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Scientific Research

Effects of the Novel Anti-Asthenic Drug Ladasten on Behavior and T-Cell Subsets Alterations in a Social Defeat Animal Model of Depression

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ABSTRACT

The aim of the present study was to investigate the effects of the anti-asthenic drug ladasten on behavioral patterns and T-cell subsets in blood, spleen, and thymus in socially stressed male C57Bl/6 mice. Mice subjected to social defeat stress (SDS) for 25 days developed a depressive-like phenotype. The submissive SDS animals were assigned to one of two treatment groups: one group was treated with ladasten (30 mg/kg, i.p.) for up to 5 days, and the other one was administered vehicle as a control. Twenty four hours after the last injection, behavioral parameters were tested, and trunk blood and tissue samples were collected. SDS mice from the vehicle-treated group showed a subordinate and passive avoidance behavior with significantly decreased spontaneous locomotor activity (SLA) and exhibited impaired parameters in the forced swimming test (FST). Changes in behavioral status were correlated with an increase spleen weight, a decrease in thymic index and a shift in the CD4/CD8 balance toward T-cytotoxic cells. The behavior parameters were reversed in the group treated with ladasten compared to the untreated SDS group and were similar to those of unstressed mice. Treatment of socially stressed mice with ladasten normalized the amount of T-lymphocyte cells in the blood, spleen, and thymus. These findings support the notion that depression is accompanied by cell-mediated immune activation and that targeting this pathway may be a new therapeutic approach for treatment. Furthermore, our data support further investigations of ladasten as a potent anti-depressive drug which can be used alone as well as in combination with other anti-depressants.

KEYWORDS

Ladasten; Social Defeat Stress; Depression; Cytokines; T-Cells

1. Introduction

An enlarging body of evidence suggests the presence of interactions among the immune system, the central nervous system and the endocrine system, where these systems can be influenced by physiological and social factors [1]. Based on evidence, including the findings of clinical depression studies and animal models, the new "5-HT" (serotonin) hypothesis of depression, which states that the T cell-mediated immune (CMI) activation contributes to depressive symptoms and neurodegenerative process, was formulated [2-4]. The long-term activation of CMI not only contributes to depressive symptoms but also is accompanied by a Th-1-like shift away from Th-2 and Th-3-like cells, leading to the development of immune deficiency [2,5]. Some authors consider the lack of full recovery of immune reactivity in the treatment of depression with conventional drugs to be an important predisposing factor for the recurrence of depressive episodes [6,7].

On the other hand in recent decades it was postulated

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that dopamine might play a role in depression and the action of antidepressants. In particular, it was postulated that depression was due to an imbalance and more specifically to decrease in extracellular dopamine levels [8].

Based on this hypothesis, it has been suggested that the clinical efficacy of anti-depressants may be augmented by co-administration of immunomodulatory and dopaminergic agents [4,7,8]. We suggest considering the anti-asthenic drug ladasten as a new drug candidate. Clinical investigations have confirmed that ladasten improves psychological parameters of attention, success of operational performance, and sensomotor capabilities [9-11]. In compliance with ICD-10, ladasten was found to be effective in the treatment of patients with Neurasthenia F48.0, a psychogenic disorder [12]. The drug was shown to possess psychotropic, immunomodulatory, and anti-inflammatory properties as well as anxiolytic activities [11, 13-15].

It is known that the dopaminergic and serotonergic systems play an important role in the effects of ladasten [16,17]. Ladasten leads to a rapid and long-lasting increase in tyrosine hydroxylase (TH) mRNA expression in the ventral tegmental area (VTA) and TH mRNA and protein in hypothalamus. The increase in the levels of tyrosine hydroxylase mRNA in the VTA correlates well with the ladasten-mediated increase of L-DOPA and dopamine (DA) content in the nucleus accumbens. This *de novo* synthesis of catecholamines is regarded as the key mechanism of the psychotropic activity of ladasten [16,18,19]. Early studies demonstrated that ladasten exerts central dopaminomimetic effects which were related to DA release from the pre-synaptic endings of free-moving rats [20,21]. *In vitro* experiments have shown that 50 - 500 μkM ladasten significantly decreases the uptake of serotonin by the isolated cerebral synaptosomes of rats and, to a lesser degree, inhibits the uptake of dopamine [22].

Thus, the investigation of the effects of ladasten in an animal depression model is a topical problem.

Social defeat is an ethologically relevant stressor that utilizes the natural establishment of social rank in male rodents [23]. The social defeat stress is a model that is often used to evaluate anti-depressant drug activity in laboratory animals [24-26]. Chronic stress is induced by social dominance of an aggressor over an intruder mouse; the intruder is the subject under study [27-29]. Among the symptoms observed in the subordinate male (intruder) are weight loss, increased heart rate, sleep disturbances, and increased body temperature as well as disturbances of the hypothalamic-pituitary-adrenal axis, including an increase in corticosterone levels and changes in immune responses [30]. The social defeat stress model has been suggested to have clinical relevance in major depressive disorders in humans. This model can be used to screen new psychotropic drugs under simulated clinic conditions and to investigate the mechanisms underlying stress-induced immune dysfunction to provide a basis for the development of immune therapy for depression [28,30,31].

This study was undertaken to analyze the effects of ladasten in a mouse model of social defeat stress simulating the stage of major depression. Behavioral patterning and changes in T-lymphocyte subsets ($CD3^+$, $CD4^+$, $CD8^+$) were analyzed in male C57Bl/6 mice. We show here that behavioral patterns in SDS mice can be reversed by treatment with the anti-asthenic drug ladasten and that this treatment is associated with somewhat normalized T-cell subsets.

2. Materials and Methods

2.1. Animals

Fifty adult male C57Bl/6 mice weighing 18 - 20 g were used in this study. The animals were obtained from Stolbovaya, the nursery of laboratory animals of the Russian Academy of Medical Science (RAMS), and adapted for 2 weeks prior to testing in the Zakusov Institute of Pharmacology RAMS vivarium. Mice were maintained in an environmentally controlled facility with a 12 h light-dark cycle (lights on at 08:00 hours) and a temperature of 22˚C ± 2˚C. All mice were maintained on a standard diet with food and water available *ad libitum*. The experimental protocols were in compliance with the European Communities Council Directive (86/609/EEC).

2.2. Drug Treatment and Social Defeat Stress

The anti-asthenic drug ladasten (N-(2-adamantyl)-N-(para-bromophenyl)-amine) was designed and developed at the Zakusov State Institute of Pharmacology RAMS [11,13] and received from the Technological Division of the Institute of Pharmacology RAMS (Moscow).

The present study adapted the sensory contact model, which was developed by N. Kudryavtseva and colleagues and is currently widely accepted as a valid chronic social defeat paradigm [27,28].

Twenty-five days after the beginning of the SDS experience, submissive mice received daily intraperitoneal (i.p.) injections of 30 mg/kg ladasten or saline for 5 days. Ten submissive animals were assigned to each treatment group. The choice of the dosage of ladasten (30 mg/kg for 5 days daily) was based on previous experimental results [11,13]. The day after the last injection, we tested the behavioral consequences of chronic defeat stress and the effect of ladasten.

2.3. Behavioral Tests

Spontaneous locomotor activity (SLA) was evaluated in

an infrared actimeter (IR, PanLab, Spain). Parameters measured in this study during the 3-min testing period were the following: locomotor (fast/slow activity) and stereotyped (fast/slow) movements; traveled distance (and %); maximum, minimum and mean speed; time (and %) spent moving fast, slow and resting; and number and mean duration of rearing.

The Forced Swimming Test (FST) with video-based analysis system (OpenScience, Moscow, Russia) was used to evaluate the depression state of the mice [32,33]. Mice were individually placed in cylinders (height = 30 cm, diameter = 13 cm) containing 10 cm water (22˚C) from which they could not escape. The mice were placed in water for 5 min, and the duration of swimming was recorded on video. Images were analyzed with "Real-time" (OpenScience, Moscow, Russia) software. The latency to the first period of immobility and passive behavior (immobility) time were recorded during the test. Depressed mice were less active (exhibited less swimming, climbing/struggling) and floated in the cylinders, showing behavioral despair. Parameters measured in this study were the latency to immobility and the duration of immobility (floating + freezing).

2.4. Analyses of Blood (Spleen, Thymus) T-Cells by Flow Cytometry Method

The thymus and spleen were weighed, and the results were expressed as relative tissue weight, *i.e.*, mg tissue/ 10 g mouse weight). Cell suspensions were prepared from the spleen or thymus by pushing the tissue through a nylon mesh using the plunger of a 2-ml syringe in cold PBS containing 0.1% BSA. Whole blood or 100 µL of splenocytes (thymocytes) were stained with fluorescence-labeled monoclonal antibodies against CD3 (FITC-labeled), CD4 (PE-labeled), or CD8 (PECy5-labeled) (eBioscience, Austria) according to the manufacturer's instructions. After incubation for 30 min at 4˚C in the dark, the red blood cells were removed by lysis buffer. Samples were centrifuged at 300 - 400 × g at 4˚C for 5 min, the supernatant was discarded and the pellet was washed twice with 1mL PBS containing 0.1% BSA. Re-suspended stained cells were analyzed using the EPICS XL 4 flow cytometer (Beckman Coulter, USA); 10,000 - 15,000 events were acquired; and the data were analyzed using the WinMDI 2.7 software.

2.5. Statistical Analysis

Data were analyzed using two-way ANOVA, followed by Newman-Keuls post hoc test for multiple comparisons and the results are presented as mean ± SEM. When variances were not equal as assessed by a Levene test, non-parametric statistics were conducted using a Kruskal-Wallis test followed by Dunn's multiple comparison test and results are presented as median (low/upper quartile). Statistical significant was set at $p < 0.05$.

3. Results

Mouse SLA was analyzed after SDS stress; the data are presented in **Table 1**. The results show that mice in the SDS group that received saline for 5 d showed a significant decrease in locomotor activity (by 23%), movement stereotypes (by 56%), maximum velocity (by 57%) and total traveled distance (by 65%). The number of fast movements in stressed mice diminished by 4-fold whereas the resting time increased 1.3-fold.

Thus, our data demonstrate a significant decrease in the motor and exploratory activity of defeated mice as well as evidence of a behavioral deficit and development of a depressive-like phenotype, which were in line with previous findings [28,29].

Compared to control non-defeated mice, the mice from the FST social defeat group exhibited significantly shorter latency to the first float period (by 44%) and longer time of immobility (by 20%). These data demonstrate that defeated mice had symptoms of depressed mood and behavioral despair (**Table 2**).

Table 1. Effects of ladasten on SLA parameters in SDS C57Bl/6 mice in IR actimete.

	Groups	Spontaneous locomotor activity (SLA) parameters								
		Locomotors activity	Stereotype movements	υ_{max} cm/s	υ_{mean} cm/s	Distance, cm	Time of rest, %	Slow movements, %	Fast movements, %	Number of rearings
30 days of SDS mice **Non-defeated mice**	Vehicle-control mice	477.2 ± 35.9	100.0 (47.0/163.0)	28.9 (17.6/38.5)	3.9 ± 0.8	673.9 (388.4/958.7)	48.4 ± 3.7	35.7 ± 4.3	12.8 (6.9/24.4)	47.0 (37.0/59.0)
	Ladasten 30 mg/kg, i.p.	387.7 ± 34.1	64.5 (44.0/92.0)	14.9 (14.0/16.3)	2.7 ± 1.2	**290.7*** (208.0/345.4)	**64.9 ± 3.1***	37.6 ± 3.2	10.2 (6.0/14.6)	19.5 (13.0/28.0)
	Vehicle	**366.4 ± 43.1***	**43.5*** (24.0/56.0)	**12.3*** (10.5/17.5)	2.3 ± 0.7	**238.5*** (173.9/421.6)	**62.5 ± 4.7***	34.1 ± 4.6	**3.2*** (1.9/5.1)	29.5 (23.0/38.0)
	Ladasten, 30 mg/kg, i.p.	421.8 ± 39.2	60.5 (57.0/69.0)	19.7 (16.3/37.3)	5.6 ± 1.6	383.5 (37.3/477.8)	54.5 ± 4.3	34.7 ± 4.4	5.5 (4.0/13.2)	49.0 (28.0/58.0)

Comment: υ_{max} и υ_{mean}—maximal and mean velocity of mice in test cm/s. Data expressed as mean ± standard error of the mean (SEM) or median (low/upper quartile); *Significant between-group difference in parameter compared with vehicle-control group (Kruskal-Wallis test followed by Dunn's multiple comparison test, $p < 0.05$, n = 10 per group).

Table 2. Effects of ladasten on the latency and immobility time in the FST and thymus and spleen weights indexes.

Parameters	Non-defeated mice		30 days of SDS mice	
	Vehicle-control mice	Ladasten 30 mg/kg, i.p.	Vehicle	Ladasten, 30 mg/kg, i.p.
Latency to the first immobility, s	74.6 ± 7.8	75.7 ± 8.4	**41.6 ± 8.4***	57.1 ± 15.7
Immobility time, s	201.1 ± 9.3	191.4 ± 9.9	**245.7 ± 9.9***	204.1 ± 15.6$^{\#}$
Thymic index, mg/10g body weigh	24.4 ± 1.4	22.8 ± 1.7	**14.8 ± 2.2***	19.8 ± 1.5$^{\#}$
Spleen index, mg/10g body weigh	31.1 (29.1/33.5)	36.8 (35.7/39.6)	**62.5**$^{\$\$}$ (38.3/75.3)	**51.3**$^{\$}$ (38.2/55.9)

Comment: Data expressed as mean ± standard error of the mean (SEM); *Significant between-group difference in parameter compared with vehicle-control group; $^{\#}$Significant between-group difference in parameter compared with vehicle-SDS group; (two-way ANOVA, followed by Newman-Keuls post hoc test for multiple comparisons; $^{*}p < 0.05$, $^{\#}p < 0.05$, n = 10 per group); Data expressed as median (low/upper quartile); $^{\$}$Significant between-group difference in parameter compared with vehicle-control group; (Kruskal-Wallis test followed by Dunn's multiple comparison test, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, n = 10 per group).

Ladasten injections alleviated SDS behavioral effects and attenuated symptoms of depression. The defeated group that received ladasten showed behavior similar to that of non-defeated control mice, which were housed and treated similarly but were not exposed to aggressors.

The exposure to social defeat stress for 30 days led to a 1.6-fold decrease in thymus weight whereas spleen weight increased 1.7 fold (**Table 3**). In comparison to control, stressed animals showed a 10 % decrease in the number of $CD4^{+}CD8^{+}$ double-stained immature thymocytes, whereas there were increases in subset percentages of single $CD4^{+}$ (T helper cells) and single $CD8^{+}$ cells (T cytotoxic cells) by 28% and 45%, respectively (**Table 3**). These data demonstrate marked changes in the populations of T lymphocytes following social defeat stress for 30 days. These observations are in good accordance with earlier studies showing the redistribution of T-cell populations in the thymus resulting in high levels of the suppressor/cytotoxic T-lymphocyte subset [1,4,31,34,35]. There was a 41% decrease in the number of $CD3^{+}$ T lymphocytes in the spleen of stressed mice due to the decreased number of both helper and cytotoxic T cells by 40% and 31%, respectively. The total percentage of circulating blood $CD3^{+}$ T cells diminished under stress from 24.5% in control animals to 20.2% in defeated animals (18 %, $p < 0.05$). However, the $CD4^{+}/CD8^{+}$ ratio was not affected by the stress.

Administration of ladasten to defeated mice prevented thymus weight loss. Although ladasten did not reverse splenomegaly, it partially restored spleen weight (by 18%). The T-lymphocyte subsets present in the blood, spleen and thymus of ladasten treated mice remained in similar proportions to those of the unstressed control animals.

4. Discussion

Our data are consistent with previous studies which have demonstrated that social defeat stress in mice after repeated experiences of aggression results in profound behavioral changes reminiscent of chronic stress disorder and major depression [28-30]. Chronic social defeat stress for 30 days leads to defeat-induced social avoidance and depression in mice [23]. The administration of the anti-asthenic drug ladasten for 5 days prevented the expression of avoidance behaviors in response to social defeat stress.

Earlier findings also completely align with our results, showing a decrease in the population of T cells in primary and secondary immune organs and a stress-induced shift in the $CD4^{+}/CD8^{+}$ ratio toward T-cytotoxic subsets associated with the development of depression in mice exposed to SDS [31,35]. The degree of change in the pool of T cells in blood was not the same as that observed in the spleen and the thymus. The most dramatic stress-associated changes in the T-cell population were displayed in the thymus, whereas there were no significant changes in the blood. The observed extreme sensitivity of immature $CD4^{+}CD8^{+}$ to acute and chronic stress is a well-known phenomenon [35,36]. However, the results of our experiment seem to disagree with the results of Dominguez-Gerpe, who observed the decrease of two glucocorticoid-resistant populations of $CD4^{+}$ and $CD8^{+}$ T cells in blood of stressed mice [35]. The observed differences in the peripheral T-cell population may depend on the duration of SDS as well as on the animal strain used. On the basis of the results seen in the present study and in a previous study, we hypothesize that the observed changes in the peripheral T-cell pool were because of the migration of naïve T-cells from the thymus for maintaining the blood population [30].

Thus, stress induced by social defeat is a strong modulator of immune status, from the activation of CMI to immune deficiency, and abnormalities in immune function are considered as the underlying mechanism of depression [1,4,6].

The administration of ladasten led to the recovery of T-cell subsets in blood, spleen, and thymus and averted

Table 3. Effects of ladasten on T-cell subsets in blood, spleen, and thymus in SDS C57Bl/6 mice.

Parameters	Non-defeated mice		30 days of SDS mice	
	Vehicle	Ladasten, 30 mg/kg, i.p.	Vehicle	Ladasten, 30 mg/kg, i.p.
Thymus				
$CD4^+CD8^-$	6.8 ± 0.3	8.5 ± 0.6	**9.5 ± 0.8***	6.2 ± 0.4
$CD4^+CD8^+$	85.2 ± 1.9	75.2 ± 1.4	**76.8 ± 2.6***	83.2 ± 1.9
$CD4^-CD8^+$	2.4 ± 0.3	4.5 ± 0.5	**4.4 ± 0.4***	3.1 ± 0.2
Spleen				
$CD3^+$	33.9 ± 1.8	38.9 ± 1.8	**21.6 ± 1.8***	29.6 ± 1.3
$CD4^+CD8^-$	22.2 ± 1.6	25.1 ± 1.1	**13.3 ± 1.5***	19.5 ± 1.7
$CD4^-CD8^+$	11.5 ± 0.8	13.8 ± 0.9	**7.9 ± 0.6***	11.8 ± 0.9
Blood				
$CD3^+$	24.5 ± 1.2	23.8 ± 1.3	**20.2 ± 1.7***	24.5 ± 2.9
$CD4^+CD8^-$	14.4 ± 1.6	14.9 ± 0.9	11.5 ± 1.8 (p = 0.08)	15.2 ± 2.0
$CD4^-CD8^+$	10.1 ± 0.6	11.2 ± 1.1	9.5 ± 0.9	10.5 ± 1.8

*Significant between-group difference in parameter compared with vehicle-SDS group; (two-way ANOVA, followed by Newman-Keuls post hoc test for multiple comparisons; *p < 0.05, n = 10 per group).

the immune imbalance seen in defeated mice.

It is well established that dopaminomimetic drugs exert stimulatory effects on the immune system, as well as antidepressant activity [8,37]. The influence of ladasten on the levels of TH mRNA and protein as well as on DA content may be linked to the regulation of neuroendocrine function that, in turn, provides peripheral effects of the drug, such as protection from stress, metabolic status and immune modulation [16-18,21].

The affinity of ladasten to the central monoaminergic (MAergic) systems and its ability to regulate gene expression in the brain were suggested to explain the effect of ladasten on behavior as well as peripheral immune-competent cells, which express neurotransmitter receptors. Previous studies have shown the direct effects of ladasten on the functional activity of lymphocytes, such as the stimulation of antibody production and inhibition of cytotoxic activity of T cells [16,22]. However, the exact cellular mechanism through which ladasten elicits its neuroimmunomodulation effects is unknown and requires further experimental and clinical study.

Thus, the results obtained in this study confirm the notion that depression is accompanied by disturbances in cell-mediated immune activation, including the reduction in T cells and their redistribution in immune organs. Administration of the anti-asthenic drug ladasten restores the balance of T-cell subsets in the blood, spleen and thymus and diminishes the behavioral symptoms of depression in stressed mice. The data reveal the pharmacological properties of ladasten in the SDS model of depression and support further investigations of ladasten as a potent anti-depressive drug that can be used alone as well as in combination with other anti-depressants.

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