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Role of Supramammillary Nucleus Derived Substance P in Regulating Adult Hippocampal Neurogenesis and Depression and Anxiety-Like Behaviour

Jiayinuo Li

RCF Experimental School, Beijing, China
angela.li2024al@gmail.com

Abstract. Accumulating evidence showed the adult hippocampal neurogenesis (AHN) has played an essential role in regulating cognitive functions and emotions, while the other potential regulator such as the neuropeptides has still received less attention.. In the nestin-GFP mice, our data showed that the SP-overexpressed SuM neurons inhibited the proliferation of radial neural stem cells (rNSCs) and reduced the cell densities of immature and proliferating neurons in the dentate gyrus (DG). All of these processes were accompanied by depression and anxiety-like behaviours, including the reduced exploration, the lower sucrose preference, and the prolonged immobility. However, the neurokinin-1 receptor (NK1-R) antagonist could reversed those behavioural deficits. Together, our study demonstrated that SP was an essential inhibitor of AHN and emotional behaviours, while the NK1-R antagonism may provide a potential therapeutic strategy for depression and anxiety-like behaviours.

Keywords: adult hippocampal neurogenesis, supramammilary nucleus, substance P , neurokinin-1 receptor, depression and anxiety-like behaviours

1. Introduction

It is well known that anxiety and depression are prevalent mental disorders around the world. According to the World Health Organization, an estimated 3.8% of the population (including about 280 million adults) experiences depression, and 300 million individuals suffer from anxiety. More than 720,000 people die due to suicide every year. These disorders impair quality of life and daily functioning, leading to increased risks of self-harm, and can ultimately lead to suicide. As a result, these disorders impose a heavy economic burden due to reduced productivity and increased healthcare costs. Thus, there is an urgent need for effective treatments, highlighting a critical public health concern. However, existing medications fail to provide sufficient relief for all patients. For instance, aselective serotonin reuptake inhibitors (SSRIs) are widely used nowadays [1]. SSRIs functions by blocking the reuptake of serotonin in the synaptic gap, which makes serotonin remains between neurons for a longer time to promote transmission of the signals. This increases serotonin availability and improves mood in depressed patients. However, there is usually a delay of 2 - 6 weeks before clinical effects appear, which is why many patients choose to discontinue treatment.

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Besides, up to 30-40% of patients show only partial or no response. Moreover, there are several side effects such as weight gain, sexual dysfunction, and sleep disturbances [2]. Such adverse effects often lead to poor treatment adherence. Psychological interventions, such as cognitive behavioural therapy (CBT), are effective but still have limitations. They are usually expensive and require long-term commitment. These challenges emphasise the need for new therapeutic strategies. To address this challenge, researchers have shifted their focus toward the neurobiological mechanisms underlying mood regulation. They have found that disabilities in emotional regulation are closely associated with neuroplasticity, especially in the hippocampus. It is widely recognised for its role in memory formation, spatial navigation, and emotional processes. Within the hippocampus, radial neural stem cells (rNSCs) continue to generate new neurons in the dentate gyrus (DG). The supramammillary nucleus (SuM) projects to the DG as well as to the medial septum and lateral hypothalamus, which makes the SuM significant in linking arousal, reward, and memory circuits. This process is known as adult hippocampal neurogenesis (AHN). An increasing number of studies have demonstrated that AHN plays an important role in cognitive functions and emotional regulation [3,4]. In particular, stress resilience and mood regulation can be improved by enhancing AHN. However, the underlying neuromodulators that regulate AHN are not fully understood. Although in previous studies, they have shown that signallings sent from the SuM neurons to DG in the hippocampus can activate rNSCs and promote their proliferation, which is driven by glutamate, not GABA, neuropeptides like substance P (SP) have gained attention as potential regulators of hippocampal plasticity [5]. While glutamatergic inputs can enhance AHN, SP may act as an opposing signal [6]. It is being released from the SuM and functioning via neurokinin-1 receptor (NK1-R), modulating stress, pain and emotions [7,8]. Although the involvement of SP in emotional regulation has already been recognized [9,10], there remains a knowledge gap as to whether SP influences anxiety and depression-like behaviour by modulating AHN. Therefore, discovering the mechanism by which SP modulates AHN and its effects on anxiety and depression may deepen our understanding of the pathophysiology of emotional disorders and pave the way for new therapeutic targets.

2. Experimental design

This experiment was designed to investigate the role of SP in regulating AHN through the SuM-DG pathway and its effects on depression and anxiety-like behaviour. Nestin-GFP mice were randomly divided into three groups. The control group was established for better comparison and received vehicle injection and saline treatment; the over-expression (OE) group was established to examine the effect of increased SP signalling and received stereotaxic injection of AAV-hSyn-SP. AAV is commonly used for efficient gene delivery into neurons. It has low immunogenicity and long-term expression, making it ideal for in vivo studies. The hSyn promoter can ensure neuron-specific expression, avoiding non-specific effects in glial cells. Spread of virus can be tracked by co-expressing a fluorescent marker like GFP. While the receptor antagonist (RA) group was included to evaluate the effect of blocking the receptor of SP, NK1-R. L822429 is a selective antagonist of NK1-R and it has been shown to reduce anxiety and depression-like behaviour. Repeated daily injections allowed for short-term, reversible blockade of SP signalling. Then, behaviour tests were carried out to test for behaviour of the mice. In open field test (OFT), total distance travelled by mice was measured to reflect their locomotor activity and reduced centre exploration may result from anxiety or less motivation. In elevated plus maze (EPM), number of entries in open arm was used to evaluate level of anxiety. In sucrose preference test (SPT), total fluid intake was measured to

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calculate sucrose preference of the mice. In forced swim test (FST), immobility time was analysed the level of depression.

2.1. Animal model

Nestin-GFPA mice were chosen as the experimental model to visualise rNSCs and neural progenitors in the DG, which allowed tracking of neurogenesis and cell proliferation via fluorescence imaging. 8-10-week-old male mice were selected to reduce hormonal variability.

2.2. Viral injection

For the OE group, as shown in Figure 1a, AAV-hSyn-SP was stereotaxically injected into the SuM region under anaesthesia [11]. The injection was performed at a rate of 30-50 nl/min with a volume of 0.3-0.5μl per site to avoid diffusion. Then allowed 3-4 weeks recovery for stable viral expression. All operations were carried out under sterile conditions to minimise the risk of infection.

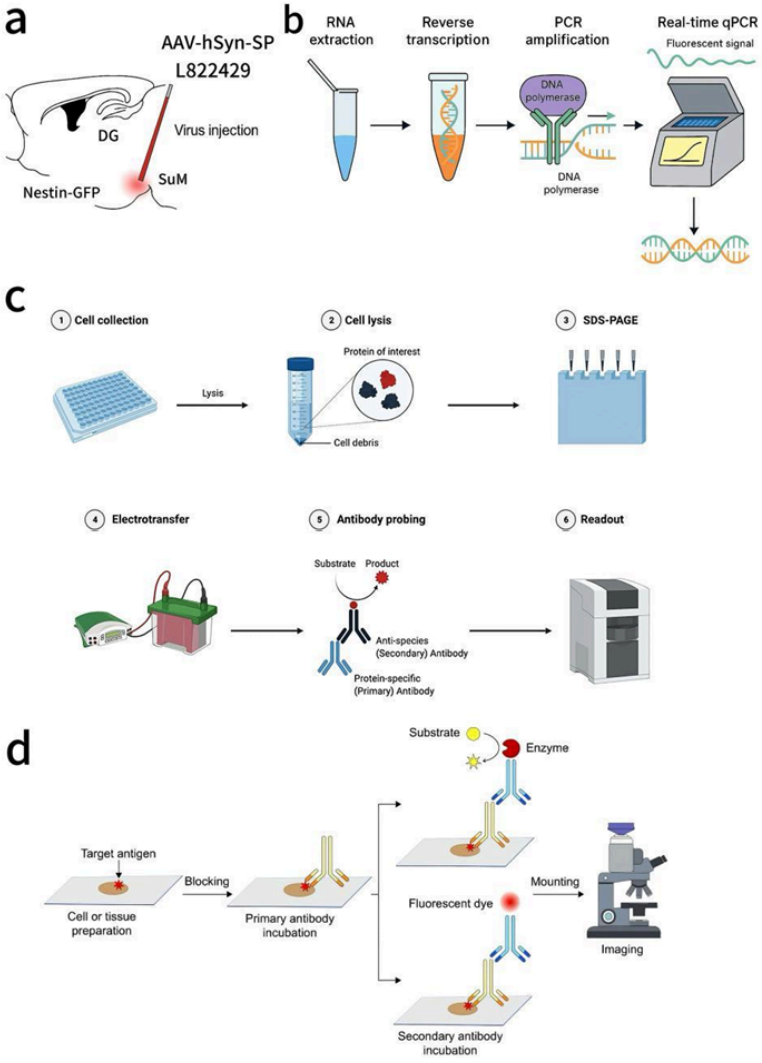

Figure 1. Experimental techniques

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Diagram of AAV-hSyn-SP and L822429 injected stereotaxically into the SuM region. (b-c) Testings for level of mRNA (b) RT-qPCR and expressed SP (c) Western blot. (d) Procedure of immunostaining.

2.3. Drug administration

For the RA group, in Figure 1a, L822429 was injected into the SuM region once per day after the viral recovery period (Day 21-28). The control and OE group received an equal volume of saline during the same period to ensure that all groups undergo similar injection procedure and eliminate stress-related confounding factors.

2.4. RT-qPCR shown in Figure 1b

Step 1: Extract and purify RNA from tissues.

Step 2: RNA was converted into complementary DNA (cDNA) using reverse transcriptase.

Step 3: Perform PCR amplification using specific primers and fluorescent dyes.

Step 4: Monitor the amplification in real time via fluorescent signals. Fluorescence intensity correlated with the amount of DNA, which allowed us to quantify gene expression levels accurately.

2.5. Western blot shown in Figure 1c

Step 1: Harvest cells or tissues containing SP.

Step 2: Break open cells with lysis buffer to release proteins, debris was removed by centrifugation.

Step 3: Separate proteins by size using gel electrophoresis.

Step 4: Transfer separated proteins from the gel to a PVDF or nitrocellulose membrane.

Step 5: Block non-specific sites on the membrane. Add primary antibody to bind the target protein. Add enzyme-linked secondary antibody for detection.

Step 6: Visualise protein bands using a chemiluminescence imaging system.

2.6. Behaviour tests

Behaviour tests were conducted during Day 28-35. A 24-hour interval was maintained between each behaviour test to avoid fatigue. Mice were provided with adequate water and food except during the sucrose preference test.

2.6.1. Anxiety tests

In Figure 2a, open field test was carried out in a 50*50 cm square chamber, consisting a peripheral and central zone.The mice were placed in the centre of the chamber, and their free exploration was recorded for 5-10 minutes using a tracking system. Time spent in two zones reflects the level of anxiety.

In Figure 2b, the plus maze was consisted of two open arms and two closed arms (each 30*5 cm), elevated 50 cm above the ground. The mice were placed on the central platform facing an open arm and allowed to explore freely for 5 minutes. We recorded the time spent and number of entries into open and closed arms.

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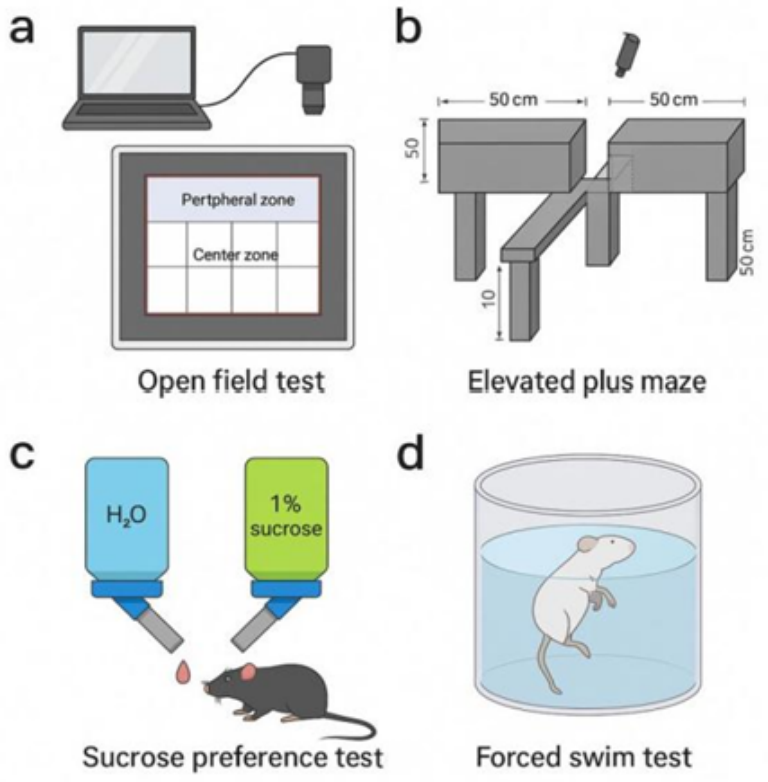

Figure 2. Experimental set up. (a-b) Anxiety behaviour tests. Open field test (a) and elevated plus maze (b). (c-d) Depression behaviour tests. Sucrose preference test (c) and forced swim test (d)

2.6.2. Depression tests

In sucrose preference test, 24 hours before official testing, two bottles were filled with water. This allowed the mice to become familiar with the setup and reduces novelty-induced stress. In Figure 2c, one of the bottles was switched into 1% of sucrose solution, switching positions daily to prevent side bias. Sucrose preference was calculated by using the formula sucrose intake/total fluid intake *100% [12].

In forced swim test, A transparent cylindrical tank with 20-25 cm diameter, 15-20 cm water depth, and 25±1°C water temperature. In Figure 2d, each mice was placed into the water tank individually for 6 minutes. The entire procedure was video-recorded, and immobility time during last 4 minutes was analysed [13].

2.7. AHN measurement

After behaviour tests were finished (Day 35), the mice were anesthetised and transcardially perfused with saline and 4% paraformaldehyde (PFA). In Figure 1d, Nestin$^+$ (rNSCs), DCX$^+$ (immature neurons), and Ki67$^+$ (proliferating cells) were labelled by using immunostaining [14]. Images of the DG were captured under a con-focal microscope and cell densities were analysed.

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3. Expected results

3.1. Possible result 1: SP reduced AHN, and this reduction was partially restored by NK1-R antagonism

In Figure 3, Overexpression of SP reduced cell densities of Nestin$^+$, DCX$^+$, and Ki67$^+$ cells, while NK1-R antagonist treatment restored the levels partially and approach to the control group level, suggesting reversibility of SP-induced inhibition, which was consistent with the hypothesis that SP suppresses neurogenesis via NK1-R signaling.

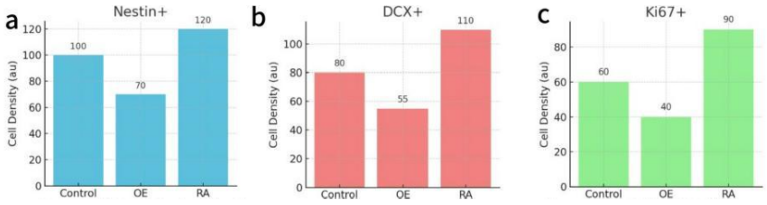

Figure 3. Bar charts for results of immunostaining in three groups. Cell densities of rNSCs (a), immature neurons (b) and proliferating cells (c) in the DG

3.2. Possible result 2: SP overexpression reduced exploration and induced anxiety-like behaviour, whereas RA treatment enhanced locomotion and open-arm exploration

In open field test, SP overexpression group spent significantly less time in the central zone and showed reduced locomotion compared to the control group. RA treatment increased centre exploration and overall activity. Those data was shown in Figure 4.

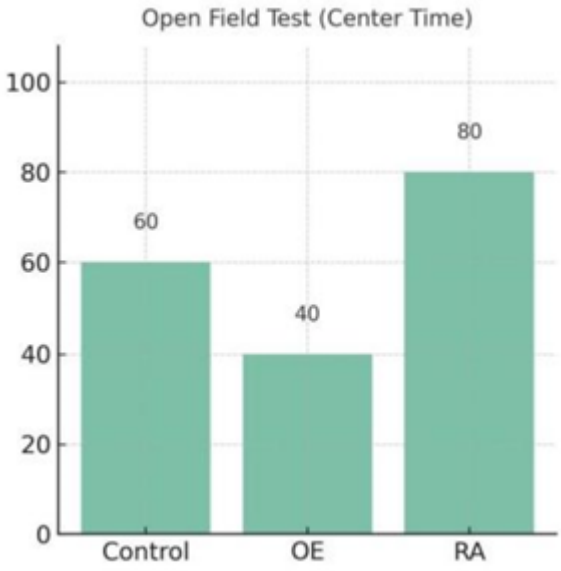

Figure 4. Open field test results. Time spent in centre zone was measured

In elevated plus maze test, as Figure 5 indicated, SP overexpression mice spent less time and made fewer entries into open arms, whereas RA mice entered open arms more frequently.

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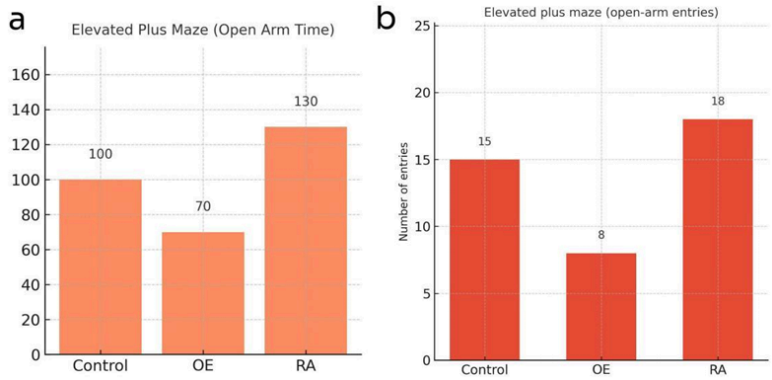

Figure 5. Elevated plus maze test results. Time spent in open arms (a) and open-arm entries (b) were tested

3.3. Possible result 3: SP overexpression induced anhedonia and behavioural despair, while RA treatment alleviated these effects by restoring sucrose preference and reducing immobility, indicating an antidepressant effect

In sucrose preference test, demonstrated in Figure 6, OE mice showed lower sucrose preference comparing to RA group. RA group restored preference closer to control level.

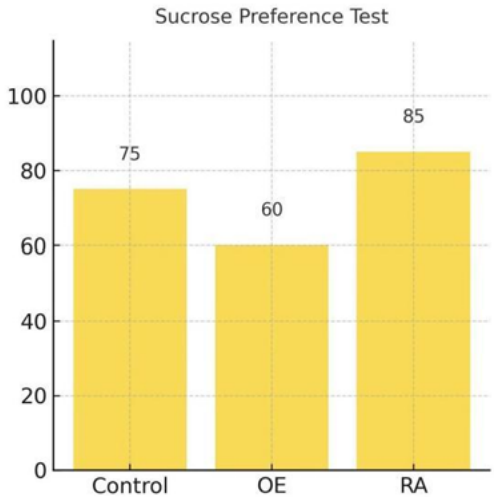

Figure 6. Sucrose preference calculated by using the formula sucrose intake/total fluid intake *100%

In forced swim test, OE mice showed longer immobility duration, whereas RA mice exhibited longer struggling time and reduced passive floating, shown in Figure 7. Changes in the results further supported the antidepressant-like effect of NK1-R antagonism.

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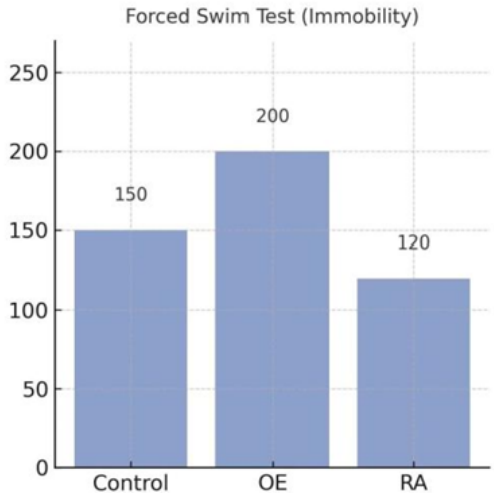

Figure 7. Forced swim test results. Immobility time was recorded

4. Discussion

Our expected results indicated that SP over-expression significantly reduced the density of Nestin$^+$, DCX$^+$, and Ki67$^+$ cells, while NK1-R antagonist had reverse effects. Behaviourally, SP over-expression induced depression and anxiety-like behaviour, as shown by performance in OFT, EPM, SPT, and FST. The reduced locomotion in central zone and open arms in OE group indicated increased anxiety, while low sucrose preference and longer immobility time suggested increased depression. These interpretations align with previous studies showing that stress-related substance P inhibits hippocampal plasticity and contributes to mood disorders. The reverse effects observed in RA group highlights the antidepressant effects of NK1-R antagonist. One possible mechanism is that SP activation of NK1-R increases the release of stress hormone via the HPA axis, thereby inhibiting rNSCs proliferation in the DG [15,16]. SP may also increase the expression of inflammatory cytokines. Neuroinflammation has been shown to suppress AHN [17]. Alternatively, AP might inhibit hippocampal brain-derived neurotrophic factor (BDNF), which affects neuroplasticity [18-20]. As previous papers have investigated that glutamatergic signalling from the SuM neurons can enhance AHN [5], the contribution of neuropeptides like SP may provide a complementary regulatory pathway in modulating the DG. Despite these insights, our study has several limitations that need to be addressed. Our interpretations are based on expected results only, lacking actual experimental data. Moreover, only short-term effects will be tested, long-term impacts remain unknown. AAV injection may cause local inflammation as well. The underlying mechanism is only partially understood, and the molecular pathway by which SP affects AHN is still unclear. To strengthen the evidence, further studies should perform molecular analyses to identify down stream targets of SP signalling and explore therapeutic potential of NK1-R antagonists in combination with classical antidepressants.

5. Conclusion

In summary, our study showed that SP-NK1-R signalling within the SuM-DG pathway played a crucial role in regulating AHN and modulating anxiety and depression-like behaviours. Based on our expected outcomes, SP overexpression suppressed the rNSC proliferation and reduced the density of immature and proliferating neurons in the DG, ultimately contributing to mood-related

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deficits. Conversely, NK1-R antagonist restored both neurogenic and behavioural parameters to control levels, highlighting its potential role as the therapeutic strategy for anxiety and depression-like behaviours. These findings emphasised the importance of neuropeptides, particularly SP in emotional regulation. Future research would aim to validate these effects on chronic stress models and further explore the long-term potential therapy through targeting the SP-NK1-R signalling.

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