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Exploring the influence of cannabinoid system activation on axon sprouting: A study of ATRX, STK24, GDF10, RTN4, and PTEN proteins

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Axonal damage in the central nervous system (CNS) often results in long-term neurological impairments due to the limited regenerative capacity of neurons. Identifying mechanisms and therapeutic agents that promote axon sprouting is essential for advancing treatments for neurological disorders. Cannabinoids, through their interaction with CB_1 and CB_2 receptors, have been implicated in neuronal development and regulation. Numerous studies have demonstrated that proteins analyzed in this study, including ATP-dependent helicase (ATRX), Serine/threonine-protein kinase 24 (STK24), Growth differentiation factor 10 (GDF10), Reticulon 4 (RTN4), and Phosphatase and tensin homolog (PTEN), play a crucial role in axon sprouting. The objective of this study is to determine whether the cannabinoid system, in conjunction with ATRX, STK24, GDF10, RTN4, and PTEN proteins, collectively influences axon sprouting. Therefore, the effect of Δ-9-THC on the expression of ATRX, STK24, GDF10, RTN4, and PTEN proteins is examined. For this purpose, the neuronal cell line model (SH-SY5Y) was grown in culture and treated with Δ-9-THC. The amounts of related proteins were measured by ELISA method and compared with control group. The administration of Δ-9-THC significantly ($P < 0.05$) increased the levels of ATRX, STK24, and GDF10 proteins, whereas it had no significant effect on RTN4 and PTEN proteins. Given the stimulating role of ATRX, STK24, and GDF10 proteins in axon sprouting, it is reasonable to speculate that the activation of the cannabinoid system may enhance axon sprouting. We anticipate that these findings will contribute to future studies aimed at addressing nerve cell losses in conditions such as stroke, ischemia, Alzheimer's, and Parkinson's.

Keywords: Axon growth, Cannabinoid receptors, Delta-9-THC, ELISA, SH-SY5Y cell line

The central nervous system of a healthy adult is characterized by a vast network of harmoniously interconnected nerve cells. These nerve cells, at the end of their developmental stage, become highly specialized and generally remain post-mitotic. Recent findings indicate that the brain possesses the capability to generate new nerve cells, and these neurons can establish connections with existing nerve cells[1,2]. Damage to the central nervous system in adult mammals frequently results in enduring impairments with restricted potential for improvement. The primary reason for the lack of successful recovery lies in the limited ability of the adult brain and spinal cord to facilitate the regrowth and restructuring of axonal connections[3]. Axon growth can be stimulated by various treatments. The most straightforward growth response occurs when an axon extends from the cut end itself[3]. The neuroanatomical definition of axonal sprouting also prompts questions about the cells and molecules that regulate this process. Proteins that are active during the completion of nervous system development in the embryonic period have been the focus of extensive studies to shed light on this matter.

The regulation of DNA structure and gene expression through epigenetic mechanisms plays a significant role in brain development, the differentiation of neural stem cells, and the adaptability of neural circuits in response to activity[4]. ATP-dependent helicase (ATRX) is among the genes that experience substantial induction in neurons during the process of sprouting after a stroke and is also heightened in young developing neurons. When ATRX function is compromised during brain development, it results in neural cell death and disrupts the migration of neural precursor cells, leading to significant alterations in brain development[5].

Many studies show that axon growth involves a purine-sensitive protein kinase. Multiple studies have demonstrated that Serine/threonine-protein kinase 24 (STK24), one of these kinases, undergoes swift

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activation when embryonic cortical neurons or PC12 cells are exposed to neurotrophins. Furthermore, blocking the expression or activity of STK24 has the effect of preventing these cells from extending their axons in response to inosine or trophic factors[6]. Recent studies have revealed that growth differentiation factor 10 (GDF10) is induced in peri-infarct neurons in mice, non-human primates, and humans. Additionally, it has been demonstrated that GDF10 promotes *in vitro* axonal growth in neurons from mice, rats, and humans through transforming growth factor beta receptor I (TGFβRI) and transforming growth factor beta receptor II (TGFβRII) signaling[7]. In studies on axon sprouting, researchers investigate proteins and receptors that inhibit axon growth, as well as proteins that promote axonal growth. The primary role of these proteins is to halt axon growth when it encounters other axons. It is believed that inhibiting these proteins can potentially enhance axon sprouting. Two such proteins, Reticulon 4 (RTN4), which is the ligand for Nogo receptor 1, and Phosphatase and tensin homolog (PTEN), have been the focus of research for potential treatments[8,9]. Cannabinoids function by interacting with cannabinoid receptors found within the central nervous system[10,11]. Within the central nervous system, there exists an endogenous cannabinoid system, which encompasses two primary types of receptors known as CB_1 and CB_2. The involvement of this system in the development, control, and proliferation of neurons in the central nervous system has been substantiated by numerous studies[12,16].

The proteins ATRX, STK24, GDF10, RTN4, and PTEN, which are examined in this study, are involved in the regulation of neuronal development[6,17-19]. Elevated levels of ATRX, STK24, and GDF10 proteins promote axon sprouting, whereas increased levels of RTN4 and PTEN proteins inhibit axon sprouting. However, it remains uncertain whether there is any connection between the cannabinoid system and ATRX, STK24, GDF10, RTN4, and PTEN proteins and whether they collectively contribute to neuron proliferation and development. Hence, the primary objective of this study is to investigate the impact of the cannabinoid system on proteins influencing neuronal regeneration and to assess the therapeutic potential of Δ-9-THC in treating conditions characterized by neuronal damage, such as Alzheimer's disease, Parkinson's disease, and ischemia.

Materials and Methods

Cell culture

The undifferentiated SH-SY5Y neuroblastoma cell line (a thrice-cloned subline of the neuroblastoma cell line SK-N-SH, which was established in 1970 from a metastatic bone tumor from a 4 year old cancer patient) was procured from ATCC. Cell line authentication was conducted through STR DNA profiling utilizing Powerplex 16 and cell lines used were tested for the presence of mycoplasma. Cell line was incubated at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, high glucose, 4500 mg/L glucose, L-glutamine, and sodium bicarbonate, without sodium pyruvate) enriched with 10% fetal bovine serum (Sigma-Aldrich), 20 mM glutamine (Sigma-Aldrich), penicillin (Sigma-Aldrich), and streptomycin (Sigma-Aldrich) for a period of 24 h. Following this initial 24 h period, the culture medium was replaced with the same medium, but with the addition of 10μM retinoic acid (Sigma-Aldrich). This process was repeated every 48 h until the end of the first week, thereby enabling differentiation of the SH-SY5Y neuroblastoma cell line. After differentiation, the mediums of the flasks were changed every 48 h and the flasks in the experimental group were given Δ-9-THC (Sigma-Aldrich, stock solution 1.0 mg/mL in methanol) (1, 5, 10, 20 μM) (dissolved in DMSO 1%) with the medium. This process was repeated 5 times and differentiated cells were kept in culture (37^0C temperature and 5% CO_2) for 10 days. After the cells were treated with Δ-9-THC for 10 days, the cells were collected for total protein isolation and quantification of the relevant proteins.

Cell homogenization

The cells were treated with a solution consisting of Radio-Immunoprecipitation Assay buffer (3 mL/g), phenylmethanesulfonylfluoride (30μL/g), sodium vanadate (30μL/g) and protease inhibitor (30μL/g) and homogenates were obtained by breaking the cells on ice with an ultrasonic lysis device. The homogenates were centrifuged at 10,000 RPM for 10 min, the supernatants were taken and the pellets were discarded.

Protein quantification

The quantification of proteins in the homogenized cell samples was carried out using the Bradford method to standardize ELISA experiments. To create the standards, concentrations of 1.5625, 3.125, 6.25, 12.5, and 25 μg/mL were prepared using bovine

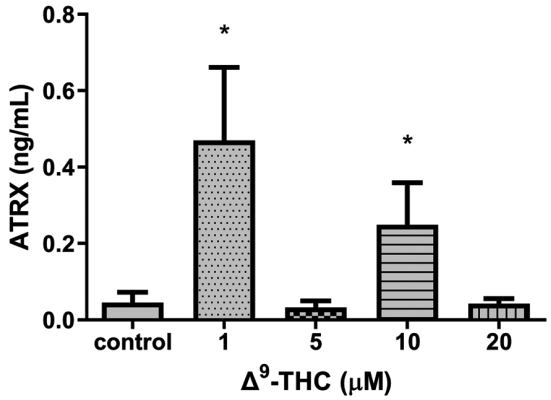

Fig. 1 — The impact of Δ-9-THC treatment on ATRX expression. [n=6 for each group; *: For control $P < 0.05$]

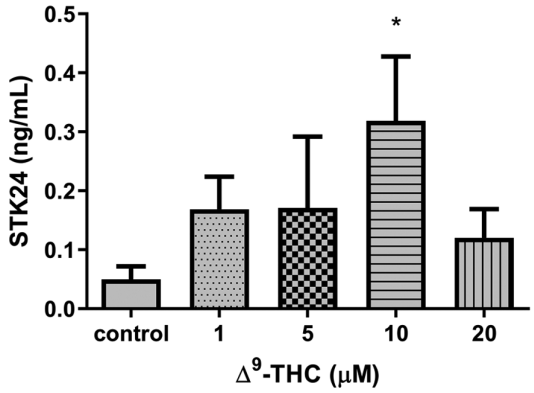

Fig. 2 — The impact of Δ-9-THC treatment on STK24 expression. [n=6 for each group; *: For control $P < 0.05$]

serum albumin. Subsequently, 10 µL was extracted from each sample and diluted to a total volume of 100 µL with distilled water. Following this, 1 mL of Bradford solution was added to both the standards and the samples, and they were thoroughly mixed by vortexing. Absorbance values were then measured at a wavelength of 595 nanometers using a spectrophotometer. Protein quantification was performed based on the standard curve that was generated.

ELISA experiments

For the ELISA test, the procedure specified in the supplied kit (Sunred Biotechnology) was applied. ELISA kits employ the sandwich enzyme immunoassay. Linear standard curves are drawn according to the manufacturer's recommendations for each specified protein.

Statistical analysis

The results were presented as means±Standard Error of the Mean (S.E.M.), with 'n' indicating the number of cell cultures employed for each distinct group. To assess differences in the outcomes between the control and Δ-9-THC treatment groups, a Student's *t-test* was conducted using Graph pad Prism software (Graphpad, California, USA). *P*-values below 0.05 were regarded as statistically significant.

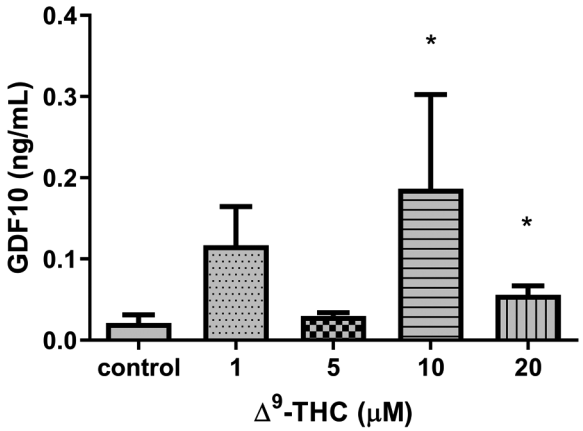

Fig. 3 — The impact of Δ-9-THC treatment on GDF10 expression. [n=6 for each group; *: For control $P < 0.05$]

Results

ELISA ATRX protein quantification

The effects of Δ-9-THC on ATRX protein levels were evaluated using ELISA. A significant increase in ATRX levels was observed in the 1 µM and 10 µM treatment groups compared to the control ($P < 0.05$). However, no statistically significant changes were observed in the 5 µM and 20 µM treatment groups (Fig. 1). These results indicate that lower and moderate concentrations of Δ-9-THC can upregulate ATRX expression, whereas higher doses may not yield the same effect.

ELISA STK24 protein quantification

The effects of Δ-9-THC on STK24 protein levels were evaluated using ELISA. A significant increase in STK24 levels was observed in the 10 µM treatment group compared to the control ($P < 0.05$). However, no statistically significant changes were observed in the 1 µM, 5 µM and 20 µM treatment groups (Fig. 2). This suggests that STK24 expression may require a specific threshold concentration to be upregulated effectively.

ELISA GDF10 protein quantification

The effects of Δ-9-THC on GDF10 protein levels were evaluated using ELISA. A significant increase in GDF10 levels was observed in the 10 µM and 20 µM treatment groups compared to the control ($P < 0.05$). However, no statistically significant changes were observed in the 1 µM and 5 µM treatment groups (Fig. 3). The dose-dependent increase at 10 µM and

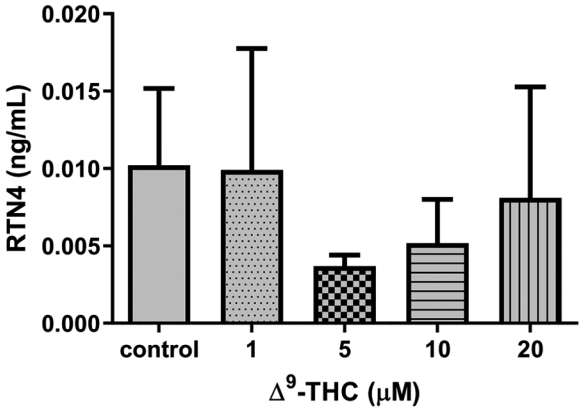

Fig. 4 — The impact of Δ-9-THC treatment on RTN4 expression. [n=6 for each group; all groups $P > 0.05$]

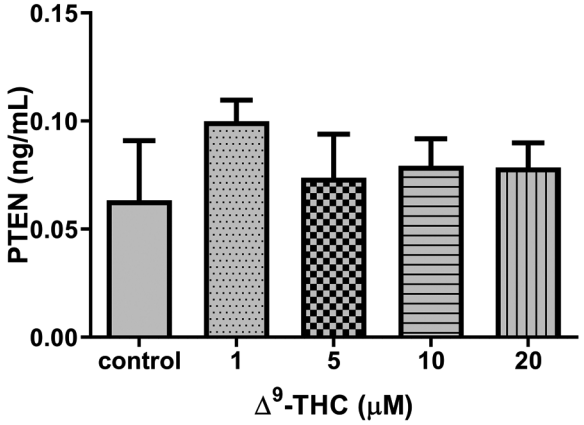

Fig. 5 — The impact of Δ-9-THC treatment on PTEN expression. [n=6 for each group; all groups $P > 0.05$]

above implies that GDF10 expression may be more sensitive to higher concentrations of Δ-9-THC.

ELISA RTN4 protein quantification

No significant changes were observed in RTN4 protein levels across any treatment groups, indicating that Δ-9-THC does not influence the expression of this inhibitory axonal sprouting factor (Fig. 4).

ELISA PTEN protein quantification

Similar to RTN4, PTEN protein levels remained unaffected by Δ-9-THC treatment at all tested concentrations, suggesting no modulatory effect of Δ-9-THC on this axon growth inhibitor (Fig. 5).

Discussion

Axon sprouting can be stimulated by various treatments following diseases that lead to neuronal damage[20,21]. The neuroanatomical definition of axonal sprouting also prompts questions about the cells and molecules involved in regulating this process. The proteins that play a role in the development of the nervous system during the embryonic period have been extensively investigated and are among the most studied in efforts to elucidate this phenomenon. ATRX, STK24, GDF10, Reticulon 4, and PTEN proteins are known to play roles in the regulation of neuronal development[22-26]. However, their relationship with the cannabinoid system remains unknown. The primary objective of this study was to uncover this relationship. We examined how the stimulation of the cannabinoid system affected ATRX, STK24, and GDF10, but no significant relationship could be identified for RTN4 and PTEN.

ATRX is expressed in the developing and adult nervous system, and it is particularly important in the regulation of axon sprouting. Axon sprouting is the process by which new axons grow from existing neurons. It is essential for neural development and repair, and it is also thought to be involved in learning and memory[27]. Studies have shown that ATRX deficiency leads to impaired axon sprouting in both animals and humans. For example, mice with ATRX deficiency have fewer axons in the hippocampus, a brain region that is important for learning and memory. People with ATRX deficiency can also have intellectual disabilities and other neurological problems[28]. One way that ATRX regulates axon sprouting is by regulating the expression of growth factors, which are proteins that promote axon growth. ATRX deficiency can lead to decreased expression of growth factors, which can impair axon sprouting[29]. Also, ATRX is a chromatin remodeling protein involved in the regulation of gene activity. Since Δ-9-THC triggers an increase in ATRX expression, it has the potential to influence the epigenetic control of genes associated with axonal growth. Modifications to the structure of chromatin could result in shifts in gene expression patterns that promote axon sprouting or the activation of factors that encourage axonal growth.

STK24 is a serine/threonine kinase that regulates various cellular processes, including cell survival and cytoskeletal dynamics. It may influence axon sprouting indirectly by modulating intracellular signaling pathways related to axonal growth. Activation of STK24 could lead to changes in the cytoskeleton that facilitate axon extension.

GDF10 is a member of the TGF-beta superfamily of growth factors. GDF10 functions as a neurotrophic factor and supports the growth, differentiation, and

survival of neurons. If Δ-9-THC induces GDF10 expression, it might promote axon sprouting through its role in neuronal differentiation and growth. GDF10 could potentially enhance the survival and growth of neurons, which are essential for successful axon sprouting.

According to the results, there was a significant increase in ATRX, STK24, and GDF10 levels with a 10 μm Δ-9-THC application. ATRX levels also increased at 1 μm and GDF10 levels also increased at 20 μm Δ-9-THC application. However, ATRX and STK24 levels show no significant increase at a 20 μm dose and GDF10 and STK24 levels show no significant increase at 1 μm dose. This may be due to expression being triggered by different activation pathways and specific intracellular signaling cascades that are activated only at certain Δ-9-THC concentrations. Low and high concentrations (1 μM and 20 μM) may involve one pathway, while moderate concentrations (10 μM) activate another, with both being less effective at other concentrations. Therefore, it can be concluded that a 10 μm Δ-9-THC application is the optimal dose to effectively increase all three of these proteins.

The collective effect of these proteins on axon sprouting likely depends on their specific roles and interactions within the complex network of molecular processes involved in neuronal development and axonal growth. This is the first study to examine the expression of ATRX, STK24, GDF10, RTN4, and PTEN proteins after neuronal cells are stimulated by Δ-9-THC. As a result, the authors could not compare the findings with other studies, which is a limitation. Additionally, the study has limitations. First, it was conducted using the SH-SY5Y neuroblastoma cell line, which, although a widely used *in vitro* model, may not fully capture the complexity and functional dynamics of the human brain. Furthermore, the investigation was limited to only five proteins, potentially overlooking other critical factors involved in cannabinoid-induced axonal prouting.

Conclusion

Our results demonstrated that Δ-9-THC significantly increased the levels of ATRX, STK24, and GDF10, while having no significant effect on RTN4 or PTEN. Given the established roles of ATRX, STK24, and GDF10 in promoting axon sprouting, these findings suggest that Δ-9-THC may support neural repair processes through selective protein modulation. These insights contribute to the understanding of cannabinoid system interactions and may inform future research on therapeutic approaches for neurodegenerative conditions and neuronal injury. In conclusion, there are numerous promising therapeutic targets in axon sprouting research. This study has identified the stimulating effect of Δ-9-THC on proteins involved in neuron regeneration, and we believe it holds promise for the treatment of diseases causing neuronal damage, such as Alzheimer's, Parkinson's, and ischemia.

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Conflict of interest

The authors declare no conflict of interest.

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