Indian Journal of Biochemistry & Biophysics
Vol. 61, January 2024, pp. 32-38
DOI: 10.56042/ijbb.v61i1.5286

Interplay between NMDA receptor modulation and Na/K ATPase activity under the social isolation-induced stress in the hippocampus of male rats

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Received 31 August 2023; *revised 28 November 2023*

Living organisms exhibit heightened susceptibility to prolonged social isolation, leading to the onset of diverse pathological processes culminating in various diseases. The nervous system, notably sensitive to such alterations, may manifest neurodegenerative changes. Of particular interest in these conditions is the role of neurotransmitters within the central nervous system (CNS). Thus, our focus was directed towards the glutamate NMDA receptor, known for its pivotal involvement in synaptic plasticity and memory regulation.

Understanding the interplay between glutamate and its receptors, notably the NMDA receptor, with the Na^+/K^+-ATPase - an essential player in cellular homeostasis - remains insufficiently explored, especially under social isolation. Consequently, our study aimed to elucidate this relationship within the male rat brain's hippocampus.

Experimental subjects underwent either 30 days of isolation or remained in a communal cage as control animals. Assessing the expression levels of glutamate NMDA-receptor and Na^+/K^+-ATPase subunits *via* the western blot method.

Under prolonged social isolation, a surge in the phosphorylated NR2B subunits of the NMDA receptor indicated heightened receptor activation. Simultaneously, a substantial reduction in Na^+/K^+-ATPase activity was observed, potentially linked to decreased α1-subunit expression. These alterations hint at the NMDA receptor's activation and subsequent changes triggered by elevated intracellular Ca^{2+} as likely causes for the diminished Na^+/K^+-ATPase activity amidst prolonged social isolation.

Keywords: Mg^+-ATPase, Na^+/K^+-ATPase subunits, NMDA receptor, Oxidative stress

Numerous studies have established a close association between prolonged social isolation and the development of chronic stress. This chronic stress is linked to various health issues, including cardiovascular and digestive system pathologies, hormonal imbalances, and nervous system alterations. Moreover, extended social isolation can lead to neurodegenerative diseases, resulting in cognitive and behavioural changes, reduced pain sensitivity, increased aggression, impaired motor and spatial responses, memory and learning deficits, and other related processes[1,2]. Importantly, these effects often mirror symptoms seen in psychiatric conditions like depression[3,4].

Neurobiological changes due to social isolation affect various brain regions, notably the prefrontal cortex, amygdala, and hippocampus. Abnormalities in behaviour and biochemistry, linked to dopamine changes, were observed in socially isolated rats, possibly stemming from NMDA (N-methyl-D-aspartate) receptor disruption[5]. Social isolation contributes to aggressive behaviour, involving the prefrontal cortex. The hippocampus, like the prefrontal cortex, is sensitive to stress-induced isolation, evident in altered serotonergic and GABAergic (gamma-aminobutyric acid) systems. Similarly, the amygdala, another brain region, is implicated in isolation; prolonged isolation leads to changes, such as quantitative shifts in brain-derived neurotrophic factor (BDNF), in its inhibitory neurons after 24 days[6].

The NMDA receptor holds a particular interest in the context of social isolation. This receptor, an ionotropic glutamate receptor, plays a vital role in synaptic plasticity and the formation of memories. Unique to this receptor is its permeability to sodium,

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potassium, and calcium ions. Notably, calcium ions serve as secondary messengers, capable of modulating cellular metabolism in response to external signals. A defining feature of these receptors is their capacity to induce quantitative changes in intracellular Ca^{2+} ions. Consequently, the NMDA receptor assumes a critical role in regulating excitatory potential and is integral to cognitive function [7].

The heightened activation of this receptor leads to increased calcium transmembrane potential, releasing intracellular calcium stores, depolarizing the mitochondrial membrane, and causing a sustained rise in cytoplasmic ionized calcium. This sequence triggers irreversible cellular reactions, eventually resulting in elevated free radical levels and an increased risk of nerve cell damage. Notably, the activity of NMDA receptors is influenced by various molecules, ions, and enzymes[7], and, in turn, the NMDA receptor's activation level affects various enzyme functions. For instance, the interaction between the NMDA receptor and plasma membrane Na^+/K^+-ATPaseis well-documented[8,9]. Cardiotonic steroids, such as ouabain, can impact the NMDA receptor. For example, a two-day injection of Endobain E, a Na^+/K^+-ATPase inhibitor, in laboratory rats increases NMDA receptor expression in the hippocampus and cerebral cortex. However, the precise nature of their interaction under extreme conditions, like stress induced by social isolation, remains unclear.

Considering this, this study aimed to assess NMDA receptor activation during 30 days of social isolation-induced stress and its relationship with Na^+/K^+-ATPase enzyme activity in these circumstances.

Materials and Methods

Ethics approval

The study design was approved by the internal review board of the institution. The scientific research was carried out at Ivane Javakhishvili Tbilisi State University, Faculty of Exact and Natural Sciences, Department of Biology, in tight coordination of chairs of Biochemistry and Physiology.

Animals and social conditions

The study involved male white laboratory rats, weighing 150±15 grams, provided with standard laboratory chow and water. The vivarium was maintained at a temperature of 20-21°C, humidity at 47% ± 2, and preserved the natural circadian rhythm. In our study, ten rats were individually housed in cages measuring 380 × 180 × 190 mm (SI group). At the same time, control animals (10 individuals) were kept together in a cage of dimensions 595 × 500 × 200 mm (group C) for 30 days, as per Ieraci *et al*[1]. After 30 days of social isolation, the rats were decapitated, and their brains were removed under cold conditions. The hippocampus was then extracted and stored at -40°C.

Open field test

To assess stress-related behavioural shifts, an open-field test was performed. A circular enclosure measuring 1.5 m in diameter was employed for this experiment. The floor was divided into 12 outer squares and 6 inner squares, with a well-lit centre. Over 5 days, animals underwent 5-minute tests. Various behaviours, including vertical posture, and centripetal and horizontal movements, were observed for motor activity evaluation. Grooming duration and faeces count were also noted using visual observation [10].

Obtaining plasma membranes from hippocampal cells

The isolated hippocampus from the rat brain was rinsed in 0.9% NaCl solution under cold conditions and homogenized in 0.32 M sucrose solution (0.32 M sucrose + 0.5 M EDTA + 0.2 M PMSF). The obtained homogenate was centrifuged at 1000 g for 10 min. The supernatant was centrifuged again at 25 000 g for 30 min. The pellet was dissolved in 40 mM Tris-HCl solution (pH 7.4) and stored at -25°C.

Determination of Na^+/K^+-ATPase and Mg^{2+}-ATPase activity

Na^+/K^+-ATPase activity was calculated as the difference between total ATPase activity (Na^+/K^+, Mg^{2+}-dependent) and Mg^{2+}-dependent ATPase activity. Total ATPase activity was assayed at 37°C in an incubation medium consisting of 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 20 mM KC1, 4 mM $MgCl_2$, 3 mM disodium ATP and 80–100 µg protein of the plasma membrane, total 1 mL. Ouabain (1 mM) was added to the medium to determine the activity of the Mg^{2+}-ATPase. The values of Mg^{2+}-ATPase (1) with NaCl, KCl and $MgCl_2$, and (2) with $MgCl_2$ only, were compared. Specific Na^+/K^+-ATPase and Mg^{2+}-ATPase activities were computed by subtracting the blank from the overall activity and expressed in µmol Pi/mg protein/min[11].

Estimation of the kinetic parameters of the Na^+/K^+-ATPase and Mg^{2+}-ATPase

We defined the nature of changes in kinetic parameters of Na^+/K^+-ATPase and Mg^{2+}-ATPase (K_m, V_{max}) based on the affinity of the enzyme for the substrate, ATP and the change in the maximum reaction rate. To this end, we studied the kinetic parameters under variable ATP concentrations of 0.5–5.0 mm, respectively. We used the obtained results to measure the kinetic parameters using the Johansen and Lumry equations[12]:

$$V_{max} = \frac{\Sigma\frac{v^2}{s_2}\Sigma v^2 - \left(\Sigma\frac{v^2}{s}\right)^2}{\Sigma\frac{v^2}{s_2}\Sigma v - \Sigma\frac{v^2}{s}\Sigma\frac{v}{s}} \text{ and } K_m = \frac{\Sigma\frac{v^2}{s_2}\Sigma v^2 - \left(\Sigma\frac{v^2}{s}\right)^2}{\Sigma\frac{v^2}{s_2}\Sigma v - \Sigma\frac{v^2}{s}\Sigma\frac{v}{s}}$$

Where v is the enzymatic reaction velocity and s is the substrate concentration. The standard deviations of V_{max} and K_m were determined using the equations:

$$\delta^2(V_{max}) = \frac{v^2\delta^2_{exp}\sum w}{\sum w\sum ws^2 - (\sum ws^2)} \text{ and}$$

$$\delta^2(K_m) = V^2\delta\frac{2}{exp}\left[\frac{\sum ws^2 + 2Km\sum ws + Km^2\sum w}{\sum w\sum ws^2 - (\sum ws^2)}\right]$$

Where $\omega = v^2/s^2$, $\delta^2_{exp} = SS/(n-2)$ and $SS = \sum(v^2e^2)/s^2$, e is the standard deviation of the experimental measurements.

Western blotting analysis

Samples were subjected to 8 or 10 % SDS-PAGE, transferred to a nitrocellulose membrane, and probed with appropriate antibodies by standard procedures as described by Meparishvili *et al*[13]. The immunoreactive bands were developed and detected using enhanced chemiluminescence. For quantitative comparisons, images were scanned with a densitometer.

We have not normalized dataconcerningany other housekeeping protein in brain tissue samples, because it cannot be guaranteed that such proteins are not affected by our experimental condition. We have controlled gel loading by Ponceau S staining and Image J software analysis.

Statistical analysis

All statistical analyses were conducted using SPSS software (version 23, SPSS, Chicago, IL). One-way ANOVA was used to assess group differences in all physiological and biochemical values. Tukey HSD or Games-Howell post hoc test was performed to assess the differences between groups. The values are expressed as the mean ± SEM. P values less than 0.05 were considered statistically significant.

Results

The study of the psycho-emotional state of experimental animals under the conditions of long-term social isolation

Prolonged social isolation in animals represents a significant psychological and emotional stress factor [14]. Thus, as an initial phase of the study, we examined the impact of isolation on the animals' psychological and emotional well-being, assessed through research and fear reactions. Table 1 illustrates that the protocol induced notable physiological alterations, particularly an increase in the number of animals exhibiting heightened anxiety and emotional tension, as evidenced by increased fear responses and reduced exploratory behaviour[10].

The study of NMDA receptor subunits and their phosphorylation level under 30 days of social isolation

In the subsequent experiment, we assessed the expression of NMDA receptor subunits and their phosphorylated forms in plasma membrane samples from the hippocampus of both the control (C) and socially isolated (SI) animal groups. Figure 1 presents the results of Western blotting analysis for these proteins.

In probing the NR-1 subunit of the NMDA receptor, 30 days of social isolation yielded no consistent alteration in its expression level. However, there was a significant increase in the expression of its phosphorylated form (p-NR-1 (Ser 896)). Expression levels of NR-2B and NR-2A subunits within the NMDA receptor showed heterogeneity. As evident from (Fig. 1), 30 days of social isolation led to changes in the expression of the NR-2B subunit and

Table 1 — Physiological characteristics of animals under the stress caused by 30-daysocial isolation

Reactions	Physiological characteristics	Control (0-day stress)	30-day stress
Fear reactions	Defecation	3.6 ± 0.9	5.6 ± 0.3*
	Duration of grooming (sec)	3.7 ± 0.5	17.2 ± 1.4**
	Number of freezing movements	3.2 ± 0.8	9.8 ± 2.5**
	Duration of freezing movements (sec)	14.4 ± 2.6	21.4 ± 2.4**
Research reactions	Vertical posture	11.6 ± 2.4	3.2 ± 0.7**
	Duration of position in the centre (sec)	4.0 ± 0.7	2.2 ± 1.1*
	Number of positions in the centre	1.9 ± 0.4	0.8 ± 0.1*
	Number of centripetal movements	5.3 ± 1.0	0.6 ± 0.1**
	Number of horizontal movements	4.0 ± 1.5	0

$^*P \leq 0.05$; $^{**}P \leq 0.01$

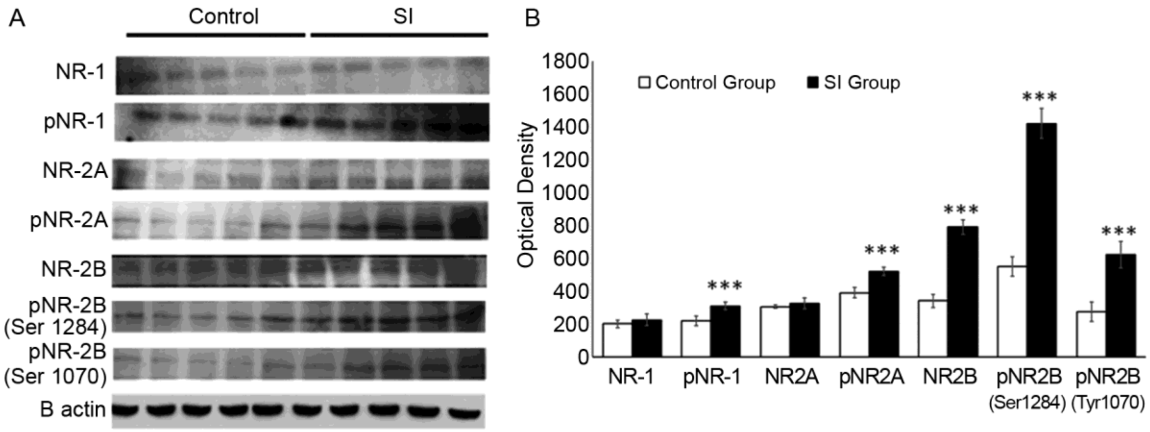


Fig. 1 — (A) Detection of NR-N1, p-NR- N1 (ser896), NR-A2, p-NR-A2 (Ser897), NR- 2B, p-NR-2B (Ser1284, Tyr1070) subunits of NMDA-receptor in the hippocampus by Western blotting analysis.; and (B) – Quantitative indicators of proteins; y-axis – the exposure index of optical density; Control–samples from the control group; SI – the samples from socially isolated rats. Data are shown as mean ± SEM. ****P*< 0.001 compared to the control

its two phosphorylated isoforms, namely p-NR-2B (Ser1284) and p-NR-2B (Ser1070). In these experimental conditions, the expression levels of the NR-2B subunit and these two isoforms were heightened. Concerning the NR-2A subunit of the receptor and its phosphorylated form p-NR-2A (Ser897), alterations were observed specifically in the phosphorylated form.

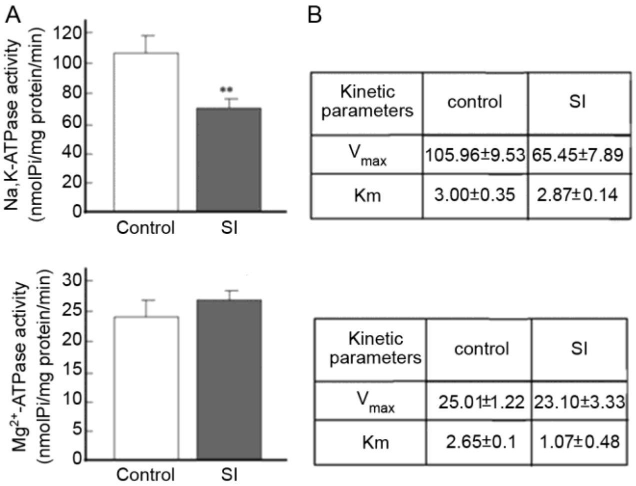
Changes in the activity of Na^+/K^+-ATPase and Mg^{2+-}ATPase of the plasma membrane of hippocampal cells under the conditions of social isolation

In light of these discoveries, the subsequent phase of the experiment investigated the effects of 30-day social isolation stress on the activity of Na^+/K^+-ATPase and Mg^{2+}-ATPase within the hippocampal plasma membranes of the experimental subjects. The resulting data are illustrated in (Fig. 2).

The data indicates a substantial alteration in hippocampal plasma membrane Na^+/K^+-ATPase activity, with a notable 45% reduction under the conditions of chronic stress induced by 30 days of social isolation (Fig. 2A). This change is primarily characterized by a slight decrease in the enzyme's V_{max}, while K_m remains unchanged. In contrast, Mg^{2+}-ATPase activity remained unaltered (Fig. 2B).

Quantitative changes in$Na^{+/}K^+$-ATPase subunits in the hippocampal plasma membranes under the conditions of 30 days social isolation

The expression levelsof Na^+/K^+-ATPase subunits in the hippocampal plasma membranes were studied in the following experiment in rats from the SI- and C-groups. The findings are shown in (Fig. 3).



Kinetic parameters	control	SI
V_{max}	105.96±9.53	65.45±7.89
Km	3.00±0.35	2.87±0.14

Kinetic parameters	control	SI
V_{max}	25.01±1.22	23.10±3.33
Km	2.65±0.1	1.07±0.48

Fig. 2 — (A) Changes in the activity of Na^+/K^+-ATPase and Mg^{2+}-ATPase in the plasma membrane of hippocampal cells under the conditions of social isolation; and (B) Changes in the enzyme kinetic parameters (Vmax, Km) under the conditions of social isolation. Data are shown as mean ± SEM. ****P*< 0.001 compared to the control

The results reveal unequal expression levels of enzyme subunits during the 30 days social isolation period. Specifically, a statistically significant reduction in the expression of the α1-subunit of Na^+/K^+-ATPase co exists with a notable increase in the expression of the α3-subunit. No significant changes were observed in the case of other subunits.

Discussion

The data presented in our study demonstrates that subjecting animals to social isolation for 30 days results in chronic stress, leading to observable

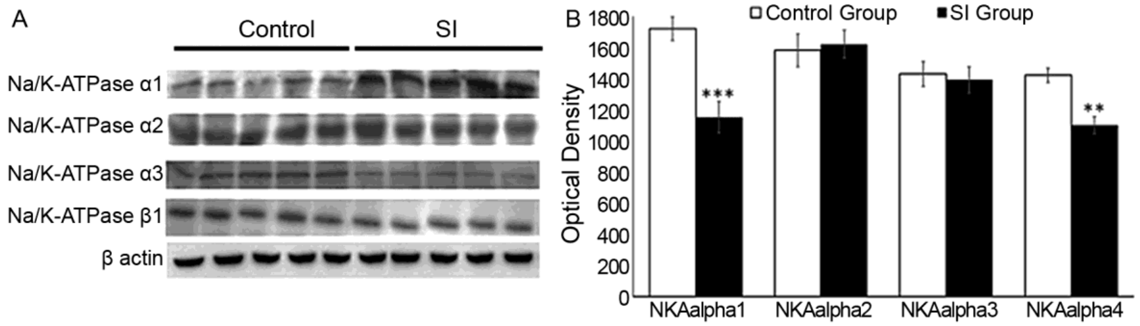


Fig. 3 — (A) Detection of the α1, α2, α3 and β1 subunits of Na^+/K^+-ATPase in the hippocampal plasma membranes by Western blotting; and (B) – Quantitative indicators; ordinate – the percentage of detected proteins. β-Actin is shown as a comparison control. The procedure and conditions of the method are given in the "Materials and Methods" section. Data are shown as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the control

changes in behaviour, such as decreased exploratory activity and heightened fear reactions (see Table 1). These outcomes align with existing research on physiological changes induced by chronic stress, even when considering different durations of social isolation (45, 60, 90 days)[15,16]. Hence, it appears that chronic stress may initiate within a relatively short period of 30 days.

Previous studies have established that varied durations of social isolation (45, 60, 90 days) disrupt an organism's psychological and emotional state, impacting neurotransmitter systems in the brain[17,18]. This emphasizes the importance of examining several systems including glutamate NMDA receptor. Our investigation reveals that 30-day social isolation not only affects physiological parameters but also introduces alterations in cell signalling. Specifically, we observed uneven changes in the expression levels of NMDA receptor subunits and their phosphorylated forms in the socially isolated group compared to the control group. Notably, there's an increase in the phosphorylated isoform of the NR1 receptor subunit while the expression of the non-phosphorylated subunit remains constant. The NR2A receptor subunit also demonstrates variability under these conditions, showing a marked increase in its phosphorylated isoform. Conversely, the NR2B subunit of the NMDA receptor and its phosphorylated forms exhibit elevated expression levels (refer to Fig. 1).

Notably, similar alterations in hippocampal and cerebral cortex cells have been reported in studies involving longer social stress conditions (90, 120 days)[19-23]. These changes are attributed to increased glutamate release and its subsequent interaction with postsynaptic receptors, actively contributing to stress-related processes.

Activation of the NMDA receptor is known to regulate intracellular sodium (Na^+) and potassium (K^+) levels, with subsequent restoration involving Na^+/K^+-ATPase[24]. Literature supports the connection between the NMDA receptor and Na^+/K^+-ATPase, particularly within neurons. It is believed thatNa^+/K^+-ATPase and the NMDA receptor may form complexes to rebalance ions following neuronal excitation. Pharmacological agents that regulate NMDA receptor activity also affect Na^+/K^+-ATPase function[23,24]. Additionally, interactions between NMDA receptors and PSD-95, PLC γ, PI3K, and tubulin have been observed, with these entities interacting with the α-subunit of Na^+/K^+-ATPase[22, 26-28]. However, contradictory data suggests a lack of a direct connection between these functional structures[28].

Given this context, our study aimed to explore the relationship between these two entities under the 30-day social isolation-induced stress.

It is well-established that oxidative processes can influence Na^+/K^+-ATPase activity, particularly through the oxidation of SH-groups, resulting in structural alterations and reduced enzymatic activity[29]. Markedly, our previous research indicates that 30-day social isolation induces oxidative stress and its associated effects[30]. Our findings, depicted in (Fig. 2), corroborate existing literature by illustrating a significant reduction in Na^+/K^+-ATPase activity during 30-day social isolation. In contrast, Mg^{2+}-ATPase activity remained unaffected throughout the study.

Both the α1- and α3-subunits of Na^+/K^+-ATPase are believed to play a role in the interaction between the pump and the NMDA receptor[24]. It is proposed that the inhibition of Na^+/K^+-ATPase by NMDA receptor activation relies on intracellular calcium (Ca^{2+}) levels, potentially activating protein kinase C and subsequently decreasing enzymatic activity[30-32].

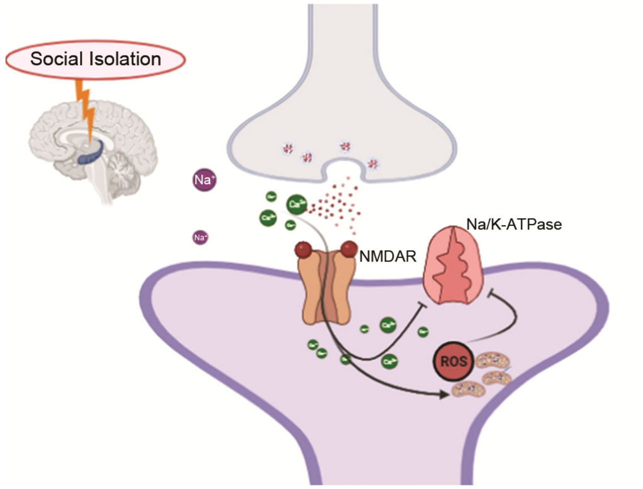


Fig. 4 — Possible impact of prolonged social isolation on Na/K-ATPase in the hippocampus *via* NMDA receptor activation and oxidative stress upregulation

In summary, our findings suggest a negative relationship between the functional status of the NMDA receptor and Na$^+$/K$^+$-ATPase activity during 30-day social isolation. This implies that NMDA receptor activation in the hippocampus during social isolation coincides with reduced Na$^+$/K$^+$-ATPase activity. Importantly, these alterations seem to predominantly affect specific subunits of Na$^+$/K$^+$-ATPase, particularly the α1- and β-subunits, as illustrated in (Fig. 3). This is in contrast to the α2 and α3 subunits, which remain unchanged. Previous studies from our group also indicate an elevation in Ca^{2+} levels during 30-day social isolation[33].

The α1-subunit plays a crucial role in the catalytic and transport functions of Na+/K+-ATPase, housing binding sites for cations, ATP, and the enzyme inhibitor ouabain. Conversely, the β subunit is essential for the enzyme's proper functioning, serving as a chaperone that ensures the correct orientation of the α1-subunit in the membrane, thus maximizing its activity[8,22].

In conclusion, our findings shed light on the impact of 30-day social isolation, revealing its potential to induce chronic stress and molecular changes in the central nervous system. Although the precise mechanisms governing the interaction between the NMDA receptor and Na$^+$/K$^+$-ATPase under stress induced by social isolation remain unclear, it is proposed that this interaction involves Ca-related alterations (Fig. 4) rather than a direct protein-protein interaction, as was proposed by some researchers. These findings carry implications for understanding the molecular basis of stress-related pathologies and lead to further research into specific molecular mechanisms and potential preventive measures.

Conflicts of interest

All authors declare no conflict of interest.

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