

CAN BLOCKING ANGIOTENSIN-CONVERTING ENZYME TYPE 2 IMPAIR BEHAVIOUR AND NOCICEPTION IN HEALTHY MICE?

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Abstract

The renin-angiotensin system (RAS) is an important regulator of functions in various systems, including the brain. A classic pathophysiological consequence of RAS imbalance is hypertension, but the system is also involved in the control of behaviour and the development of neurodegenerative diseases and psychiatric disorders. Angiotensin-converting enzyme type 2 (ACE2) is an important member of the protective arm of the RAS. We hypothesized that ACE2 blockade may alter the systemic balance controlling brain functions. The present study aimed to characterize the effect of systemic acute ACE2 blockade on some behavioural parameters and nociception in mice.

Experiments were performed on male ICR mice treated acutely with the selective ACE2 inhibitor MLN-4760. We used a battery of behavioural tests: the “Elevated Plus Maze” for anxiety behaviour, the “Open Field” for exploratory behaviour, the “Hot Plate”, and formalin tests for thermal and chemical nociception, respectively. The results showed that acute treatment with the ACE2 inhibitor MLN reduces normal anxiety-like behaviour in a novel environment, as expressed by increased time spent in the open arms of the Elevated Plus

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Maze and increased distance in the central area of the Open Field Test. Short-term blockade of ACE2 produced antinociception in the acute thermal test and the inflammatory second phase of the formalin test.

In summary, we can conclude that ACE2 is involved in the regulation of complex brain functions, such as motivational behaviour and pain perception.

Key words: RAS, ACE2, anxiety, nociception, mice

Introduction. Angiotensin-converting enzyme type 2 (ACE2)'s main physiological function is the enzymatic regulation of the balance in the renin-angiotensin system (RAS), by regulating the levels of the biologically active octapeptide angiotensin II (Ang II), converting it into Ang 1-7 [1]. Ang II is the major active peptide in RAS that binds to its receptors AT1 and AT2 and participates in maintaining homeostasis, regulation of the functions of blood vessels, arterial pressure, local blood flow, natriuresis, and trophic responses to various stimuli [2]. The heptapeptide Ang 1-7 binds to the G-protein-coupled receptor, the Mas receptor (MasR), which has effects opposite to those of Ang II, related to vasodilation and the prevention of plastic vascular changes, as well as anxiolytic and neuro-protective effects [3]. In this way, the axis with the beginning of ACE2 appears protective and balances the entire regulatory functions of RAS. ACE2 is widely distributed in all organs and systems. Recent research shows high density and specific enzyme distribution in the rat brain. Its levels are particularly high in the cerebral vasculature, all components of the blood-brain barrier, the olfactory bulb, the hypothalamic nuclei, the dopaminergic areas of the midbrain, and the brain stem nuclei that control respiratory functions [4].

In addition to its regulatory functions, ACE2 was identified as a membrane receptor for the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to enter cells [5, 6]. SARS-CoV-2 has posed a major challenge to humanity and causes concomitant damage to the respiratory, cardiovascular, and nervous systems, which is a prerequisite for some of the late consequences of infection (PASC, post-acute complications of SARS-CoV-2), such as fatigue, shortness of breath, cough, pain, cognitive problems, difficulty concentrating [7]. Restoring impaired homeostasis in patients from vulnerable groups with chronic diseases such as hypertension, diabetes, and cancer has proven difficult and is often accompanied by complications.

Based on literature data, we hypothesized that blocking ACE2, similar to acute viral invasion, would induce adaptive changes in peptides controlled by its enzymatic activity and the regulatory mechanisms mediated by them, which would cause an imbalance in the RAS. The present study aims to characterize the changes induced by selective ACE2 blockade on basic behavioural parameters and pain sensitivity in experimental animals.

Materials and methods. Animals and treatment. The experimental animals were ICR mice weighing 20 g. They were kept under standard conditions: a light regime of 12/12 h (light/dark), a temperature of $21 \pm 1^\circ\text{C}$, 40–50% air

humidity, and ad libitum availability of food and water. Each experiment was carried out with test groups of 6–8 mice. MLN-4760 (an ACE2 inhibitor) dissolved in 1% DMSO was intraperitoneally injected 15 min before each test at a 1 mg/kg body weight dose. All animal experiments were carried out following the Declaration of Helsinki Guiding Principles on Care and Use of Animals, EC Directive 2010/63/EU, and were approved by the Ethics Committee of the Bulgarian Food Safety Agency (No. 389/2023–2029).

“Elevated plus maze” test. The elevated plus maze test was performed via a black-painted wooden apparatus having two closed arms ($25 \times 5 \times 14$ cm) perpendicular to two open arms (25×5 cm) with a central platform (5×5 cm). The apparatus was placed 18 cm above the floor level. At the beginning of the test, each mouse was placed on the central platform facing an open arm. During each five-minute measurement, a video tracking system (SMART PanLab software) detected the distance travelled by the animal and the time spent in each arm [8].

“Open field” test. The test was conducted in a square opaque box ($50 \times 50 \times 60$ cm) equipped with a video tracking system (SMART PanLab software). The square floor area was digitally mapped into a peripheral and central zone. The test begins by placing a mouse in the centre of the box. During the five-minute measurement, the travelled distances through the zones and the time spent in them were detected for each animal [9].

“Hot plate” test. The test was carried out using an apparatus (Ugo Basile) with a metal heating plate surrounded by a transparent cylinder. Each measurement begins after placing the animal on the heated plate (55°C). The nociceptive threshold was determined as a latency time from the initial moment of the thermal nociceptive stimulus to the moment of the animal’s reaction, usually expressed by licking the hind paw or jumping. The test’s cut-off time was 30 s, regarding possible damage to the paws [10].

Formalin test. The test was performed in transparent observation chambers ($15 \times 15 \times 15$ cm) and started immediately after the subcutaneous injection of an irritant ($10\ \mu\text{l}$ of 5% formalin) in the right hind paw. The nociceptive threshold was determined by the time (in seconds) of the pain behaviour (licking the injected paw). The general duration of measurement for each mouse was 40 min, subdivided into two phases: acute phase (0–15 min) and inflammatory phase (20–40 min) [11].

Results. Data analysis showed that acute inhibition of ACE2 produced significant antinociception increasing the thermal nociceptive threshold in the “Hot plate” test ($F_{1,18} = 5.147$, $p = 0.036$), and inhibiting the pain behaviour during the 2nd phase of the Formalin test ($F_{1,16} = 25.866$, $p < 0.001$) (Fig. 1A, B). The injection of MLN-4760 increased the total ambulation ($F_{1,24} = 8.684$, $p = 0.007$; Fig. 2A), the trajectory length ($F_{1,24} = 8.163$, $p = 0.009$; Fig. 2B), and the number of re-entrances in the central area ($F_{1,24} = 5.023$, $p = 0.035$; Fig. 2C) in the “Open field” test. The enzyme inhibition did not influence the proportion of distance but

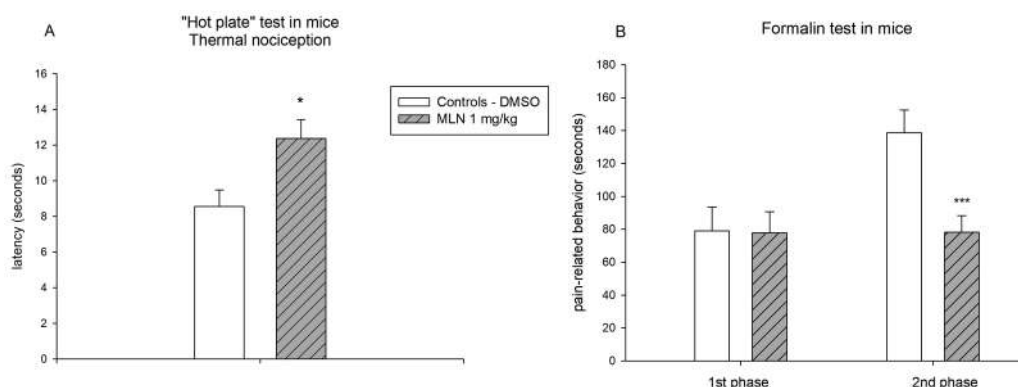


Fig. 1. Effects of a single intraperitoneal (IP) injection of the selective ACE2 inhibitor MLN-4760 (1 mg/kg) on (A) noxious heat threshold (latency to the hind paw licking) measured by "Hot plate" test, and (B) pain-related behaviour (time spent in licking the injected paw) evaluated by Intraplantar-formalin test in ICR mice. Each bar showed mean $\pm$ SEM in seconds ($n = 8$). * $p < 0.05$, and *** $p < 0.001$ compared to vehicle (DMSO) treated mice

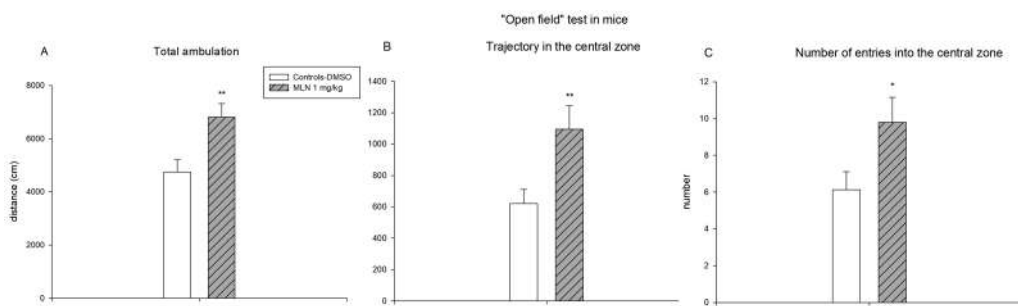


Fig. 2. Effect of a single intraperitoneal (IP) injection of the selective ACE2 inhibitor MLN-4760 (1 mg/kg) on (A) exploratory ambulation (total trajectory length), (B) activity in the aversive central area (trajectory length in the centre), and (C) number of centre entries measured by the "Open field" test in ICR mice. Each bar represents the mean $\pm$ SEM ($n = 8$). * $p < 0.05$ and ** $p < 0.01$ compared to mice treated with vehicle (DMSO)

significantly increased relative time spent in the aversive open arms ($F_{1,18} = 5.297$, $p = 0.034$; Fig. 3A, B) in the "Elevated plus maze" test. ACE2 inhibition did not significantly influence the motor coordination studied in the "Rota-rod" test (mean 204.429 ± 45.278 compared to the controls 237.889 ± 25.330).

Discussion. The present results showed that acute selective inhibition of ACE2 can significantly change mice's normal exploratory and pain behaviour. These changes are associated with increased exploratory behaviour and decreased anxiety-like behaviour in a novel, neutral environment. The results suggest that acute ACE2 inhibition leads to the expression of specific emotion-based behaviour with reduced vigilance upon exposure to an unfamiliar environment. Additionally, the ACE2 blocker elevates the phasic reaction threshold to high temperature-induced pain and reduces pain behaviour induced by a local inflammatory agent.

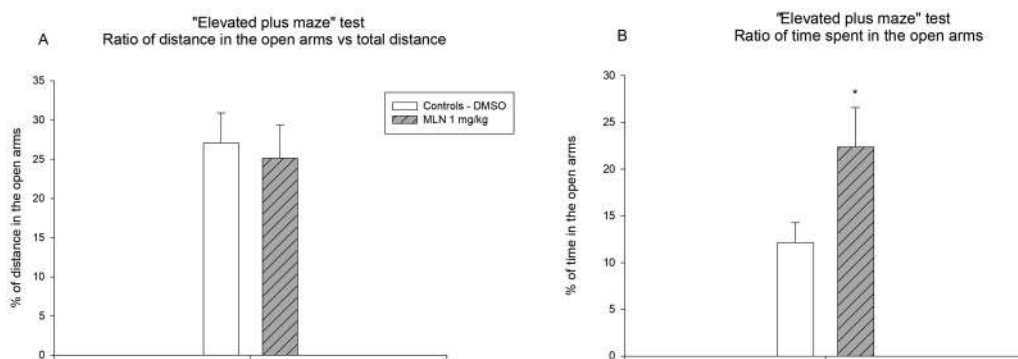


Fig. 3. Effect of a single intraperitoneal (IP) injection of the selective ACE2 inhibitor MLN-4760 (1 mg/kg) on anxiety-like behaviour, expressed as (A) the ratio of the trajectory in the open arms to the length of the total trajectory (in %), (B) the ratio of the time in the open arms to the total test time (in %), studied by the “Elevated plus maze” test in ICR mice. Each bar represents the mean $\pm$ SEM ($n = 8$). * $p < 0.05$ compared to vehicle-treated (DMSO) mice

Although controversial in many aspects, the involvement of angiotensin receptors in the control of emotionally driven behaviour has been established by studying AT1, AT2, and Mas receptors in the amygdala [12,13].

ZHU et al. [14] have shown that local injection of Ang 1-7 into the ventral hippocampus abolished a chronic restraint stress-induced anxiety behaviour by modulating the glutamatergic NMDAR–nNOS–NO pathway and stimulation of serotonergic metabolism. Transgenic rats with overproduction of Ang 1-7, which is equivalent to the long-term consequences of the treatment, show increased activity in the aversive area (open arms of the elevated plus maze). The Mas receptor mechanism of the anxiolytic effects of Ang 1-7 was confirmed using a selective Mas receptor antagonist, which abolished the behavioural changes induced by this genetic manipulation [15]. Chronic MLN-4760 treatment on spontaneously hypertensive rats has not influenced the typical hyperactivity of the rat’s strain [16].

MLN-4760-induced disruption of the Ang II conversion to Ang 1-7 may lead to a dysregulation in the balance of the RAS and overactivation of AT1 and/or AT2 receptors. Like modulation of other physiological functions, the two angiotensin receptors exhibit functional antagonism in regulating emotional behaviour. For example, selective inhibition of AT1 receptors in the hippocampal CA1 has been shown to produce an anxiolytic effect [17], while blocking or ablation of AT2 receptors increases anxiety-like behaviour [12,18]. In addition, ACE2 is involved in the degradation of other peptides, such as dynorphin, which is widely distributed in the central nervous system and has been shown to play a critical role in modulating anxiety-like behaviour [19].

ACE2 mRNA and protein were found in human DRG samples, colocalizing with nociceptor neuronal markers. It is also expressed in the mouse spinal cord, where it is localized on neurons and microglia but not on astrocytes [20]. The

study of the participation of ACE2 in pain modulation is still underdeveloped and restricted to its modulatory effects at the spinal level. Substantial data showed that spinal ACE2 contributes to the suppression of formalin-induced nociception, and the enzyme degradation is involved in the pathogenesis of painful diabetic neuropathy [21].

Ang 1-7 is the major product of ACE2 with a relatively short half-life (≈ 10 s), which limits its therapeutic utility. The ACE2 activity can be maintained pharmacologically, resulting in lower Ang-II levels and providing sustained Ang-(1-7) concentrations. However, the effects of short-term selective inhibition of ACE2 on the processing of nociceptive information have not yet been studied. The present data indicate that short-term systemic ACE2 inhibition can increase the pain threshold to thermal nociception and suppress pain behaviour in the inflammatory phase of the formalin test. The “Hot Plate” test is widely used to assess the analgesic potential of various groups of drugs that have an impact on supraspinal pain control [10].

These preliminary data do not reveal the potential mechanism, but we can speculate that antinociception results from the rapid imbalance in the RAS produced by the inhibition of this key enzyme. We may speculate that a different mechanism of the acute ACE2 inhibition includes the endogenous dynorphin in the regulation of thermal or chemical nociception. Endogenous dynorphin can inhibit the acute nociceptive response by acting on kappa opioid receptors, but this peptide can interact with multiple sites on the NMDA receptor complex, with excitatory responses leading to nociceptive and even toxic effects in pathological conditions such as chronic pain. Although the current data are indicative of a regulatory role for ACE2 in the control of brain functions, further studies are needed to elucidate the specific ligands and receptors involved in the observed disturbances in normal behaviour and nociception.

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