural experiments with zebrafish, this experimental procedure of morphine addiction development in zebrafish was developed on the basis of the results of many experiments carried out (unpublished data). A procedure was selected that used the lowest doses of morphine inducing morphine addiction but not being toxic to animals. During the presented experiment, we assessed whether DMSO would influence the behaviour of animals – this effect was ruled out (unpublished data).

The presented studies showed that the GLP-1 peptide is involved in the addictive effect of morphine in *Danio rerio*. In these experiments, linagliptin was used in two concentrations (0.01 and 0.1 mM), which were selected on the basis of preliminary experiments. The maximum concentration of the substance that had no effect on *Danio rerio* motility (unpublished data) and was devoid of toxic effects on the organs (a visual assessment) was first determined. Morphine addiction was induced by placing 1-day-old larvae in a morphine solution for 14 days. Then, the fish were placed in a naloxone environment (0.01 mM). The intensity of morphine withdrawal symptoms was assessed on the basis of changes in the locomotor activity of the fish. An increase in locomotor activity compared to the control group confirmed the development of morphine withdrawal symptoms. In order to determine the role of the GLP-1 peptide, on day 15 of the experiment, the fish were placed in a linagliptin solution (concentration: 0.01 and 0.1 mM) for 30 minutes. Accordingly, the locomotor activity of the zebrafish treated with linagliptin was reduced, compared to the zebrafish showing morphine withdrawal symptoms. The obtained results confirmed the hypothesis concerning the participation of the GLP-1 peptide in the addictive effect of morphine. The obtained results also confirmed the impact of the DPP-4 inhibitor, linagliptin, on the morphine addictive effect. It may be suspected that the effect of linagliptin is exerted via an increase in the GLP-1 peptide activity, but further experiments are needed to confirm this hypothesis.

Literature data also suggest the potential of zebrafish as a model for drug abuse [24,25]. In the literature, zebrafish have been used to analyze the rewarding properties of different drugs being abused, including heroin and morphine [26,27], amphetamine [25,28], cocaine [29] and salvinorin A [30]. These findings are consistent with other literature data confirming that zebrafish dopaminergic projections to the basal forebrain parallel the mammalian mesolimbic system implicated in drug addiction [31]. A group of scientists examining morphine withdrawal symptoms in zebrafish in the novel tank diving test found no significant changes in the behaviour of these animals during morphine withdrawal. However, elevated cortisol levels were observed in the withdrawal group. Increased cortisol levels indicate that zebrafish may have experienced increased anxiety. However, the authors explain the lack of significant behavioural effects by the probability of a common morphine tolerance development as a result of a constant dose of morphine administration for 1 week [22]. In this study, the authors observed significant morphine withdrawal symptoms expressed as increased locomotor activity in zebrafish probably because of the increasing morphine dose administration, due to which the tolerance was balanced.

To date, only one research concerning the involvement of GLP-1 in brain disorders in zebrafish has been published. The GLP-1 analogue, liraglutide, has been shown to be active on the zebrafish brain and may counteract some of the effects induced by stress [32].

Therefore, in the light of previous reports, the presented research results are innovative and indicate a further direction of the research concerning the involvement of the incretin system in physical morphine dependence.

Numerous literature data concerning the involvement of the GLP-1 peptide in the effect of addictive substances, carried out in rodents, are available. They confirmed that injections of the GLP-1 peptide analogue, exendin-4, into the nucleus accumbens and into VTA decreased food intake [19], suggesting that the GLP-1 peptide receptors in the reward system structures mentioned are involved in the action of rewarding addictive substances, including morphine, which exerts a pharmacological effect through the μ opioid receptors located in the mesolimbic system. Recent reports confirm the GLP-1 peptide receptor interaction with the opioid system – exendin-4 has been shown to inhibit the recovery of heroin-[33] and oxycodone-seeking [34] behaviour in rats, and to inhibit the binge-like feeding induced by μ-opioid receptor stimulation of the nucleus accumbens in rats [35].

After analyzing the current scientific reports, it can be assumed that the reduction of morphine-induced withdrawal symptoms by the administration of linagliptin, as observed in this experiment, was probably caused by the direct effect of stimulation of GLP-1 receptors in VTA. The presence of GLP-1 receptors in the structures of the mesolimbic system, including VTA and nucleus accumbens, has already been demonstrated in 1999 [36]. Based on the conducted experiments, it is difficult to determine the detailed mechanisms of interactions taking place in the brain. It can be assumed that this is an effect of the co-localization of receptors for the GLP-1 peptide and opioid receptors in the structures of the mesolimbic system. The presented research gives a specific further direction to scientific experiments on morphine addiction.

In summary, the role of the DPP-4 inhibitor in the addictive effect of morphine was demonstrated for the first time in this study. A selective, long-lasting DPP-4 inhibitor, linagliptin, was used to indirectly stimulate GLP-1 receptors. Linagliptin reduced the locomotor activity of the morphine-dependent *Danio rerio* – the locomotor activity test, according to the literature data [21], can be analyzed in the context of the assessment of morphine addiction. The obtained results suggest the importance of GLP-1 in the addictive effect of morphine.

CONCLUSIONS

It seems that the recently discovered GLP-1 peptide may play a role in the treatment of addictions. Now, it is commonly known that receptors for the GLP-1 peptide are located not only in the gastrointestinal tract, but also in the heart, kidneys and lungs and in the brain. Taking into account the fact that receptors for the GLP-1 peptide are located in the structures of the mesolimbic system, it can be assumed that increasing their activity may affect the addictive substances action. This experiment and the other available published data suggest the involvement of GLP-1 in the rewarding and reinforcing effects of addictive drugs. It can be assumed that drugs affecting the incretin system may be potentially used in the treatment of addiction.

ABBREVIATIONS AND SYMBOLS

DMSO – dimethylsulfoxide
DPP-4 – dipeptidyl peptidase-4 inhibitor
E3 medium – embryo medium
GIP – glucose-dependent insulinotropic peptide
GLP-1 peptide – glucagon-like peptide 1
SEM – standard error of the mean
VTA – ventral tegmental area (VTA)
WHO – World Health Organization

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