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Research report

Lunasin-induced behavioural effects in mice: Focus on the dopaminergic system

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HIGHLIGHTS

- For the first time central effects of peptide lunasin are studied.
- Lunasin possesses markedly expressed neuroleptic/cataleptic effect in mice.
- Lunasin does not influence ketamine and bicuculline effects in mice.
- Binding assay demonstrates modest affinity of lunasin for the dopamine D1 receptor.
- Lunasin antagonises apomorphine effect on cAMP accumulation.

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ABSTRACT

The present study for the first time is devoted to identify central effects of synthetic lunasin, a 43 amino acid peptide. A markedly expressed neuroleptic/cataleptic effect was observed at low (0.1–10 nmol/mouse) centrally administered doses in male C57Bl/6 mice. Lunasin considerably reduced the amphetamine hyperlocomotion but weakly apomorphine climbing behaviour. No influence on ketamine and bicuculline effects was observed. Binding assay studies demonstrated modest affinity of lunasin for the dopamine D_1 receptor ($K_i = 60 \pm 15$ μM). In a functional assay of cAMP accumulation on live cells lunasin antagonised apomorphine effect on D_1 receptor activation ($pEC_{50} = 6.1 \pm 0.3$), but had no effect in cells expressing D_2 receptors. The obtained data suggest that lunasin's action at least in part is provided via dopaminergic D1 receptor pathways. However, other non-identified mechanisms (probably intracellular) may play an important role in lunasin's central action. Nevertheless further studies of lunasin are promising, particularly taking into account a necessity for novel type of antipsychotic drugs.

1. Introduction

Lunasin is a biologically active 43 amino acid peptide (SKWQHQQDSCRKQKQGVNLTPCEKHIMEKIQGRGDDDDDDDDDD), which was first discovered by Dr. Alfredo Galvez [1,2] by isolating it from soybeans. Afterwards lunasin was found in cereal grains [3–7] and other plants [8,9]. Since its discovery, many health-promoting effects of lunasin and its mechanisms of action have been described. The cancer-preventing activity is explained by lunasin's ability to inhibit histone acetyltransferase [10–13], anti-inflammatory effects by suppression of NF-κB, cyclooxygenase-2, iNOS, PGE2 and interleukine-1β production in macrophages [14,15], cholesterol-lowering properties by inhibition of the expression of HMG-CoA reductase gene and upregulation of the LDL-receptor gene [16] and the immune-modulating action by the activation of NK cells [17]. Lunasin protects DNA from oxidation [18] and increases the glutathione peroxidase activity, and shows 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity [4]. However, despite lunasin's medical importance, its exact three-dimensional structure is not very clearly characterized, though using the method of classical molecular dynamics the conformational profile of three α-helical bundles has been demonstrated [19]. Previously a cell adhesion motif RGD and a carboxyl acid tail of nine aspartic acid residues were identified [11].

Studies in animals [20] and human beings [21] have shown that lunasin administered orally can enter target tissues, even brain tissue [20]. However up to now, we have not found any data about lunasin's influence on the central nervous system functions. Therefore, the present study for the first time is devoted to the examination of the synthetic full sequence (43 amino acids) of

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lunasin on behaviour after its central (intracisternally) administration in mice. Pharmacological properties in vitro (ligand binding and signal transduction modulation) of lunasin on dopamine receptors D_1 and D_2 were also evaluated.

2. Materials and methods

2.1. Animals

Male C57Bl/6 mice were obtained from the Laboratory of Experimental Animals, Riga Stradins University, Riga, Latvia. Animals weighing 22 ± 2 g were housed under standard conditions (21–23 °C, 12-h light–dark cycle) with unlimited access to food and water. Experimental groups consisted of 6–8 mice. All experimental procedures were carried out in accordance with the EU Directive 2010/63/EU and local laws and policies on the protection of animals were used for scientific purposes, and were approved by the Animal Ethics Committee of the Food and Veterinary Service (Riga, Latvia). All efforts were made to minimize animal sufferings and to reduce the number of animals used.

2.2. Materials

Synthetic lunasin was purchased from CASLO Laboratory ApS (Technical University of Denmark, Denmark), reference drugs amphetamine, apomorphine, ketamine and bicuculline from Sigma/Aldrich (St. Louis, USA). [^{3}H]SCH23390 and [^{3}H]raclopride were from PerkinElmer (Massachusetts, USA), as well as all the equipment and materials used for counting radioactivity. The biological activity of lunasin on live cells was assessed using ^{T}Epac^{VV} cAMP biosensor [22]. Cell culture media and supplements were from PAA (Westborough, USA); foetal bovine serum was from Sigma/Aldrich (St. Louis, USA).

2.3. Intracisternal injection

Lunasin was dissolved in sterile water for stock solution, and then diluted for necessary concentrations with the artificial cerebrospinal fluid (aCSF). The peptide was injected into cisterna magna (intracisternally, i.c.) in conscious mice via a J-shape needle connected to a Hamilton syringe as described previously [23,24]. Lunasin was tested at doses 0.1, 1.0 and 10.0 nmol/mouse/5 μl. The mostly used dose was 0.1 nmol/mouse. For the control group, mice received aCSF i.c. (5 μl).

2.4. Open field test

2.4.1. Influence on locomotion

Locomotor behaviour was evaluated in open field (wooden square box 50 cm × 50 cm × 20 cm) using video-tracking programme with software *Panlab Smart Version 2.5*. Lunasin was injected at doses 0.1, 1.0 and 10.0 nmol/mouse/5 μl. i.c. 10 min before testing. Horizontal locomotor activity was quantified as the total distance travelled in centimetres during 5 min testing.

2.4.2. Influence on amphetamine-induced hyperactivity

The influence of lunasin at a dose 0.1 nmol/mouse on hyperlocomotion induced by amphetamine (injected 2.5 mg/kg i.p. 20 min before peptide) was assessed in open field as in Section 2.4.1.

2.4.3. Influence on ketamine-induced locomotion

Ketamine was administered at a dose 15 mg/kg i.p.10 min before i.c. injection of lunasin at dose 0.1 nmol/mouse. Locomotion was registered as in Section 2.4.1.

2.5. Influence on apomorphine-induced climbing behaviour

The experimental procedure was carried out according to that described elsewhere [25,26]. Animals received injection of apomorphine (5 mg/kg s.c.) dissolved in 0.1% sterile ascorbic acid solution, or 0.1% ascorbic acid solution for control, 10 min before i.c. administration of lunasin. After 5 min, the mouse was placed in wire-mesh cage and observed for climbing behaviour at a 5 min interval for 30 min. The climbing behaviour was scored as follows: 0, all four paws on the floor; 1, one paw on the wall of the cage; 2, two paws on the wall of the cage; 3, three paws on the wall of the cage, and 4, four paws on the wall of the cage. Thus, the climbing scores can vary from 0 to 24 for each animal.

2.6. Catalepsy test

The catalepsy test was performed 5, 30 and 60 min after lunasin administration by placing both forepaws of the mouse over a horizontal bar (diameter 0.2 cm), elevated 6 cm from the surface of the desk. The time required for the animal to move all four paws (latency) was scored in seconds [27]. The influence of apomorphine (5 mg/kg s.c. 10 min before lunasin injection) and amphetamine (2.5 mg/kg i.p. 20 min before lunasin injection) on peptide-induced catalepsy was also tested.

2.7. Influence on bicuculline-induced seizures

Seizure threshold was determined according to the method described previously [28,29]. The seizure threshold was determined by intravenous infusion (Syringe Infusion Pump, Model SP100iZ; World Precision Instruments, USA) of bicuculline (dissolved in saline to a final concentration of 2 mg/ml) at a constant rate of 0.5 ml/min and by recording the bicuculline dose that caused the first myoclonic jerk of the head and neck. Bicuculline dose per mg/kg bodyweight in the presence and absence of lunasin was calculated. Lunasin at dose 0.1 nmol/mouse was administered 10 min before bicuculline infusion.

2.8. Radioligand displacement assay

The affinities of lunasin for dopamine receptors were estimated by their ability to displace the specific binding of and [^{3}H]SCH23390 for D_1 and [^{3}H]raclopride for D_2 receptors. The used competitive radioligands [^{3}H]SCH23390 and [^{3}H]raclopride have high affinity for D_1-like (K_d: 0.5 nM) and D_2-like (K_d: 0.7 nM) dopamine receptors, respectively. The experiments were performed on cell membrane suspensions of HEK293 cells stably expressing recombinant dopamine D_1 or D_2 receptors [30].

The cell membrane suspensions were prepared and the competition assays were performed as described previously [31]. For eight-point competition assay, the concentration of radioligand was fixed to near K_d value (1 nM) and concentration of lunasin was varied from 50 μM to 1 nM.

2.9. cAMP biosensor assay

The biological activity of lunasin was tested by its ability to either activate or inhibit cAMP synthesis in HEK293 cells stably expressing recombinant dopamine D_1 or D_2 receptors [30]. For monitoring the changes in cAMP in live cells ^{T}Epac^{VV} biosensor [22] was used. For the expression of ^{T}Epac^{VV} cAMP biosensor in HEK293 cells BacMam system was generated and used and described previously [32].

Cyclic AMP levels in live cells were measured as described by Mazina et al. [32] with minor modifications. Fluorescence from ^{T}Epac^{VV} biosensor was measured using PHERAstar plate reader (BMG Labtech GmbH) at 37 °C. Background fluorescence intensities were detected in non-stimulated cells by excitation at 427(20) nm (mTurquoise excitation) and measuring dual emission of the excited fluorophores at 480(20) nm (mTurquoise emission) and 530(20) nm (Venus emission).

2.10. Statistics

The behavioural data were analysed with GraphPad Prism 5 software (GraphPad Software Inc., CA): one-way ANOVA with Bonferroni's comparison test for selected pairs of columns as post hoc analysis. The results were expressed as mean ± SEM. A significance level was set at $p < 0.05$.

All in vitro pharmacological data were normalized to 100% response values and analyzed by means of non-linear least squares regression analysis using the GraphPad Prism 5. Changes in FRET and the corresponding biological activities of the ligand were calculated as described previously [32] using MATLAB 7.8.0 (R2009a) (MathWorks, Natick, Massachusetts, USA). The results are represented as mean ± SEM of at least two independent experiments carried out in triplicates.

3. Results

In open field test, the influence of lunasin on locomotor activity was observed by video tracking at the doses 0.1, 1.0 and 10 nmol/mouse injected i.c. The peptide caused a considerable decrease in locomotor activity ($F(3,24) = 10.18$, $p < 0.0002$) by reducing the total length of track. The more pronounced effect was observed at the lowest tested dose of 0.1 nmol/mouse (reduction of track length by 75% vs. control, $p < 0.001$); at the higher tested doses of 1.0 and 10.0 nmol/mouse the effect was even less than that of the lowest one (reduction of track length by about 63% vs. control, $p < 0.01$) (Fig. 1).

For further experiments we used the lowest active dose of 0.1 nmol/mouse. This dose significantly (by about 40%) inhibited hyperlocomotion induced by amphetamine ($F(3,23) = 28.7$, $p < 0.0001$) (Fig. 2A), and reduced (by 15%) the apomorphine-induced climbing effect ($F(3,20) = 43.8$, $p < 0.0001$) (Fig. 2B). No significant influences on ketamine-induced locomotor activity were observed (Fig. 3A), however ketamine per se reduced horizontal activity in open field test ($F(3,26) = 12.15$, $p < 0.0001$). Lunasin also did not change bicuculline-induced convulsions (Fig. 3B).

At a dose 0.1 nmol/mouse lunasin caused a pronounced catalepsy ($F(3,20) = 55.59$, $p < 0.0001$) (Fig. 4), which started in 5 min after lunasin injection and lasted for 30 min ($F(3,20) = 67,37$,

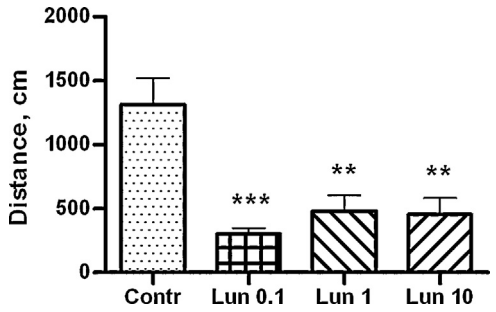

Fig. 1. Influence of lunasin on locomotor activity in open field test in C57Bl/6 mice. Lunasin (Lun) at doses 0.1, 1.0 and 10 nmol/mouse/5 μl or aCSF for control (Contr) was injected i.c. 10 min before testing. Horizontal locomotor activity was quantified as the total distance travelled in centimetres during 5 min testing. Data are expressed as mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. Contr.

$p < 0.0001$) (and even 60 min, not shown). Pre-treatment with apomorphine or amphetamine completely prevented lunasin-induced catalepsy (Fig. 4).

Radioligand displacement assay studies demonstrated modest affinity of lunasin for the D_1 receptor with $K_i = 60 \pm 15$ μM (Fig. 5 circles), however no effect on 1 nM [^{3}H]raclopride binding to D_2 receptors could be detected at peptide concentrations up to 100 μM (data not shown).

In cAMP assay lunasin inhibited the cAMP formation initiated by 10 nM apomorphine in cells expressing dopamine D_1 receptor activation with apparent pEC_{50} value 6.1 ± 0.3 ($n = 5$), whereas D_1-specific antagonist's SCH 39166 had $pEC_{50} = 7.61 \pm 0.03$ ($n = 5$) in this system. No effect on cAMP accumulation (neither agonistic nor antagonistic) was evident in cells expressing D_2 receptors at lunasin concentrations up to 100 μM.

4. Discussion

The ability of lunasin, a 43-amino acid natural peptide, to penetrate the blood–brain barrier [20] evokes our interest to assess its influence on the functions of the central nervous system. For the first time, we have found that the synthetic lunasin induced very clearly expressed motionless and catalepsy, even at small doses, such as 0.1 nmol/mouse. Taking into account the well-established phenomenon of catalepsy, which is provided mostly via dopamine receptor antagonism, first of all we studied the interactions of lunasin with drugs related to dopaminergic system. When we pre-treated mice with apomorphine, a dopamine receptor agonist, which activates both D_1 and D_2 subtypes of dopamine receptors, and which causes a considerable climbing behaviour, surprisingly we found only 15% reduction of climbing activity when lunasin was injected.

The binding of lunasin to dopamine D_1 and D_2 receptors assessed in a radioligand displacement assay on HEK293 cell membranes showed a significant binding to D_1 receptors ($K_i = 60 \pm 15$ μM), but no binding to D_2 receptors.

The cAMP assay in live HEK293 cells expressing D_2 receptors showed neither agonistic nor antagonistic properties of lunasin, while in cells expressing D_1 receptors it inhibited the activation of 10 nM apomorphine with average efficacy $EC_{50} = 0.8$ μM.

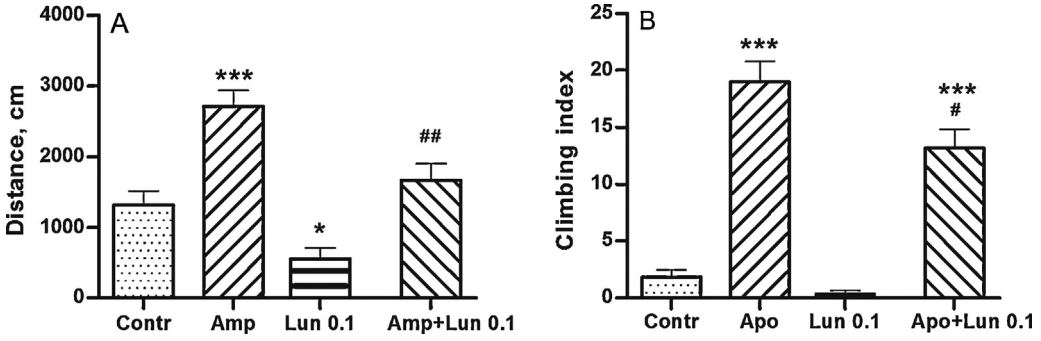

Fig. 2. Influence of lunasin on amphetamine-induced hyperactivity (A) and apomorphine-induced climbing behaviour (B) in C57Bl/6 mice. Amphetamine (Amp) 2.5 mg/kg i.p. administered 20 min before lunasin at dose 0.1 nmol/mouse/5 μl (Lun 0.1) or aCSF (Contr) for control i.c. injection. Locomotor activity in open field test was assessed 10 min after i.c. injection and scored as the total distance travelled in centimetres during 5 min testing.
Apomorphine (Apo) 5 mg/kg s.c. administered 10 min before Lun 0.1 or aCSF for control i.c. injection. The climbing behaviour was observed starting 5 min after the i.c. injection, and scored at 5 min interval for 30 min. Data are expressed as mean ± SEM. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. Contr; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. Amp (A) or Apo (B).

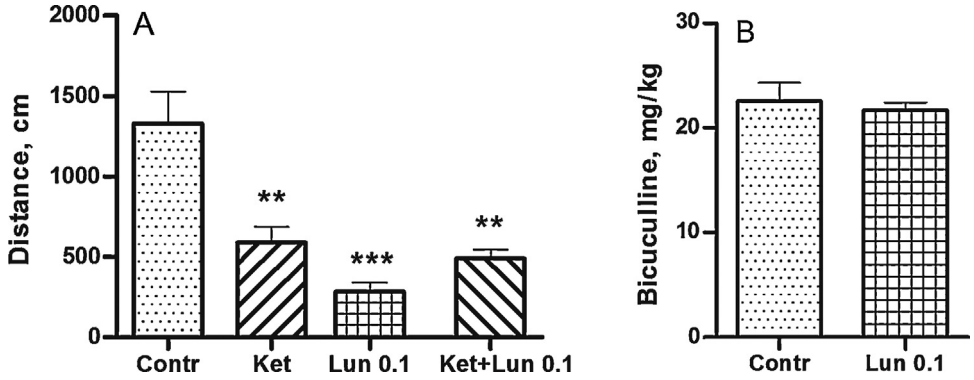

Fig. 3. Influence of lunasin on ketamine-induced locomotion in open field test (A) and bicuculline-induced seizures (B) in C57Bl/6 mice. Ketamine (Ket) was administered 15 mg/kg i.p. 10 min before lunasin 0.1 nmol/mouse/5 μl) (Lun 0.1) or aCSF (Contr) for control i.c. injection. Locomotor activity in open field test was assessed 10 min after i.c. injection and scored as the total distance travelled in centimetres during 5 min testing. Lun 0.1 or aCSF was administered i.c. 10 min before bicuculline 2 mg/ml i.v. infusion. Seizure threshold was calculated as bicuculline dose per mg/kg bodyweight. Data are expressed as mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. Contr.

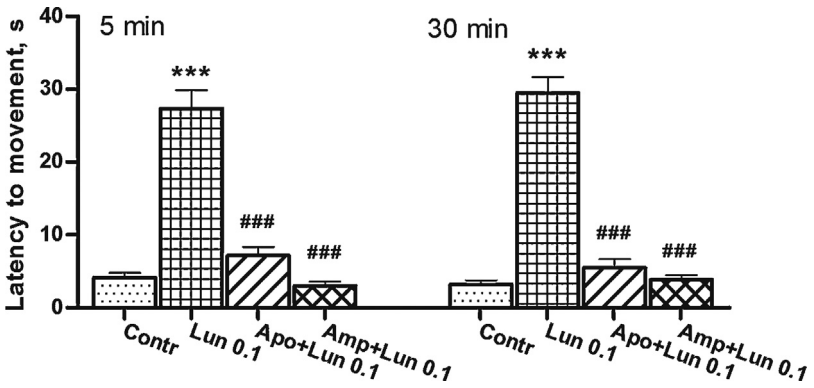

Fig. 4. Influence of amphetamine and apomorphine on lunasin-induced catalepsy, which was assessed 5 and 30 min after peptide i.c. injection in C57Bl/6 mice. Apomorphine (Apo) 5 mg/kg s.c. was injected 10 min before lunasin (Lun 0.1 nmol/mouse/5 μl) and amphetamine (Amp) 2.5 mg/kg i.p. 20 min before Lun 0.1 or aCSF for control i.c. injection. The time required for the animal to move all four paws was scored in seconds (latency). Data are expressed as mean ± SEM. $^{***}p < 0.001$ vs. Contr; $^{###}p < 0.001$ vs. Lun 0.1.

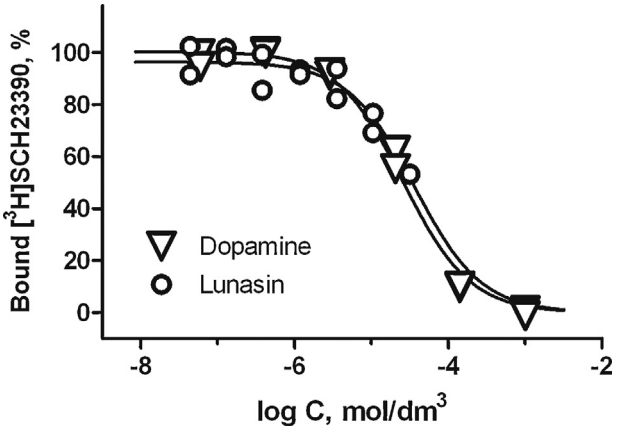

Fig. 5. Displacement of [3H]SCH23390 by dopamine and lunasin at dopamine D1 receptors in HEK293 cell membranes. Data on graph are from a single representative experiment. The affinity constant values from two independent experiments for dopamine: $Ki = 36 \pm 7$ μM and lunasin $Ki = 60 \pm 15$ μM.

The obtained data allow us to suggest that D_1 receptors rather than D_2 receptors play an essential role in lunasin's central effects. However, the dopaminergic mechanism of lunasin cannot be attributed only to the D_1 receptor-mediated action, because in catalepsy test, both apomorphine (receptor agonist) and amphetamine (dopamine releaser) completely prevented lunasin-induced catalepsy. Moreover, lunasin caused a pronounced (by 40%) inhibition of amphetamine locomotion, indicating that intracellular dopaminergic processes affected by lunasin may be involved in its central effects. In this context, the possibility of lunasin to be internalised in the cell via the RGD cell adhesion motif and C-terminal nine aspartic acid tail [33] is not to be ruled out.

The present data demonstrated that lunasin did not significantly influence the locomotor activity induced by ketamine, which is classified as an NMDA receptor antagonist, and did not change the dose of bicuculline (a GABA-A receptor GABA site ligand) to induce seizures. Therefore these findings indicate that glutamate and GABAergic systems do not play an essential role in lunasin's central effects.

5. Conclusions

The obtained results are intriguing because for the first time they demonstrated clearly expressed motionless and cataleptic action of the peptide lunasin that at least in part is provided via dopaminergic pathways, however other non-identified mechanisms may play an important role in lunasin's central action. Nevertheless further investigation of this peptide is promising particularly taking into account a necessity for novel type of antipsychotic drugs.

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