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The Influence of Polyphenols on Calcium Dynamics in Synaptosomes of Model Rats with Attention Deficit Hyperactivity Disorder of Varying Ages

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Abstract

Introduction: ADHD is characterized by disrupted neuronal excitability, often associated with dysregulated calcium (Ca^{2+}) signaling. We investigated the effects of the polyphenol ANK-2 on intracellular Ca^{2+} dynamics in synaptosomes isolated from the brains of healthy and ADHD-model rats of different ages. Materials and Methods: In the studies, rats weighing 150 - 200 g were subjected to an ADHD model by administering 6-OHDA at 2 different doses of 8 and 4 mg/kg orally for 14 days using a special probe. Also, in order to induce the ADHD model in the prenatal period in young rats, it was first established that rats of both sexes were placed together, their condition was determined using a swab, and then, on average 3 - 4 days before birth, $CdCl_2$ was dissolved (50 ppm) and administered orally to pregnant rats weighing 200 - 250 g using a special probe, as a result of which the ADHD model was manifested in the offspring. Using the calcium-sensitive fluorescent probe Fluo-4AM, we found that ANK-2 significantly reduced cytosolic Ca^{2+} levels in a dose-dependent manner. Results and discussion: In ADHD models, baseline Ca^{2+} content was markedly elevated compared to age-matched controls, consistent with hyperactive synaptic transmission. GABA (100 μM) decreased [Ca^{2+}] in by 14 % in healthy rats and 20 % in ADHD rats, suggesting enhanced compensatory GABAergic inhibition in the ADHD condition. Co-incubation with ANK-2 (10 - 100 μM) further suppressed calcium influx, indicating a synergistic enhancement of GABAergic effects. Additionally, ANK-2 potentiated the calcium-lowering effect of levetiracetam (5 μM), a selective N-type Ca^{2+} channel blocker, further reducing cytosolic Ca^{2+} levels. Conclusions: These findings suggest that ANK-2 acts through multiple mechanisms, including enhancement of GABAergic signaling, direct or indirect inhibition of voltage-gated calcium channels, and modulation of neurotransmitter release. Our results position ANK-2 as a promising candidate for correcting excitatory-inhibitory imbalance in ADHD by targeting calcium-dependent synaptic transmission.

Key words: Calcium dysregulation, ADHD, Synaptosomes, Glutamatergic hyperactivation, GABA, VGCC

Introduction

Studies of brain calcium dynamics and synaptosomes in animal models of ADHD have advanced significantly in recent years. A major focus has been on understanding how calcium signaling contributes to the neurobiological underpinnings of ADHD, particularly with regard to synaptic function and neurotransmitter release [1,2]. Advances in calcium imaging technologies, such as genetically encoded calcium indicators and miniaturized imaging devices, have enabled researchers to monitor calcium dynamics in real time in neuronal populations [3]. This allows us to observe how calcium signaling correlates with behavioral changes in ADHD models, providing information about the functional properties of neural circuits [4]. Studies have shown that synaptosomes, which are isolated presynaptic terminals, can be used to study calcium influx and neurotransmitter release in response to various stimuli [5]. Altered calcium dynamics have been observed in ADHD models, indicating potential dysregulation of excitatory and inhibitory signaling pathways [6,7].

The study of plant-derived polyphenols is gaining momentum as a potential therapeutic avenue for ADHD. Research is focused on how these compounds can modulate calcium signaling and enhance GABAergic activity, which may help restore the balance between excitation and inhibition in the brain. Polyphenols are known for their antioxidant and anti-inflammatory properties [8]. Investigating their effects on calcium dynamics in ADHD models may reveal mechanisms by which they protect against neurodevelopmental deficits and improve cognitive function.

Polyphenols are natural compounds found in a variety of fruits, vegetables and herbs [9,10]. Polyphenols are thought to aid digestion, brain function and protect against cardiovascular disease, type 2 diabetes, and various cancers when consumed daily. More than 8,000 phenolic structures have been identified, of which more than 4,000 are flavonoids, and hundreds of them are found in food plants. However, like many of the phenolic compounds found in fruits, vegetables and their derivatives [11,12]. Their potential to modulate neurotransmitter systems [13] with fewer side effects compared to synthetic drugs makes them attractive candidates for the treatment of ADHD [14].

The current state of research on brain calcium dynamics and synaptosomes in ADHD models highlights the importance of understanding calcium signaling in the context of neurodevelopmental disorders [15]. Promising projects focusing on the effects of plant-derived polyphenols may lead to new therapeutic strategies that improve calcium regulation and enhance cognitive outcomes in people with ADHD. The natural properties of polyphenols, combined with their potential to modulate neurotransmitter systems, make them an attractive area of research in the search for effective treatments for ADHD.

Materials and methods

Experimental models of ADHD “Animals”

The experiments were carried out on outbred white male rats kept on a standard vivarium diet. All experiments model experience in these 10 groups performed comply with the requirements of the World Society for the Protection of Animals and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and Ethical principles of psychologists and code of conduct [16].

For the modeling of the Attention Deficit Hyperactivity Disorder ADHD, laboratory rats of males were used, weighing 200 - 300 g. First of all, weighing and selection of animals for experiments were carried out. Then, behavioral tests are carried out: An open field, a conditioned response of passive avoidance (CRPA) and active avoidance (ACRA), swimming on the pool (Morris test). We feed animals with a standard diet with add-on for a month or 2. After a week, we repeat behavioral tests. Analyzing the data, depending on the test results, enter neurotoxin. After playing the model AD, we score the animal and take biological materials for further research.

The results of behavioral tests showed that in control groups, experimental animals on the “open field” tests were very active and overexcited, quickly moved and practical did not stand in one place. At the same time, the ADHD groups are very passive, the nervous system inhibited and the animals were delayed for a long time in one place. This slows down that after the introduction of neurotoxin into the animal’s body, the normal functioning of the nervous system is violated,

the destruction of the transmission of inpuls in neurons and the death of the cell.

In this context, the “bright phase” and “dark phase” refer to zones in a CRPA or ACRA test chamber, which is commonly used in behavioral neuroscience to assess learning, memory, anxiety, and fear-related responses in animals, especially rodents. Bright Phase - Rats naturally avoid brightly lit areas because they prefer darker, enclosed spaces for safety. Thus, being in the bright area is often associated with discomfort or aversion, making it a suitable place to start the test. Dark Phase - A dark compartment that rodents naturally prefer. However, in these tests, animals often receive a mild electric shock or aversive stimulus when entering this area during training, which creates a negative association with the dark phase. At the tests of CRPA and ACRA, the obtained experts showed that in the control group the animals were in the bright phase quickly sought to go to the dark phase, after they received fragmentation quickly moved to the bright phase. When this test was repeatedly carried out by an ec -permanent animal did not pass into the dark phase from the light. When the model ADHD groups of animals were placed in the bright phase, she did not strive to go to the dark phase, after she went in the graternation.

When this test is repeated, the experimental animal, as last time, slowly went into the dark phase and again received gratification. The data obtained witnesses that under the control group, experimental animals quickly moved into a dark phase that resembled the mink of animals, but after receiving irritation when repeated the same test did not go into the dark phase. From this we can conclude that the animals have a reaction to the gratification in the dark phase and this remained unih in memory. In the model group, the initially animals were very passive and slowly perpeted in the languid phase. When the test is repeated, the animals again went into the dark phase and received gratification. This suggests that in the model group of animals, cognitive functions, the reaction to the environment and memory, which are symptoms of ADHD, are greatly impaired.

The models were used in white outbred rats (200 - 250 g). The animals were carefully weighed and various behavioral tests were performed: Open field n = 3, CPPA and ACRA active avoidance n = 3. Laboratory animals were kept on a standard diet with various protein-rich supplements in a special vivarium for 2 months.

When the rats reached a weight of 150 - 200 g, they were subjected to the ADHD model by administering 6-hydroxydopamine at 2 different doses at concentrations of 8 and 4 mg/kg orally for 14 days using a special probe [17].

Also, in order to induce the ADHD model in the prenatal period in young rats, it was first established that rats of both sexes were placed together, their condition was determined using a swab, and then, on average 3 - 4 days before birth, cadmium chloride (1 ppm = 1 mg/L = 1 μg/mL = 1,000 μg/L) was dissolved (50 ppm) and administered orally to pregnant rats weighing 200 - 250 g using a special probe - for administering medications or feeding laboratory animals. It is a syringe with a probe needle for feeding laboratory rats. The needle is made of stainless steel in a curved state and a blunt rounded end so as not to injure the soft tissues of animals. The diameter of the needle is 16 mm and the length is 80 mm., as a result of which the ADHD model was manifested in the offspring [18]. Experimental animals were sacrificed under light ether anesthesia. Blood and internal organs were collected into different vessels and processed simultaneously.

After modeling ADHD, behavioral tests were repeated: Open field n = 3, CRPA and ACRA n = 3 [19].

Test compounds

Initially, when we studied the effects of many biologically active substances on suspensions of rat brain synaptosomes, the polyphenol ANK-2 was chosen as having the most effective effect.

In order to study the chemical composition of the leaves of *Pistacia vera* plants from the Bostanlyk district of Tashkent region, plant material was collected and dried. A plant species growing in mountainous areas was used to conduct our scientific research. The compound we isolated has a molecular mass of 1,243 and a molecular formula of $C_{55}H_{40}O_{34}$. Since this plant belongs to the *Anacardiaceae* family, we have conventionally named it polyphenol ANK-2. All these polyphenols were presented from the Institute of Bioorganic Chemistry named after Academician A.S. Sadykov. All processes were carried out according to the standard mode. A reliable method for the isolation and

purification of the total polyphenols from 1 kg of air-dried plant leaves was developed using sequential chloroform and 70% aqueous acetone extraction, followed by liquid-liquid partitioning with chloroform and ethyl acetate. The final polyphenol precipitate, obtained in a 12% yield, was thoroughly purified and characterized. High-performance liquid chromatography (HPLC) analysis revealed 24 distinct fractions absorbing at 254 nm, indicating a diverse polyphenol profile. Subsequent mass spectrometric analysis using Q-TOF LC-MS in negative ion mode enabled the structural elucidation of the isolated compounds. Identification was performed by interpreting MS and MS/MS spectra and cross-referencing public chemical databases (ChEBI, ChemSpider, MolInstincts, Phenol-Explorer). The combined analytical approach confirmed the presence of a complex mixture of bioactive polyphenolic compounds suitable for further pharmacological and biochemical studies.

Isolation of synaptosomes

Synaptosomes are obtained by two-stage centrifugation Centrifuge D1524R (LK23ABH0000154. DLAB Scientific CO.LTD. Chine) [20]. The entire isolation procedure is carried out at −4 °C. After decapitation, the brain is removed as quickly as possible and crushed on ice. The crushed tissue is homogenized at a ratio of 1:10 in the isolation medium −0.32 M sucrose solution in 0.01 M Tris-HCl buffer with the addition of 0.5 mM EDTA (pH 7.4). The obtained homogenate is exposed to a 4-stage centrifugation. The supernatant after the first centrifugation (10 min, 4,500 rpm) is carefully removed without capturing the myelin layer and exposed to further centrifugation for 20 min at 14,000 rpm. The obtained dense precipitate P2 is resuspended in the isolation medium. The obtained suspension is used further in the experiment as a coarse synaptosomal fraction (synaptosomal-mitochondrial). In the case of 4-stage isolation, the second centrifugation is carried out at 11,000 rpm for 20 min. The dense pellet of P2 is resuspended in 0.32 M sucrose solution (pH 7.4) and then carefully layered on 0.8 M sucrose solution (pH 8.0), after which it is centrifuged for 25 min at 11,000 rpm. As a result of centrifugation in a sucrose gradient, factions are separated - mitochondria settle tightly at the bottom of the tube, and synaptosomes remain in suspension in a layer of 0.8 M sucrose. This layer is carefully removed, mixed with an equal amount of isolation medium and left for 15 min to restore the ultrastructure of synaptosomal particles, after which it is exposed to further centrifugation at 14,000 rpm for 30 min. The dense final precipitate P4 is resuspended in the isolation medium and then used in the experiment as a synaptosomal faction.

Method for studying changes in $[Ca^{2+}]_{in}$ concentration in rat brain synaptosomes

Changes in $[Ca^{2+}]_{in}$ concentration in rat brain synaptosomes in suspension were calculated using the method developed by Grynkiewicz *et al*. [21]. Fluo-4 AM with high sensitivity in determining intracellular calcium concentration (1×108 cells/ml) (*N-[4-[6-[(Acetyloxy)methoxy]-2,7-difluoro-3-oxo-3H-xanthen-9-yl]-2-[2-[2-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-methylphenoxy]ethoxy]phenyl]-N-[2[(acetyloxy)methoxy]-2-oxoethyl]glycine (acetyloxy)methyl ester*) was used as a fluorescent probe.

In our experiments, 1 mg of Fluo-4 AM powdered fluorescent probe was dissolved in 135 μL of DMSO to obtain 1 mM Fluo-4 AM reagent solution. Before the experiment, the Fluo-4 AM solution in DMSO was kept at room temperature [22] and 80 μl of synaptosomes and 12 μl of Fluo-4 AM were added to 2 ml of Krebs-Ringer buffer and incubated for 30 min at 37 °C. Fluo-4 AM is a fluorescent Ca^{2+} chelator with high affinity for calcium. Fluo-4 AM can specifically detect intracellular calcium ions with high sensitivity, low cytotoxicity and high content of acetyl methyl ester AM, which has good intracellular penetrating ability. After cleavage by intracellular esterase, it remains in the cell, binding to calcium ions and causing strong fluorescence.

After incubation, the dye remaining in the medium was washed twice and removed by centrifugation in standard medium. In the experiments, a cell concentration of 5×106 cells/ml per cell was used.

In the experiments, the fluorescence of Fluo-4 AM was excited by radiation at a wavelength of 488 nm and recorded by a light flux at a wavelength of 506 nm, which was taken as the maximum fluorescence value (F_{max}). Under incubation conditions with EGTA (1 mM),

i.e. $[Ca^{2+}]_{out}$ = 0 mM, the minimum fluorescence value (F_{min}) was calculated using the following equation:

$$F_{\min} = \left[(F_{\max} - F_{Fluo-4AM} / 3\right] + F_{Fluo-4AM} \quad (1)$$

Here, $F_{Fluo-4AM}$, a Ca^{2+}-sensitive probe, represents the fluorescence value of rat brain synaptosomes under Fluo-4 AM (5 μM) incubation conditions.

$$[Ca^{2+}] = Kd\ [(F - F_{min})/(F_{max} - F)] \quad (2)$$

Here F is the fluorescence indicator at the experimental calcium concentration, Fmin is the fluorescence without calcium, Fmax is the fluorescence indicator at a saturated calcium concentration, Kd is the concentration of 450 nM in a cell-free medium for Fluo-4 AM. However, Kd is usually dependent on a number of factors in the cell, including pH, protein content, ionic strength, temperature, and viscosity. Therefore, Kd calibration is necessary to accurately measure intracellular calcium concentration [23]. In the experiments, the fluorescence intensity value was recorded using a USB 2000 spectrofluorimeter.

Statistical analysis

The measurements were carried out on a universal spectrometer USB-2000. Statistical significance of differences between control and experimental values, determined for a data series using a paired t-test, where control and experimental values are taken together, and an unpaired t-test, when taken separately. A p value < 0.05 indicates a statistically significant difference. The results obtained are statistically processed in OriginPro 2022.

Results and discussion

The results of behavioral tests on rats with ADHD model during our experiments show that in the control groups the experimental animals were very active and hyperactive in the Open Field test, moved quickly and hardly stood still. At the same time, the model showed that the ADHD groups were very hyperactive, with nervous system processes being activated, while the animals remained motionless. The results obtained indicate that after the introduction of 6-hydroxydopamine and cadmium chloride into the animals' bodies, the normal functioning of the nervous system is disrupted by a transition to a hyperactive state [24], and the transmission of impulses in neurons is also disrupted (**Figure 1**).

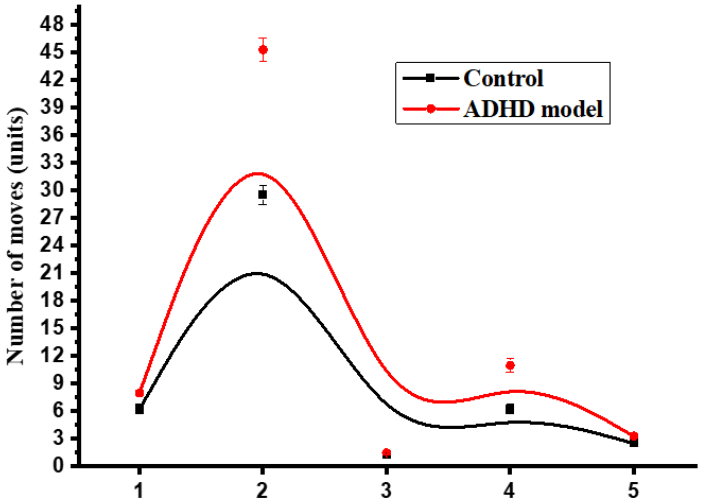

Figure 1 Determination of cognitive behavior during 3 min in the Open Field test. (1) Vertical movement, (2) Horizontal movement, (3) Washing, (4) Mink. (5) Boluses.

In our subsequent experiments, we found that the calcium content in suspensions of synaptosomes from the brains of healthy rats of different ages (4, 8, 12 weeks) differed significantly from the calcium content in suspensions of synaptosomes from the brains of rats with the ADHD model, depending on age. In this case, it was found that calcium content in healthy rats at 4 weeks of age was 15% lower than in ADHD models,

calcium content in synaptosomes of healthy rats at 8 weeks of age was 20% lower than in ADHD models, and calcium content in synaptosomes of healthy rats at 12 weeks of age was 29% lower than in ADHD models (**Figure 2**).

The results obtained are explained by the fact that over time, rats experience an increase in synaptic activity in the central nervous system and an increase in the dynamics of calcium-dependent mediators of nerve cells due to learning various skills and linear movements, while the calcium concentration decreases relatively.

In the course of experiments, we studied the effect of the polyphenolic compound ANK-2 on the calcium content in synaptosomes of the brain of healthy rats of different ages (4, 8, 12 weeks) and in suspensions of synaptosomes of rats with the ADHD model (**Figure 3**).

The results showed that exposure to the polyphenol ANK-2 resulted in a decrease in the amount of calcium in the synaptosome suspension of the brain of rats of different ages. The obtained results indicate that the polyphenol ANK-2 may have a protective effect on calcium-dependent processes.

To summarize these results, the ADHD model was initially induced in two ways: First 6-hydroxydopamine (6-OHDA) as a postnatal model, 4 and 8 mg/kg orally for 14 days. As a prenatal model, pregnant rats were injected with 50 ppm cadmium chloride ($CdCl_2$), which resulted in the development of an ADHD model in the offspring. Then, in our experiments, when measuring cytosolic Ca^{2+} in brain synaptosomes using the Fluo-4AM probe, we found a significant age-related increase in calcium in ADHD models compared to controls, namely +15% at week 4, +20% at week 8, and +29% at week 12. Rats with ADHD models consistently have higher calcium levels than healthy rats. Since Ca^{2+} is an important second messenger in synaptic vesicles and neuronal excitability, this may lead to hyperactive synaptic transmission in ADHD models. Regarding the molecular mechanisms of these processes, Ca^{2+}, being a major second messenger in neurotransmission, synaptic plasticity and neuronal excitability, alters the regulation of calcium-conducting channels in hyperdopaminergic and hypodopaminergic states in ADHD [25].

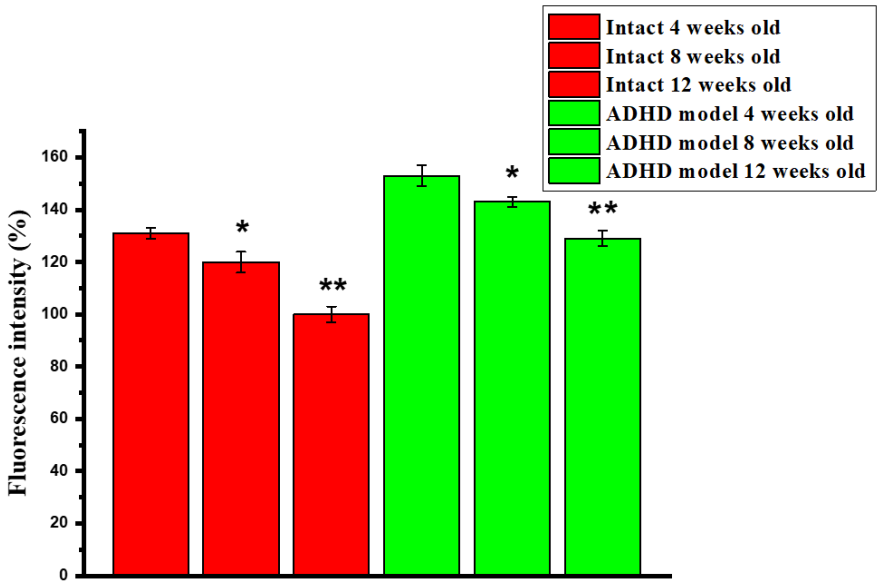

Figure 2 Calcium content in the synaptosomes of the brain of healthy and model rats with ADHD of different ages (4, 8, 12 weeks). Level of reliability. *– $p < 0.05$; **– $p < 0.01$; ***– $p < 0.001$. (n = 6).

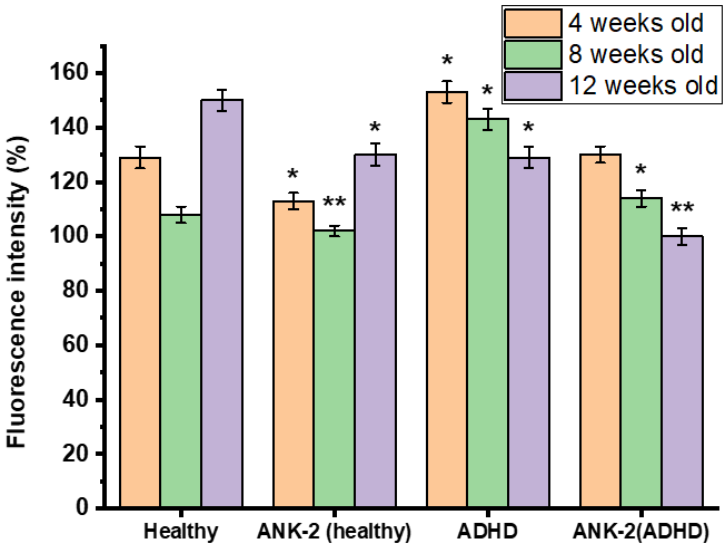

Figure 3 Calcium content in the synaptosomes of the brain of healthy and model rats with ADHD of different ages (4, 8, 12 weeks). Level of reliability. *– $p < 0.05$; **– $p < 0.01$; ***– $p < 0.001$. (n = 6).

6-OHDA selectively damages dopaminergic and noradrenergic neurons, mimicking ADHD-like hyperactivity. Decreased dopamine (DA) levels alter voltage-gated calcium channels (VGCCs) and intracellular calcium buffering, resulting in abnormal calcium influx. 6-OHDA induces dopaminergic neurodegeneration, particularly in the mesocortical and nigrostriatal pathways. This results in compensatory activation of glutamate receptors (NMDA, AMPA) and voltage-gated calcium channels (VGCC) in postsynaptic neurons, resulting in an increase in intracellular Ca^{2+}.6-OHDA is a neurotoxin that selectively destroys dopaminergic neurons, particularly in the nigrostriatal pathway [26]. This model is widely used to study neurodegenerative processes and also allows us to determine the effects of ADHD due to the role of dopamine in regulating behavior and actions.

On the other hand, cadmium chloride disrupts calcium channels (e.g., L-type Ca^{2+} channels) and calcium-dependent functions in mitochondria, which aggravates synaptic dysfunction. Cd^{2+} mimics Ca^{2+} and blocks Ca^{2+} ATPases and Na^{+}/Ca^{2+} exchangers, disrupting calcium transport. Impaired Ca^{2+} buffering and elevated synaptosomal calcium levels cause oxidative stress and mitochondrial dysfunction. Cadmium chloride is a heavy metal that can cause neurodevelopmental toxicity [27]. When administered to pregnant rats, cadmium can cross the placenta and affect fetal brain development, resulting in long-term neurodevelopmental defects in the offspring. Prenatal exposure to cadmium chloride may impair the development of the dopaminergic and GABAergic systems, which are important for attention and impulse control.

In healthy rats, calcium levels are regulated as follows: With age, calcium levels decrease as fragmentation and dissociation of synapses occurs. This reflects calcium buffering and efficient recycling of synaptic vesicles involving calbindin and mitochondria. These processes include activation of calcium-binding proteins such as calbindin and parvalbumin, activation of PMCA and SERCA pumps, and buffering of synaptic vesicles.

In ADHD models, these systems are impaired or overloaded, resulting in impaired calcium efflux, decreased plasma membrane Ca^{2+}-ATPase activity, and increased synaptosome Ca^{2+} levels due to PMCA and SERCA. Excessive calcium influx through NMDA or VGCC receptors leads to mitochondrial dysfunction, which results in impaired calcium sequencing [28]. In ADHD models, a mechanical cascade of synaptosomal Ca^{2+} overload is observed [29]. In addition, in some pathologies of Alzheimer's disease, it was found that significant changes in the nervous system and hemostasis occur simultaneously, and the basis of this pathology has been shown to be a violation of both calcium and neurotransmitter transport [30,31].

These processes suggest that as healthy rats mature, their synaptic activity becomes more efficient, resulting in reduced calcium overload through improved synaptic plasticity and inhibitory control.

In contrast, rat models of ADHD may not exhibit the same degree of maturation of synaptic efficacy, resulting in persistently elevated calcium levels. In contrast, rat models of ADHD may not exhibit the same degree of maturation of synaptic efficacy, resulting in persistently elevated calcium levels.

As a functional interpretation, the ADHD models (6-OHDA and $CdCl_2$) result in synaptic calcium overload due to impaired calcium extrusion, excessive current, or mitochondrial dysfunction. Studies of changes in calcium dynamics in ADHD models indicate impaired synaptic plasticity that regulates behavior [32]. Elevated calcium levels can lead to excitotoxicity, which further impairs neuronal function and contributes to ADHD symptoms.

In conclusion, experimental data on calcium levels in synaptosome suspensions in rat ADHD models indicate significant changes in calcium dynamics associated with dopaminergic and GABAergic dysfunction. These changes indicate disruption of synaptic plasticity and excitatory-inhibitory balance, which play a central role in the pathophysiology of ADHD.

It is known that GABA(A) receptors are ion channels that transmit fast inhibitory neurotransmitters. GABA released from synapses can activate GABA receptors at extrasynaptic sites, resulting in modulation of neuronal activity. Dysregulation of GABAergic signaling may lead to the pathophysiology of various neurodegenerative diseases [33]. GABA is the major inhibitory neurotransmitter in the central nervous system. GABA receptors help maintain the balance between excitation and inhibition in neural circuits. When GABA(A) binds, the receptor opens its Cl^- channel. The entry of chloride ions into a neuron results in hyperpolarization of the neuron and a decrease in the probability of an action potential occurring.

GABA(B) receptors activate potassium (K) channels by hyperpolarizing K^+ influx via G proteins and, at the same time, by inhibiting voltage-gated Ca^{2+} channels, thereby reducing the release of neurotransmitters from the presynaptic terminal.

Studies have found decreased GABAergic effects and altered receptor function in ADHD. In this process, mainly due to decreased GABA synthesis or release, low GABA concentrations lead to decreased activation of GABA(A) receptors, resulting in an influx of Cl^- that mediates neuronal hyperexcitability [34]. Additionally, changes in receptor subunits can alter channel kinetics (e.g., faster closing), resulting in a shorter inhibition time. In addition, disruption of presynaptic Ca^{2+} channel inhibition may result in an increase in the excitatory neurotransmitter glutamate. The result of such overstimulation is hyperactivity and impulsivity.

Taking these data into account, in our subsequent experiments we investigated the effect of the polyphenol ANK-2 on the dynamics of $[Ca^{+2}]_{in}$ in a suspension of Fluo-4AM synaptosomes in the brain of healthy and model ADHD rats against the background of GABA. In our experiments, we found that when synaptosomes were incubated with GABA (100 μM), the calcium content in the synaptosome suspension decreased by 14 % in a healthy state and by 20 % in the ADHD model, which in turn led to a decrease in the concentration of $[Ca^{2+}]_{in}$. As a result, the addition of polyphenol ANK-2 (10 - 100 μM) to it in concentrations that effectively inhibit calcium content in both cases, depending on the concentration, compared with GABA (100 μM) (**Figure 4**).

GABA is the main inhibitory neurotransmitter in the central nervous system. Its effects on GABA A and GABA B receptors are different, for example, GABA A receptors are usually hyperpolarized via the ligand-gated Cl^- channel, resulting in decreased excitability. Acting via GABA B receptors, it suppresses Ca^{2+} influx by inhibiting VGCCs via G protein-coupled receptors (Gi/o) [35,4] and consequently reduces the release of excitatory neurotransmitters glutamate from presynaptic terminals [25].

In our experiment, we found that GABA (100 μM) reduced $[Ca^{2+}]_{in}$ by 14% in healthy rats. In the ADHD rat model, this attenuation was significantly greater than 20%, possibly due to compensatory activation of GABAergic inhibition in response to dopaminergic dysfunction, a characteristic feature of the ADHD model.

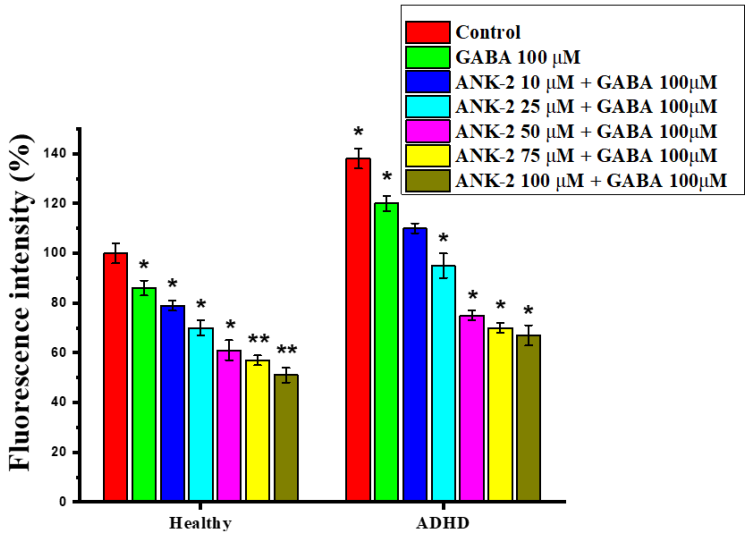

Figure 4 Effect of polyphenol ANK-2 (10 - 100 μM) on fluorescence intensity under conditions of GABA (100 μM) incubation in synaptosome suspension in healthy and ADHD models. Confidence level. *– $p < 0.05$; **– $p < 0.01$; ***– $p < 0.001$. (n = 6).

Subsequent studies have found that the polyphenol ANK-2, in addition to its inhibitory effect on GABA, further inhibits calcium influx in synaptosomes of healthy and ADHD model rats in a dose-dependent manner. This suggests that the polyphenol ANK-2 enhances GABAergic effects and may also result in greater reductions in $[Ca^{2+}]_{in}$ due to inhibition of VGCC by binding to allosteric sites on GABAergic inhibitory receptors. The polyphenol ANK-2 can also directly inhibit N-type or L-type VGCC, similar to mechanisms identified for other polyphenols such as quercetin or epigallocatechin gallate (EGSG) [36]. This may synergize with GABAergic inhibition in the synaptic environment, particularly in hyperactive states such as ADHD. Polyphenols reduce oxidative stress, which can affect calcium channel permeability and receptor sensitivity. Models of ADHD (e.g., 6-hydroxydopamine or cadmium exposure) demonstrate an imbalance in the dopaminergic-glutamatergic-GABAergic balance. In ADHD models, GABA treatment results in greater decreases in $[Ca^{2+}]_{in}$, possibly due to increased receptor sensitivity or altered intracellular Ca^{2+} buffering. The polyphenol ANK-2 normalizes synaptic calcium signaling by enhancing GABAergic inhibition and possibly directly modulating calcium channels, making it a promising candidate for neuroprotective drug development strategies in ADHD.

Levetiracetam is known to affect $[Ca^{2+}]_{in}$ concentrations by partially inhibiting calcium ion influx through N-type voltage-gated calcium channels and reducing calcium release from intraneuronal stores. This reduction in calcium influx results in a reduction in the release of the neurotransmitter glutamate from the presynaptic terminal. By reducing the release of excitatory neurotransmitters such as glutamate, levetiracetam helps reduce the excitability of neurons. By reducing the release of calcium from intracellular stores, levetiracetam further enhances its antiepileptic properties [37-39].

In our subsequent experiments, we investigated the effect of the polyphenol ANK-2 on the dynamics of synaptosomal $[Ca^{2+}]_{in}$ in the brain of healthy and model rats against the background of levetiracetam (**Figure 5**).

In our experiments, to study the effect of the polyphenol ANK-2 on calcium influx through N-type channels on synaptosomal membranes in the ADHD model, experiments were performed in the presence of levetiracetam using the fluorescent probe Fluo-4 AM. Pre-incubation of levetiracetam (5 μM) with the (Fluo-4 AM)-synaptosome suspension complex resulted in fluorescence quenching. It was found that the polyphenol ANK-2 (50 μM) incubated with levetiracetam (5 μM) significantly reduced the amount of calcium in the cytosol compared to the effect of levetiracetam (5 μM) (**Figure 5**).

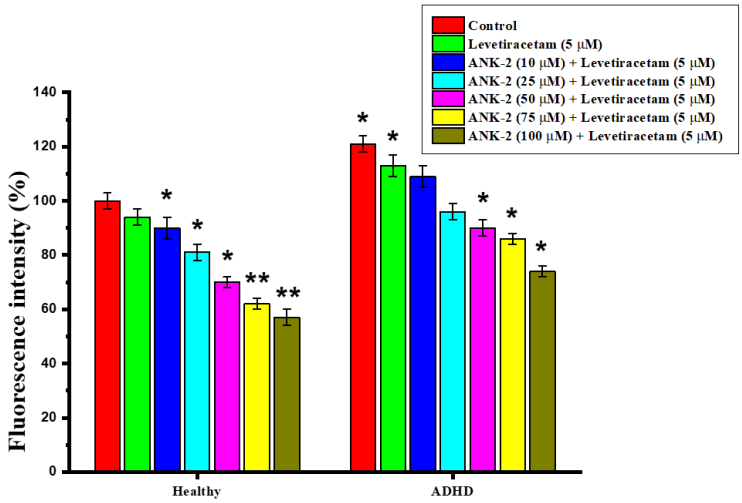

Figure 5 Effect of polyphenol ANK-2 (50 μM) on fluorescence intensity in synaptosomal suspensions of ADHD rat brains incubated with L-glutamate (50 μM) and levetiracetam (5 μM). Level of significance. *– $p < 0.05$; **– $p < 0.01$; (n = 6).

Levetiracetam primarily binds to CV2A (synaptic vesicular protein 2A), modulating the release of the neurotransmitter glutamate and inhibiting voltage-gated calcium channels, especially the N-type (CaV2.2). Preincubation with levetiracetam (5 μM) results in a decrease in Fluo-4 AM fluorescence intensity, indicating a decrease in intracellular Ca^{2+}. Levetiracetam inhibits presynaptic Ca^{2+} entry, which reduces the release of calcium-dependent neurotransmitter important in regulating hyperactivity in ADHD.

The polyphenol ANK-2 has additional modulatory effects on N-type Ca^{2+} channels, which may be mediated by direct interaction of this polyphenol with the channel structure, antioxidant effects that alter membrane fluidity and ion channel conformation, and synergistic enhancement of the inhibitory effect of levetiracetam. Co-incubation of levetiracetam (5 μM) + ANK-2 polyphenol (50 μM) further reduced cytosolic calcium levels compared to levetiracetam alone.

The polyphenol ANK-2 is a calcium channel antagonist that enhances the inhibition of N-type Ca^{2+} currents. This may result in a synergistic or additive neuroprotective effect observed in conditions of hyper-neural excitability such as ADHD.

The polyphenol ANK-2 enhances the calcium-blocking effect of levetiracetam, suggesting that it may act as a comodulator of presynaptic calcium entry through N-type channels. This may provide a new useful mechanism for regulating impulsivity in neurodevelopmental disorders such as ADHD. The calcium-suppressing property of the polyphenol ANK-2 suggests that it may enhance the inhibitory effect of GABA or directly modulate calcium channels, resulting in decreased neuronal excitability. As previously shown, the polyphenol ANK-2 can enhance GABAergic signaling, resulting in increased chloride flux through GABA A receptors. This hyperpolarization may decrease overall neuronal excitability and reduce calcium influx through voltage-gated calcium channels.

ANK-2 can also directly inhibit N-type calcium channels or other voltage-gated calcium channels, further reducing calcium influx. This action complements the effects of levetiracetam, resulting in a significant decrease in cytosolic calcium levels.

The polyphenol ANK-2 may increase the activity of the calcium-binding proteins calmodulin or parvalbumin, which helps buffer intracellular calcium levels and prevent excitotoxicity.

The results suggest that the polyphenol ANK-2 may have therapeutic potential in the treatment of ADHD symptoms by enhancing inhibitory signaling and reducing excessive calcium influx. This may help restore the imbalance between excitation and inhibition in the brain that occurs in ADHD.

Experimental data show that the polyphenol ANK-2 significantly reduces cytosolic calcium levels in the presence of levetiracetam, suggesting a potential

synergistic effect on calcium dynamics in ADHD models. This modulation may occur through enhanced GABAergic signaling and direct inhibition of calcium channels. The results may demonstrate the therapeutic potential of the polyphenol ANK-2 in correcting excitability-inhibition imbalance in ADHD models.

In order to determine the effect of the polyphenol ANK-2 used on the exchange of calcium ions in presynaptic endings as a result of its inhibition through potential-dependent sodium channels, we used the drug Lamotrigine. By stabilizing the membranes of presynaptic neurons, lamotrigine blocks the release of the excitatory neurotransmitter glutamate. This reduction in glutamate release helps prevent hyperexcitability signaling associated with various neurological diseases [30,40,41]. By modulating glutamate release, lamotrigine prevents excitotoxicity and stabilizes neural networks [42].

The experiments studied the effect of polyphenol ANK-2, used in ADHD models, against the background of lamotrigine (**Figure 6**). The experiments showed that under the influence of lamotrigine (2 μM), the fluorescence intensity decreases by 15%, which indicates a decrease in the content of cytosolic calcium, which corresponds to the known mechanism of its inhibition of sodium channels, depending on the presynaptic potential. When lamotrigine (2 μM) + glutamate (50 μM) and the polyphenol ANK-2 (50 μM) were added, the decrease in fluorescence intensity was only 10%, indicating complex interactions between the compounds.

It was found that the combined action of lamotrigine and the polyphenol ANK-2 could create a balance between the inhibitory effect (decrease in sodium/calcium flux by lamotrigine) and the excitatory effect, resulting in further attenuation of fluorescence. A 10% decrease in fluorescence intensity indicates that the combined action of lamotrigine and the polyphenol ANK-2 modulates calcium dynamics differently than the action of lamotrigine alone. The polyphenol ANK-2 was found to reduce glutamate-induced calcium influx by acting on both presynaptic and postsynaptic membrane calcium channels and partially counteracted the inhibitory effect of lamotrigine.

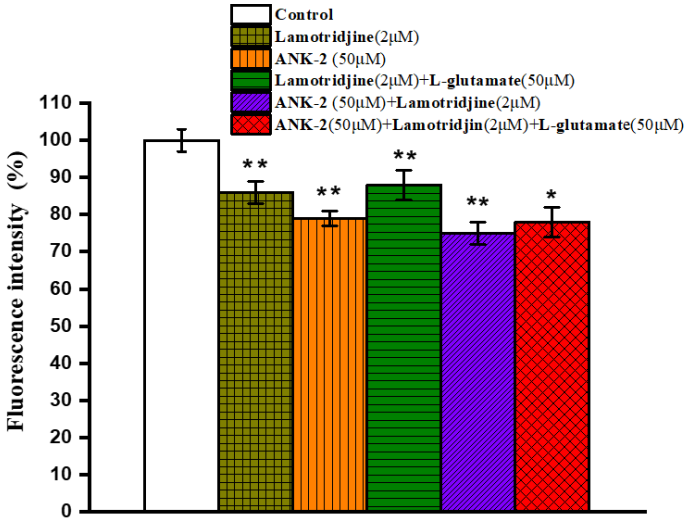

Figure 6 Effect of polyphenol ANK-2 (50 μM) on fluorescence intensity in synaptosomal suspensions of ADHD rat brains incubated with L-glutamate (50 μM) and lamotrigine (2 μM and 10 μM). Confidence level. *– $p < 0.05$; **– $p < 0.01$; (n = 6).

Conclusion

Experimental studies show that the polyphenol ANK-2 isolated from the local plant *Pistacia vera L.* significantly reduces the level of intracellular calcium in Fluo-4AM synaptosome suspensions from the brains of rats of different ages. This calcium-lowering effect can be explained by two main mechanisms, the first is that the polyphenol ANK-2 enhances the inhibitory effect of GABA, helping to reduce neuronal excitability. Second, the polyphenol ANK-2 likely acts as a direct or indirect

modulator of presynaptic calcium channels, particularly N-type Ca^{2+} channels, thereby reducing calcium influx. In addition, the polyphenol ANK-2 enhances the calcium-blocking effect of levetiracetam, which has a synergistic or comodulatory effect on calcium channel activity. This reveals a new and effective mechanism that helps regulate neuronal hyperexcitability in neurodevelopmental disorders such as ADHD. In addition, the inhibitory effect of the polyphenol ANK-2 in combination with lamotrigine leads to an increase in the effect of intracellular calcium, possibly due to cumulative inhibition of sodium and calcium currents. This effect may help restore the balance between excitatory and inhibitory signals in the brain.

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Declaration of Generative AI in Scientific Writing

The authors acknowledge the use of generative AI tools (e.g., QuillBot and ChatGPT by OpenAI) in the preparation of this manuscript, specifically for language editing and grammar correction. No content generation or data interpretation was performed by AI. The authors take full responsibility for the content and conclusions of this work.

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Kozokov Islom: Data curation, Formal analysis, Investigation, Validation, and Visualization.

Khoshimov Nozim: Conceptualization, Resources, Software, Funding acquisition, and Writing – review & editing.

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