EFFECT OF THE ANKK1 PROTEIN OVEREXPRESSION IN NEUROBLASTOMA CYTOSKELETON ORGANIZATION

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ABSTRACT

The gene *ANKK1* that codes for the Receptor Interacting Protein 5 (RIP5) has been widely associated to cognitive traits, psychiatric disorders and the dopaminergic functioning *per se.* Besides, there are some evidences that ANKK1 protein participates in neurodevelopment and plasticity in the brain, processes for which cytoskeleton dynamics is crucial. However, the function of ANKK1 is still unknown, and there is a great interest in studying this protein and the signal pathways in which it is involved. Our objective was to study a possible relation between ANKK1 function and cytoskeleton dynamics, specifically the α-tubulin post translational modifications (PTMs) that tune microtubule dynamic functions. To this purpose, we investigated the effect of ANKK1-K and ANKK1-FL isoforms overexpression upon microtubules organization in neuroblastoma cell lines. After the overexpression, we found a morphological change in neuroblastoma cells towards a more neuronal-like phenotype, which shifted into a more astrocyte-like phenotype after apomorphine treatment. Besides, we found an increase of α-tubulin tyrosination levels in SK-N-SH neuroblastoma cell line after ANKK1-FL isoform overexpression, and in SH-SY5Y neuroblastoma cell lines after the overexpression of the two ANKK1 isoforms. Finally, a α-tubulin acetylation signal change was observed in SH-SY5Y neuroblastoma cell line after the overexpression of ANKK1-FL isoform, specifically an expanded pattern is observed throughout the soma both at proximal and distal areas. Despite futher studies and replicas should be done, we think these results suggest that ANKK1 could be involved in the radial glia differentiation of neural lineages and that dopaminergic stimulation might be a modulator of the protein function or the pathways where it is involved. Regarding the study of PTMs changes, our results suggest that ANKK1 function might be implicated in neural processes which function require microtubule dynamics.

Key words: ANKK1, neuroblastoma cell line, cytoskeleton dynamics, microtubules, acetylated α-tubulin, detyrosinated α-tubulin.

RESUMEN

El gen *ANKK1*, que codifica para el *Receptor Interacting Protein 5* (RIP5), ha sido ampliamente asociado a rasgos cognitivos, trastornos psiquiátricos y al funcionamiento dopaminérgico *per se*. Además, existen evidencias de que la proteína ANKK1 participa en el desarrollo neurológico y la plasticidad cerebral, procesos para los cuales la dinámica del citoesqueleto es crucial. Sin embargo, la función de ANKK1 es aún desconocida, y es de gran interés el estudio de esta proteína y de las vías de señalización en las que participa. El objetivo del trabajo fue estudiar una posible relación entre la función de ANKK1 y la dinámica del citoesqueleto, específicamente las modificaciones post traduccionales de la α-tubulina (PTMs) que modulan las funciones dinámicas de los microtúbulos. Con esa finalidad, se investigó el efecto de la sobreexpresión de las isoformas ANKK1-K y ANKK1-FL sobre la organización de los

microtúbulos en líneas celulares de neuroblastoma. Tras la sobreexpresión, encontramos un cambio morfológico hacia un fenotipo con rasgos neuronales, que cambia a un fenotipo con rasgos de astrocito tras el tratamiento con apomorfina. Además, encontramos un aumento de los niveles de tirosination de la α-tubulina en la línea celular SK-N-SH después de la sobreexpresión de la isoforma ANKK1-FL, y en la línea celular SH-SY5Y tras la sobreexpresión de las dos isoformas de ANKK1. Por último, se observó un cambio en la señal de la acetilación de la α-tubulina en la línea SH-SY5Y después de la sobreexpresión de la isoforma ANKK1-FL, específicamente un patrón expandido en todo el soma tanto en las zonas proximal y distal. Aunque es necesario realizas réplicas y otros estudios, estos resultados sugieren que ANKK1 podría estar implicada en la diferenciación de la glía radial a distintos linajes neuronales y que la estimulación dopaminérgica puede ser un modulador de la función de la proteína o de las vías donde ésta participa. En cuanto al estudio de cambios en las PTMs, nuestros resultados sugieren que la función ANKK1 podría estar implicada en procesos neurales cuya función requiere dinámica de los microtúbulos.

Palabras clave: ANKK1, líneas celulares de neuroblastoma, dinámica del citoesqueleto, microtúbulos, α-tubulina acetilada, α-tubulina detirosinada.

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ABBREVIATIONS

ANKK1: Ankyrin repeat and kinase domain containing 1

DAPI: 4',6-Diamidino-2-Phenylindole, Dihydrochloride

DRD2: Dopaminergic Receptor D2

GFP: Green Fluorescent Protein

GTP: Guanosine-5'-triphosphate

MAP: Microtubule-Associated Proteins

$MgCl_2$: Magnesium chloride

NCAM1: Neural Cell Adhesion Molecule 1

ON: Overnight

PBS: Phosphate Buffered Saline

PTM: Post Translational Modification

PFA: Paraformaldehyde

RIP: Receptor-Interacting Protein

SNP: Single Nucleotid polimorfism

TTL: Tubulin Tyrosine Ligase

TIP: Plus-end Tracking Proteins

TTC12: Tetratricopeptide Repeat Protein 12

1. PURPOSE OF THE PROJECT

Ankyrin Repeat and Kinase Domain 1 (ANKK1) also known as receptor interactor protein 5 (RIP5), belongs to the RIP serine-threonine kinase family which is involved in survival, death, and differentiation processes. *In vitro* mRNA and protein studies have shown a potential connection between the dopaminergic system function and the ANKK1 cellular expression pattern during development and in adulthood (1) (2). From a clinical point of view, the *TaqIA* single-nucleotide polymorphism (SNP) rs1800497, located at the coding region of *ANKK1* gene, is the most studied genetic variation in psychiatry and personality traits related to plasticity and aspects of learning and memory (3). Besides, there are some evidences that ANKK1 participates in neurodevelopment and plasticity in the brain (2).

Human brain cells are enormously diverse in form and function. This diversity is achieved during neuronal and glia differentiation early in the development of central neural system (4). Besides, neural cells do not work as isolated structures but exhibit an intrincated connectivity that is on the other hand modified throughout the adult life to provide structural plasticity (5). There are evidences that both these specialized structure and connectivity correlate with neural functions (6). Changes in cytoskeleton are crucial for achieving the entire neuronal architecture and connectivity throughout the development (4).

Nonetheless, ANKK1 protein function is yet to be identified. Our purpose is to provide evidence that ANKK1 function in the brain is somehow related to neural cytoskeleton dynamics.

2. INTRODUCTION

2.1. THE CHANGING NEURAL SYSTEM

Neurons have a unique architecture with a distinct and diverse morphology. They undergo significant morphological changes leading to the maturation of an astoundingly diverse array of phenotypes. Beyond this cellular diversity, the neural system as a whole changes dramatically over human lifespan (7). Any morphological cell change is in ultimate extent on account of cytoskeleton organization and dynamics.

During neurodevelopment, it takes place the generation and differentiation of neurons and glia from radial glia cells (precursor cells), the formation of axonal pathways and synapses. From these processes, a complex neural circuit arises. During postnatal life the experience continues to shape this circuit: synaptic connections are modified as new skills and memories are acquired and others are lost. Finally, injury of neural system either by a disease or by a traumatic insult activates repairing mechanisms (7). Neuronal functional properties are critically influenced by this strict geometry (8).

Neuronal differentiation, synapse formation and plasticity and neural injury reparation depend on the ability of neurons and other non-neural cells to change their morphology (19). For instance, neurons differentiate and become polarized structures by growing multiple neurites which later on specialize in either axons or dendrites. In all these processes, neuronal cytoskeleton plays a crucial role (5).

2.2. NEURONAL CYTOSKELETON

As we already mentioned, the morphology and function of neurons depend on the cytoskeleton, which is a dynamic intracellular protein matrix of high complexity and versality. Cytoskeleton is crucial for every aspect that involves neuronal shape and motility. For instance it is the framework that controls cell polarity and migration, which define the architecture and connectivity of the nervous system; and synaptic morphology and transmission, which are the basis of learning and memory. Also, it

serves as track for intracellular transport, which creates and maintains differentiated cellular functions (4).

The cytoskeleton of neurons as well as other eukaryotic cells can be partitioned in three interacting structural complexes: microtubules (diameter 25 nm), actin filaments (diameter 8 nm) and intermediate filaments (10 nm). Each of them has a characteristic and specialized organization, structure and composition. Together, these filamentous protein assemblages impart the cell with shape and structure by interacting with each other, a multitude of other proteins, and with cellular membranes (9).

During neuronal development, for a neuron to become polarized and differentiated many dramatic changes in cytoskeleton have to occur. Outgrowth and consolidation of neurites (unspecialized projections) that become axons and dendrites require the coordinated participation of actin filaments and microtubules (10). For instance, during neurite initiation, cells surrounded by a flattened, actin-rich lamellipodium transform to produce thin, microtubule-filled neurite shafts tipped by actin-rich growth cones (11). When actin filaments in growth cones depolymerize, microtubules, which are normally confined to the central domain, extend out into a peripheral domain in an uncontrolled fashion all the way up to the leading edge (12).

2.2.1 NEURONAL MICROTUBULES

Microtubules are cytoskeletal polymers which act as both dynamic structural elements for cell motility and polarization and tracks for organelle traffic. The core structure is a polarized 50 kDa polymer composed of heterodimers of α- and β-tubulin subunits assembled into linear protofilaments that join laterally and form a cylinder with an outer diameter of 24 nm (9) (Figure 1, a).

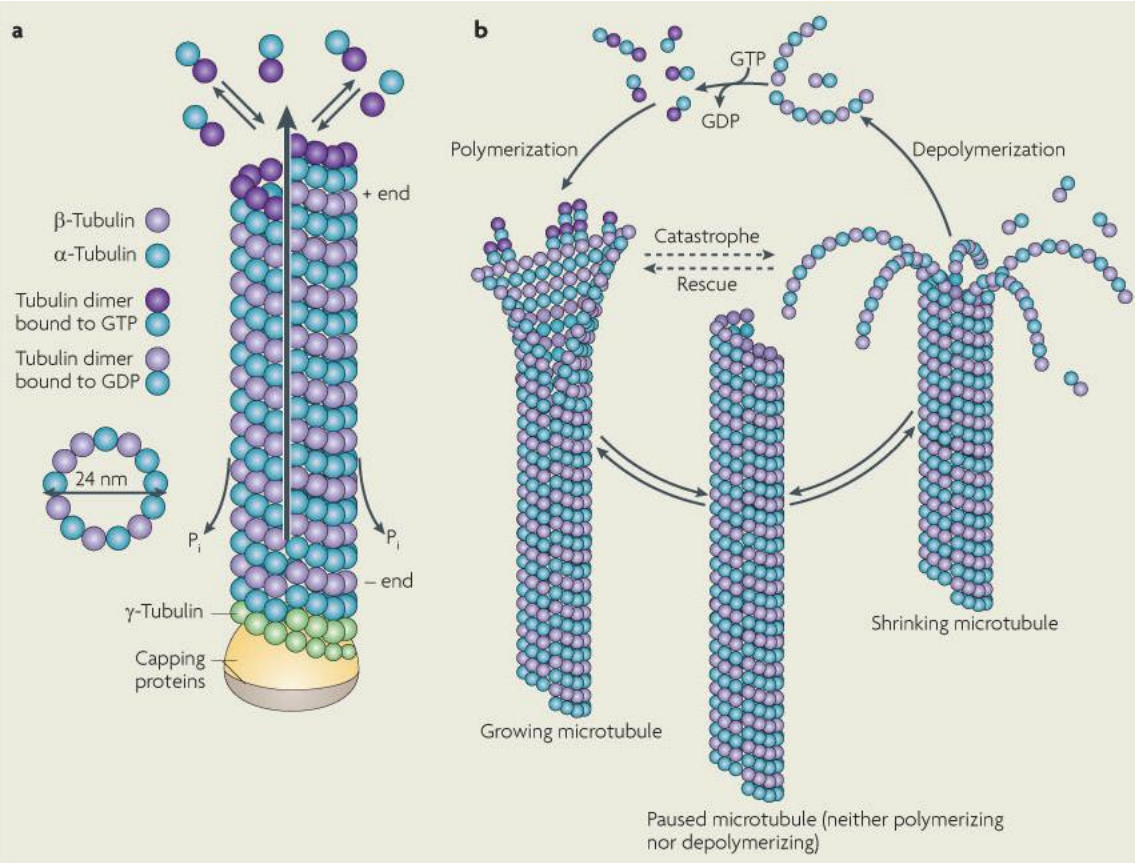

Figure 1. Microtubule structure (a) and dynamic instability behavior (b) (12).

In neurons, microtubules form dense parallel arrays (bundles) in axons and dendrites that are required for the growth and maintenance of these neurites. In growing axons, dynamic polymers are concentrated at neuritic tips in the distal part while long-lived polymers predominate in the proximal axon (10) (Figure 2).

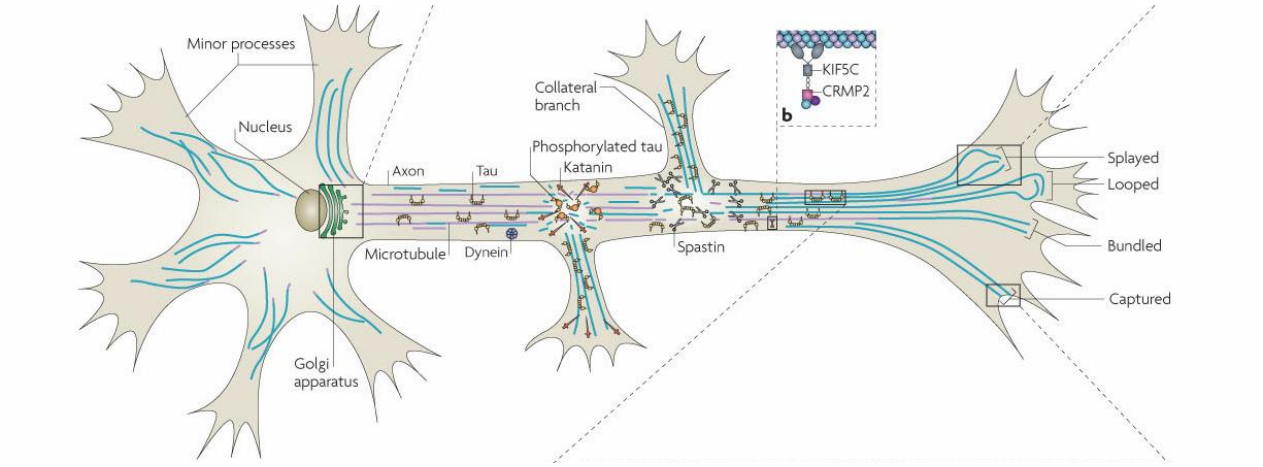

Figure 2. Microtubule organization in developing axons. Dynamic (blue) processes predominate in minor processes (short neurites) and at the distal end of the axon, whereas stable (purple) microtubules are enriched in the proximal part of the axon (12).

A distinctive property of microtubules is their ability to undergo cycles of rapid growth and disassembly, also known as dynamic instability. They exist in either polymerization (growth) or depolymerization (shrinkage) states, depending on the binding of GTP on β-tubulin during polymerization. In a neuronal context, dynamic instability allow microtubules to rapidly reorganize and to generate pushing and pulling forces that are fundamental to growth cone advance, dendrite arborization and synaptogenesis, processes that take place during neuronal differentiation and neural circuit establishment (Figure 1,b).

The observed diversity of microtubule functions lies in the cytoskeletal regulators that act on microtubules as well as the genetic and chemical diversity of tubulin itself. α- and β- tubulin are encoded by multigene families, thus creating many combinations of α/β heterodimers (11). Besides, tubulins are subject to regulated post-translational modifications (PTMs) that are specially dense and variated in complex arrays of microtubules like the ones of neurons (13). These modifications and different isoforms mark subpopulations of microtubules and selectively affect their functions (11).

2.2.1.1. MICROTUBULE POST-TRANSLATIONAL MODIFICATIONS: DETYROSINATION AND ACETYLATION.

There are a great variety of microtubule structures with specific functions. The selective modification of neuronal microtubules has been suggested to be a major regulatory mechanism in controlling several processes including neuronal differentiation and synaptic tagging, as well as being implicated in the pathogenesis of neurodegenerative disorders. One consistent hypothesis is that PTMs that occur on the α-tubulin subunit generate this functional diversity. α-Tubulin PTMs have been well reported for decades and their functional roles are beginning to be discovered (14).

α-Tubulin PTMs are known to affect to microtubule properties. First, they serve as 'road signs' for the recruitment of microtubule-associated proteins (MAPs) and plus-end tracking proteins (+TIPs). This is necessary for polarized axonal/dendritic trafficking. Second, tubulin modifications correlate well with the stability (half-life) and spatial distribution of microtubules. Stable microtubules, rather than dynamic microtubules, are known to accumulate more modifications. Moreover, different tubulin PTMs patterns are seen between stable microtubules (Figure 3). Therefore, PTMs are postulated to play a role in functions of stable microtubules (14). We will focus on the PTMs with the greatest impact on the dynamics of neuronal microtubule cytoskeleton: α-tubulin detyrosination and acetylation (16).

Detyrosination entails the C-terminal tyrosine removal of α-tubulin in microtubule polymers. This reaction can be reversed by the tubulin tyrosine ligase (TTL). Aside, detyrosinated α-tubulin can also be irreversibly converted to Δ2-tubulin by the removal of the penultimate glutamate. Retyrosination occurs on unpolymerized tubulin, thus newly polymerized microtubules are exclusively tyrosinated (Figure 3).

Detyrosination is linked to microtubule stability. Long-lived microtubules are detyrosinated in many cells and Δ2-tubulin modification is restricted to very stable microtubules. This difference in stability between tyrosinated and detyrosinated α-tubulin microtubules is not due to changes in properties of the microtubules polymer itself, but due to the modification-dependent recruitment of microtubule dynamic regulators (3). Detyrosination/tyrosination states are selectively recognized by specific

microtubule interacting proteins, which affect microtubule dynamics and interactions with the actin cytoskeleton. For instance, detyrosinated microtubules are less susceptible to depolymerizating motor proteins, thus they might be important in protecting long-lived microtubules stability. On the other hand, proteins such as plus-end tracking proteins (+TIPs) can sense the presence of tyrosinated α-tubulin.

In neurons, microtubules enriched in tyrosinated α-tubulin are expected to favor microtubule dynamic functions in the growth cone (rapid remodeling of cytoplasm), whereas microtubules enriched in detyrosinated α–tubulin are expected to favor microtubule functions in the shaft of neurites (microtubule stability and efficient cargo transport) (16).

On the other hand, α-tubulin acetylation is another PTM which acts as a broad regulator in microtubule functions. It was thought for many years to occur in the lysine 40 that is inside of the polymer. Most of the interactions between microtubules and proteins take place in the outer face of the polymer, thus it was difficult to explain the role of acetylation in microtubule functions. Recently, novel acetylation sites exposed on the outer face have been identified, opening the possibility of some of these sites to regulate microtubule- MAPs interactions.

Neurons in culture are enriched in acetylated microtubules in proximal axons, and less enriched in dendrites and growth cones. Despite this polar distribution of microtubule acetylation, this modification is not responsible for neuron polarization and axon formation (18).

Acetylation is generally considered to occur on stable microtubule assemblies, although it does not affect directly to their stability (15). Instead, acetylation plays a role in polarized protein trafficking and cargo distribution required for several neural functions such as neuronal development, differentiation and migration and cell proliferation. It stimulates anterograde and retrograde transport, because it acts as a guiding cue for kinesin-1 and dynein to the microtubules (16). Furthermore, this modification is also thought to be important for neuronal migration and differentiation during the early stages of neuronal development.

There is evidence that a cross-talk exists between the different tubulin PTMs and that this microtubule code has a potential impact on neuronal functions at several levels (16). Distinct neuronal compartments such as axons, dendrites, synapses, growth cones have a different PTMs pattern. Besides, proximal MTs ends are mainly detyrosinated and acetylated while distal MTs ends, which project into the growth cone, are tyrosinated and acetylated (Figure 3).

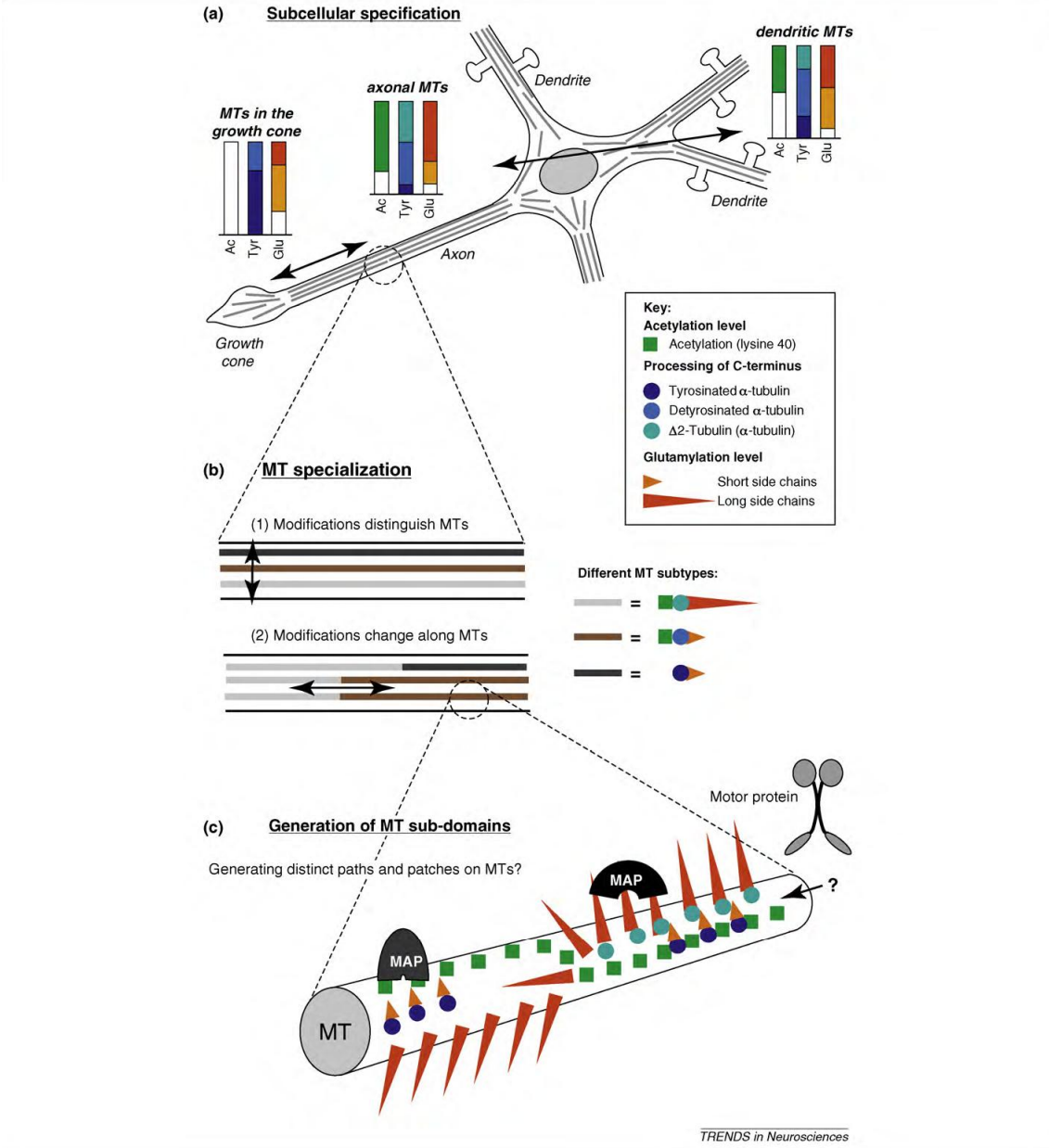

Figure 3. Schematic representation of how different patterns of tubulin PTMs might specify microtubules functions in neurons. (a) Subcellular specification. (b) MT specialization. (c) Microtubules sub-domains (16).

2.3 BASIC CONCEPTS OF ANKK1 PROTEIN

Ankyrin repeat and kinase domain containing 1 gene *(ANKK1*, chromosome 11q22-q23) encodes the protein ANKK1, which belongs to the class of receptor interacting proteins (RIP) serine/threonine kinases. *ANKK1* gene comprises 12.6 kb, contains 8 exon and is located in a cluster of genes together with neural cell adhesion molecule 1 (*NCAM1)*, tetratricopeptide repeat protein 12 (*TTC12)* and dopamine receptor 2 (*DRD2*), all of which are involved in dopaminergic transmission (Figure 4) (3).

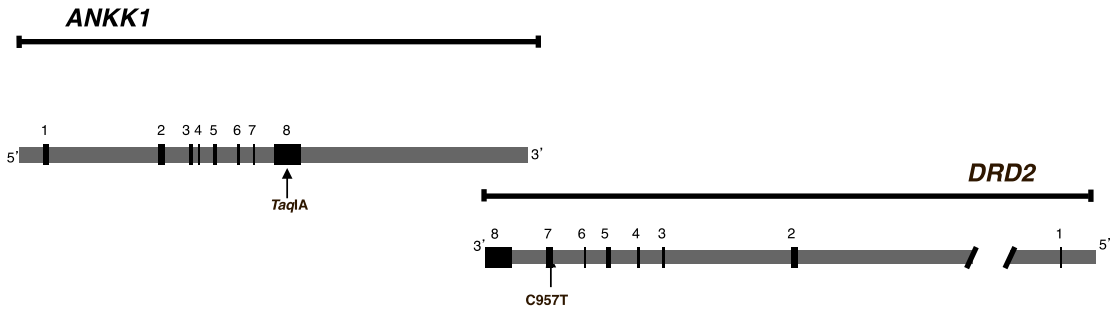

Figure 4. The genomic organization of the *ANKK1* and *DRD2* genes (3).

ANKK1 gene and ANKK1 protein have awaken much interest because of its association with cognitive traits and psychiatric disorders. Indeed, *TaqIA* polymorphism is the most studied genetic variation in psychiatry. This SNP is placed in the exon 8 of *ANKK1* nearby the termination codon of the D2 dopamine receptor gene (DRD2; chromosome 11q22-q23) (Figure 4). The SNP consists of a change of a Citosin (C) by a Timine (T), leading to a Glu713-to-Lys (E713 K) substitution in the putative ANKK1 protein. The two alleles are referred to as A2 (cytosine) and A1 (thymine) and the *TaqIA* genotypes are named A1+ (hetero or homozygous for A1) and A1- (homozygous for A2).

TaqIA SNP has been reported to be in strong disequilibrium (r^2=0.9) with the SNP rs7118900 (Ala239Thr), which causes the substitution of an alanine for a threonine in

239 position (Ala239Thr), creating an additional phosphorylation site in the kinase domain of ANKK1 (1).

2.4 ANKK1 PROTEIN FUNCTION

ANKK1 gene codes for a 765-amino acid protein. The structure of the human ANKK1 protein comprises a N-terminal kinase domain (exons 1-6), an interdomain region (exon 7) and a C-terminal 12 ankyrin repeats domain (exon 8). *In silico* evidence has become available for at least three ANKK1 isoforms with different putative functions: ANKK1-Full length (containing RIP kinase and ankyrin repeat domains), ANKK1-kinase (only the RIP kinase domain), and ANKK1 ankyrin (only the ankyrin repeat domain) (2) (Figure 5).

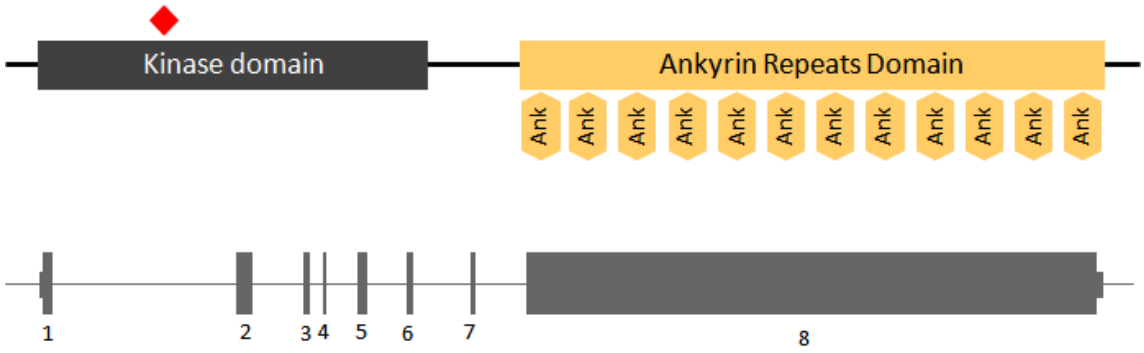

Figure 5. Structure of ANKK1 protein. A red rhombus shows the lysine residue (K) in the amino acid position 51, the active site of the kinase domain.

ANKK1 belongs to the family of RIP kinases, which participates in both pro-survival and death-inducing signal transduction pathways. This family is defined by a highly conserved kinase domain. Specificity is achieved by differences within C-terminal region. RIP4 is the RIP kinase most similar to ANKK1, due to the high homology of the kinase domain and a common C-terminal ankyrin repeats domain.

TaqIA is the most widely studied genetic polymorphism in a broad range of psychiatric disorders and personality traits closely related to dopaminergic system. The focus has been in vulnerability to addictions, including alcoholism and addiction to cocaine,

compulsive disorders and schizophrenia. All these conditions have a common feature: a learning deficit (3). Indeed, in accord with previous findings of our group, ANKK1 itself might be associated with dopaminergic transmission (1) (2). *TaqIA* might only be a marker due to its proximity to polymorphisms within DRD2 gene, or conversely it could trigger functional variations of the ANKK1 kinase, which in turn, could be implicated in dopaminergic system (3).

Studies of the ANKK1 mRNA and its expression pattern were a first attempt to uncover the biological bases of *TaqIA*-associated phenotypes (2). The results indicated that ANKK1 is expressed in astroglial cells of adult central neural system of both humans and rodents. These cells are implicated in the pathophysiology of learning processes thus these results are of great relevance. In mice, ANKK1 was also shown to be expressed in radial glia cells, which serve as neuronal and astroglial progenitors in the central nervous system (16). Together these results suggest that ANKK1 participates in neurodevelopment and plasticity in the brain. Nonetheless, no study has elucidated ANKK1 function yet or has analyzed *TaqIA* and Ala239Thr impact on the protein function.

2.5 OBJECTIVES AND HYPOTHESIS

2.5.1 OBJECTIVES

The objective of this study is to uncover a possible relation between ANKK1 function and cytoskeleton dynamics. To this purpose, we carried out a study of microtubules PTMs when ANKK1 is overexpressed in neuroblastoma cell lines. Besides, we analyzed the effect of dopaminergic treatment using the non-selective dopamine receptor agonist apomorphine.

2.5.2 HYPOTHESIS

Since ANKK1 is known to be expressed in the progenitor radial glia cells, it has been suggested to be involved in the neurodevelopment. According to his, we hypothesize that ANKK1 overexpression in neuroblastoma cell lines may induce neuronal growth

and/or differentiation. This would be reflected in the cytoskeleton dynamics and the distribution of α-tubulin acetylation and tyrosination along the microtubule cytoskeleton.

3. MATERIALS AND METHODS

3.1. BIOLOGICAL MATERIALS

3.1.1. PLASMID DNA

Plasmids containing ANKK1-K (Kinase, only kinase domain) or ANKK1-FL (Full Length, with kinase domain and ankyrin repeats domain) were cloned in our laboratory into pEGFP-N1 vector in *E.coli* (CLONTECH, California, USA) to obtain a fusion protein with Green Fluorescent Protein (GFP) tag (1). Since the tag was GFP and was inserted in the C-terminal position of ANKK1, the plasmids will be referred as ANKK1 K-GFP and ANKK1 FL-GFP.

3.1.2. CELL LINES

The cells used for all the studies were human neuroblastoma cell lines SK-N-SH (which expresses caspase-8) and SH-SY5Y, which have been extensively utilized for *in vitro* experiments requiring neuronal-like cells. SH-SY5Y cells are a subline of the parental line SK-N-SH. This parental line was subcloned three times; first to SH-SY, then to SH-SY5, and finally to SH-SY5Y. Parental SK-N-SH cells consist of two morphologically distinct phenotypes: neuroblast-like cells and epithelial-like cells. Nevertheless, SH-SY5Y consists of homogeneous neuroblast-like populations with non-polarized cell bodies. Both cell lines tend to grow in clusters and show few neuritic processes at edges of the cluster (19).

3.2. DNA MIDIPREPARATION

A flask containing 25 mL of bacterial culture grown overnight was centrifuged at 4000 rpm for 10 minutes at 4ºC and then the supernatant was discarded. Following, the plasmid DNA was isolated from bacterial culture using Invitrogen PureLink HiPure Plasmid Filter Midiprep Kit (Massachusetts, USA) and following the protocol supplied by the manufacturer. The plasmid was then quantified with the Nanodrop (ND-1000 Spectrophotometer from Thermo Scientific, Massachusetts, USA) and store at -20ºC until next use.

3.3. CELL CULTURE

Neuroblastoma cell lines were grown in a humidified incubator with 5%CO_2 at 37ºC Dulbecco´s Modified Eagle Medium- Ham's F12 (DMEM-F12) containing 10% (v/v) fetal bovine serum (FBS) supplemented with 1% penicillin–streptomycin, and 2 mM L-glutamine.

3.3.1. Trypsinisation

The cells were examined under inverted microscope to check for contamination, cell density, morphology, etc. First, the old media was removed and the monolayer was washed with 5mL of phosphate buffered saline buffer (PBS) 1X. Then PBS 1X was removed and 2 mL of trypsin, pre-warmed to 37ºC, were added per T-75 flask. The cells were then incubated with the trypsin for 3 minutes at 37ºC. When the cells lifted of the monolayer, 5 mL of fresh media were added to inactivate the trypsin. The cell suspension was transferred to a sterile 15 mL conical centrifuge tube and centrifuged for 3 minutes at 1200 rpm. After centrifugation, the supernatant was discarded and the cells were resuspended in 5 mL of fresh DMEM-F12 complete media. For a 1:5 passage, 1 mL of the cell suspension was added to a T-75 flask containing 9 mL of fresh media.

3.3.2. Cell count

After tripsinisation, centrifugation and resuspending in fresh media, the cells were counted in a hemacytometer. In an eppendorf tube, 10µL of cell suspension were added together with 10 µL of Trypan Blue (Sigma Aldrich, Missouri, USA). Following, 10 µL of the mixture were loaded onto the Neubauer chamber. The cells were counted in the four corner squares using the inverted microscope and a hand-held counter.

3.3.3. Transfection with Nucleofector

Following cell count, $1 \cdot 10^6$ cells were transferred per transfections into an eppendorf tube and centrifuged for 4 minutes at 1200rpm. The supernatant was discarded and the pellet was washed with PBS 1X and centrifuged again for 4 minutes at 1200 rpm.

The supernatant was discarded again and 1 µg plasmid per million cells was added together with 18 µL of electroporation buffer. The mix (20µL/well) was transferred to the Nucleocuvette Strip and immediately electroporation was performed in the Nucleofector 4D (Lonza, Basel, Switzerland), by selecting the specific program for the cell line. Immediately after transfections 80 µL of medium, pre-warmed to 37ºC, were added to each well of the Strip and cultured directly into p35 plates containing 4 circular cover slides each.

3.3.3.1. Buffer preparation for electroporation assays

The buffers were prepared from fresh stocks and filtered under sterile conditions. The composition of Buffer 1SM was 100 µL KCl 500 mM, 1.5 mL MgCl2 100mM, 1.2 mL Sodium Phosphate buffer pH 7.2 1M, 2.5 ml D-manitol 100mM, 2.5 mL Sodium succinate 100mM for a final volume of 10 mL (Chicaybam *et al.*, 2013).

3.3.4. Transfection with FuGene

The day before transfection, following cell count, $1 \cdot 10^6$ cells were cultured per p35 plate. The day of transfection, cells were incubated with 200 µL the transfection mix. The mix had the following composition: 2 µg plasmid, 5 µL of FuGene (Promega, California, USA) and OptiMEM media (Life technologies, California, USA) up to 200 µL. The mix was incubated for 20 minutes at room temperature (RT), and then was added to each plate drop by drop and softly mixed in cross like movements. The cells were incubated at 37ºC for 24 h.

3.3.5. Apomorphine treatment

In some cases, cells were treated 24 hours post-transfection with the dopamine receptor agonist (R)-(-)-apomorphine (Sigma, Missouri, USA) at 10 µM in complete medium with 0.5% serum, for 1 hour at 37ºC.

3.3.6. Cell collection

The cells were collected 24 hours after transfection or after treatment. The media was removed and washed with 500 µL PBS 1X. The cells were collected in PBS 1X by active

scrapping and centrifuged at 2.5 rpm for 5 minutes at 4ºC. The pellet was stored at -80ºC until next use.

3.4. PROTEIN MANIPULATION

Levels of proteins of interest were evaluated using immunodetection by Western Blot (WB). This technique is capable of identifying specific antigens recognized by monoclonal or polyclonal antibodies on proteins separated by SDS-PAGE and transferred to a nitrocellulose membrane.

3.4.1. Extraction

The cells were processed to obtain the total extracts of the protein adding lysis buffer (NaCl 250 mM, Tris HCl 50 mM, EDTA 5 mM, Triton at 0,1% and NaF 50mM) to had previously been added Phosphate inhibitor cocktail tablets (Roche, Basel, Switzerland) and using an electric stirrer. Afterwards, they were centrifuged at 13500 rpm for 15 minutes at 4ºC in order to eliminate insoluble cellular debris.

3.4.2. Quantification

The samples were quantified using the BCA Protein assay kit (Life Technologies), following manufacturer´s instructions. The absorbance measurements were done using Victor2 spectrophotometer (Perkin Elmer, Massachusetts, USA).

3.5. WESTERN BLOT

The proteins were separated in a 10 % Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel under denaturing conditions and with a concentrating gel of 6 %, which is responsible for aligning the proteins of the sample before they are separated by their molecular weight. The samples, containing 25 µg of protein, were incubated at 95ºC for 5 minutes once they were mixed with water and loading buffer (0.5 M Tris-HCl pH 6.8, 10% SDS, 10% glicerol, 50 mMDTT y bromophenol blue) to

reach 45 µL of volume. The electrophoresis was performed at 100V for 2 hours. To measure the molecular weight, a pre-stained molecular weight marker was used (Prism Ultra Protein Ladder from Abcam, Cambridge, UK). After electrophoresis, proteins were transferred to a nitrocellulose membrane for 90 minutes at 50 V at 4ºC. The membrane were washed with TBS-T20 0,1% (Tween 20) and blocked for 1 hour with 5% skimmed milk in TBS-T20 0,1% at RT in stirring. The membranes were sequentially incubated with two primary antibodies: anti tyrosinated α-tubulin or anti acetylated α-tubulin overnight (ON) at 4ºC and anti β-actin for one hour at RT , with 3 washes (10 minutes each) with TBS-T20 0,1% between each. Then membranes were incubated with the secondary antibody anti mouse IgG HRP for 1 hour at room temperature in stirring. ECL-prime Western Blot Detecting Reagent (Amersham, USA) was used for detection following manufacturer´s protocol. The results were analysed using ImageJ software (National Institute of Health, Maryland, USA) and following the criteria established by Taylor and Posch (2014). Then the results were processed with GraphPad Prism v6.0 (California, USA).

3.6. IMMUNOFLUORESCENCE

3.6.1. Fixation and permeabilization procedures

In this project, for effective detection of the microtubule PTMs, we performed a special fixation protocol that allowed the extraction of the monomeric tubulin not polymerized. 48 hours after transfection, cells were fixed and permeabilized for 20 min using the PHEM buffer (Pipes 60mM, Hepes 25 mM, EGTA 5 mM, and MgCl 1 mM; all from Sigma-Aldrich), supplemented with 0.25% glutaraldehyde, 4% paraformaldehyde and 4% sucrose. Then the cells were washed three times with PBS1X.

3.6.2. Immunofluorescence assay

The cells were blocked for 1 hour with blocking buffer (PBS 1X, inactive FBS 10% and Triton X-100 at 0,1%). After block, the cells were incubated with primary antibodies anti tyrosinated α-tubulin and anti acetylated α-tubulin ON. The primary antibody was prepared with blocking buffer diluted 1:3 in PBS 1X. Then, after the washing cycles

with PBS 1X, the cells were now incubated with the secondary antibody (prepared with blocking buffer diluted 1:3 in PBS 1X) for 1 hour at room temperature and conserved in the dark. Finally, the cover slides were fixed to the slide with Fluoromont mounting media (Sigma Aldrich, Misouri, USA), which contains DAPI, by adding 3µL per cover slide. The antibodies used for Western Blot and immunofluorescence are shown in the table below:

Table 1. The antibodies used in Western Blot (WB) and immunofluorescences (IFI)**.** Indicated by name, origin, work dilution and producer.

Primary antibody	Origin	Work dilution	Incubation time	Producer
Anti acetylated α–tubulin T6793	Mouse	1/2000 (WB) 1/10000 (IFI)	Overnight	Sigma- Aldrich
Anti tyrosinated α-tubulin TUB-1A2 T9028	Mouse	1/2000 (WB) 1/5000 (IFI)	Overnight	Sigma- Aldrich
Anti β-actina	Mouse	1/8000 (WB)	1 hour	Sigma- Aldrich
Secondary antibody	Origin	Work Dilution		Producer
Anti mouse IgG HRP	Goat	1/10000 (WB)	1 hour	Sigma Aldrich
Anti mouse Texas Red	Goat	1/1000 (IFI)	1 hour	Sigma Aldrich

2.6.2. Microscopy imaging

Immunofluorescence images were taken with a fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with 63X immersion lens. The images were then processed using ImageJ software.

4. RESULTS

4.1 Characterization of ANKK1 overexpression effect upon neuroblastoma cell lines.

To study a potential role of ANKK1 function in the cytoskeleton dynamics, we performed ANKK1 overexpression in neuroblastoma cell lines. We transfected SK-N-SH and SH-SY5Y neuroblastoma cell lines with the plasmids containing human ANKK1-K (only kinase domain, A1 isoform) or ANKK1-FL (containing kinase and ankyrin repeats domains, H1 isoform) variants using the green fluorescent protein (GFP) as a tag. We treated half of the transfected cells with the dopamine receptor agonist apomorphine (Apo). After transfection, we performed immunofluorescence and western blot studies to examine two parameters of the microtubule cytoskeleton: cellular distribution of α-tubulin acetylation and detyrosination. We also analyzed the effect of apomorphine treatment.

4.1.1. Subcellular location of overexpressed ANKK1 is both cytoplasmic and nuclear, with some differences depending on the cell line and apomorphine treatment.

In neuroblastoma cell lines, the location of overexpressed ANKK1 is both cytoplasmic and nuclear (Figures 6 to 9, B to F, II). Even so, we found differences related to the cells line and apomorphine treatment. In non-treated cells, the protein presents a non-uniform distribution in the form of clusters, regardless of the ANKK1 isoform overexpressed (Figures 6 to 9, B and C, II). In the case of SK-N-SH cells, these clusters are more evident (Figures 6 and 8, B and C, II).

Regarding the effect of apomorphine treatment upon ANKK1 overexpression in neuroblastoma, we observe an impact in the subcellular distribution of the protein such that a larger amount is observed in the nucleus (Figures 6 to 9, E and F, II).

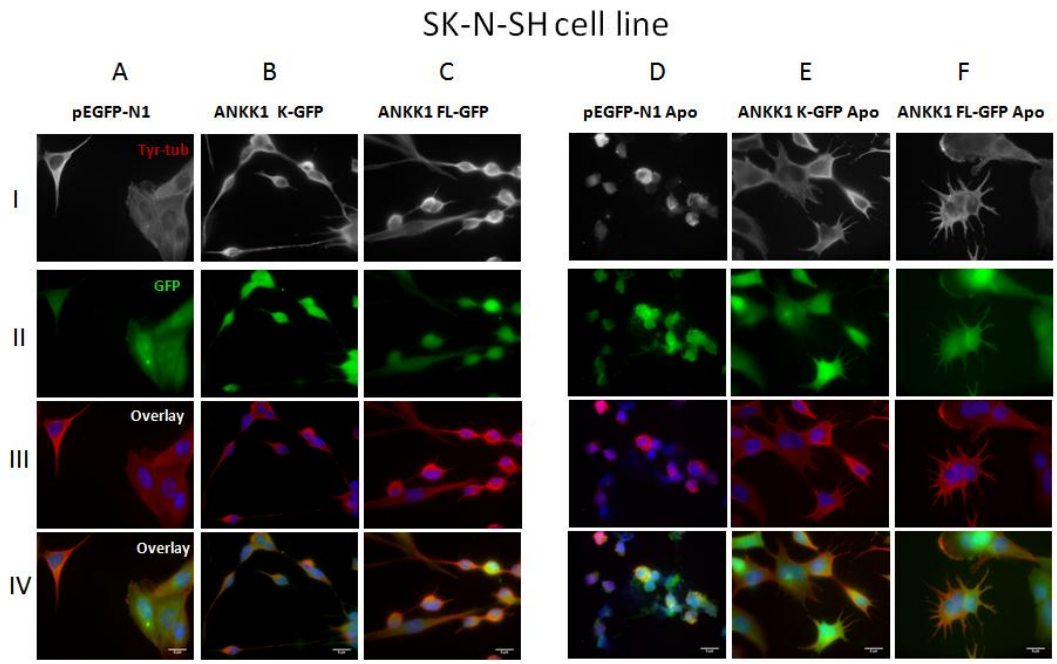

Figure 6. Immunofluorescence study in SK-N-SH cell line using anti tyrosinated α-tubulin antibody. **A, B, and C** show transfected SK-N-SH cells, with pEGFP-N1, ANKK1 K-GFP and ANKK1 FL-GFP respectively. **D, E and F** show transfected SK-N-SH cells, with pEGFP-N1, ANKK1 K-GFP and ANKK1 FL-GFP respectively and treated with apomorphine. The scale bar is 8 μm.

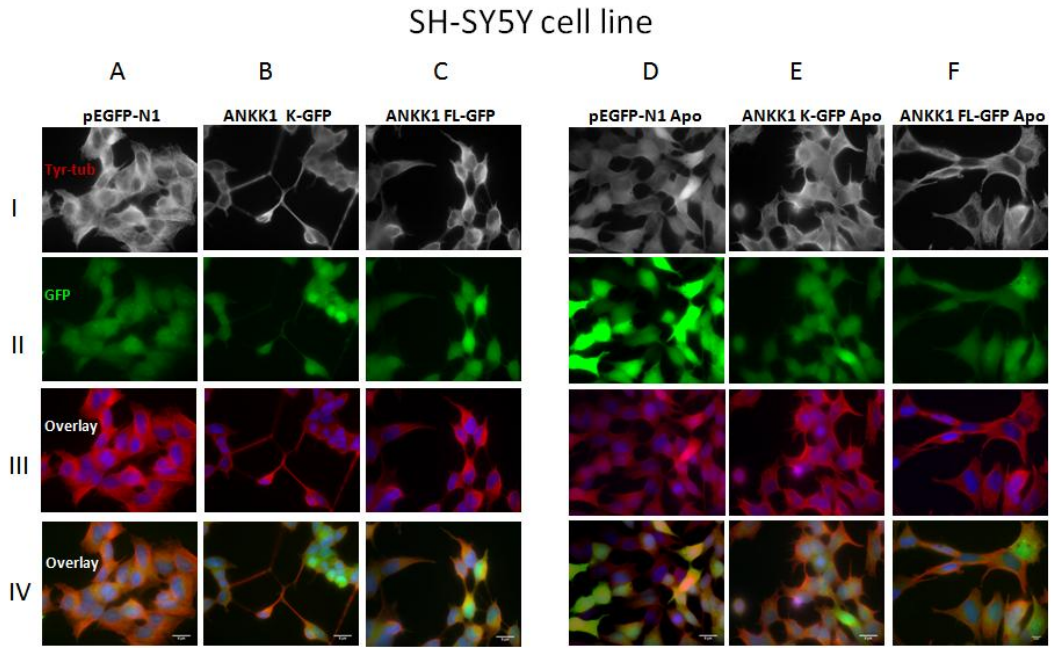

Figure 7. Immunofluorescence study in SH-SY5Y cell line using anti tyrosinated α-tubulin antibody. **A, B, and C** show transfected SK-N-SH cells, with pEGFP-N1, ANKK1 K-GFP and ANKK1 FL-GFP respectively. **D, E and F** show transfected SK-N-SH cells, with pEGFP-N1, ANKK1 K-GFP and ANKK1 FL-GFP respectively and treated with apomorphine. The scale bar is 8 μm.

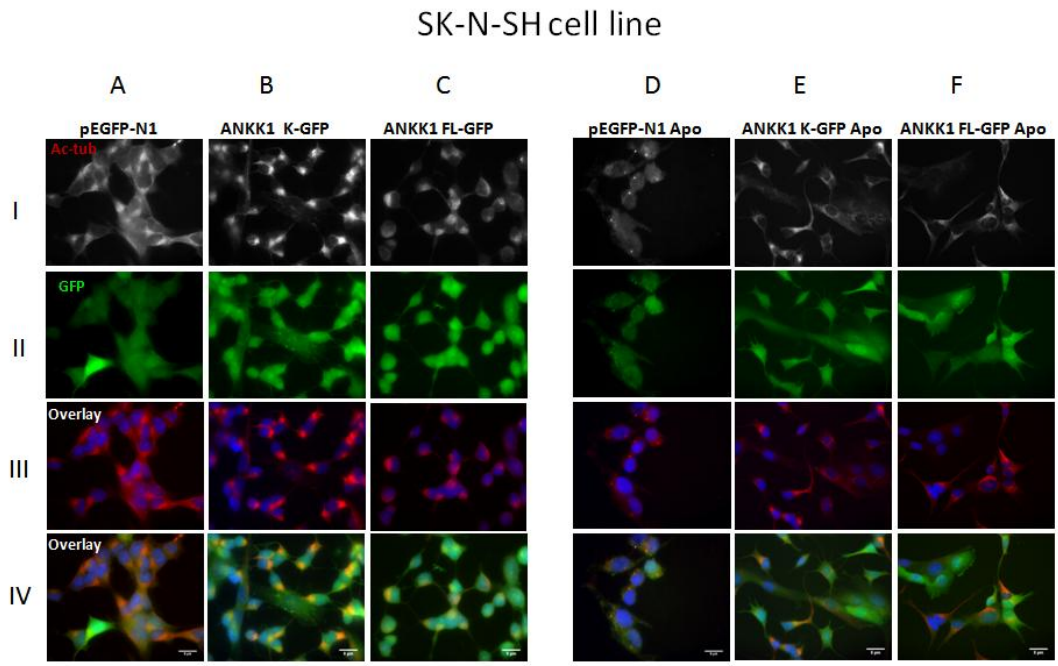

Figure 8. Immunofluorescence study in SK-N-SH cell line using anti acetylated α-tubulin antibody. **A, B, and C** show transfected SK-N-SH cells, with pEGFP-N1, ANKK1 K-GFP and ANKK1 FL-GFP respectively. **D, E and F** show transfected SK-N-SH cells, with pEGFP-N1, ANKK1 K-GFP and ANKK1 FL-GFP respectively and treated with apomorphine. The scale bar is 8 μm.

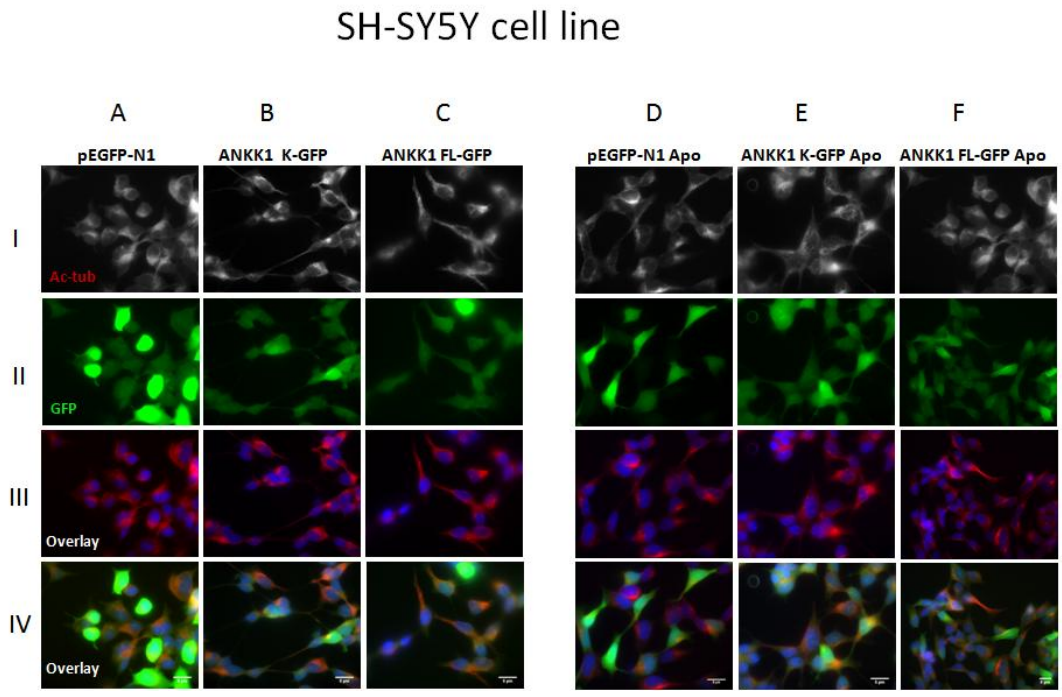

Figure 9. Immunofluorescence study in SH-SY5Y cell line using anti acetylated α-tubulin antibody. **A, B, and C** show transfected SK-N-SH cells, with pEGFP-N1, ANKK1 K-GFP and ANKK1 FL-GFP respectively. **D, E and F** show transfected SK-N-SH cells, with pEGFP-N1, ANKK1 K-GFP and ANKK1 FL-GFP respectively and treated with apomorphine. The scale bar is 8 μm.

4.1.2. ANKK1 overexpression induces a neuron-like cell morphology, which turns into an astrocyte-like morphology after apomorphine treatment.

We observe that transfected neuroblastoma cells with both ANKK1-K and ANKK1-FL isoforms show a cellular phenotype with neuron-like features, such as bipolar structure, increased connectivity and increased cytoplasmic projections akin to neurites (Figures 6 to 9, B and C, I). In contrast, apomorphine treated cells show astrocyte-like features such as absence of bipolarity, low reticular connectivity and stellate morphology with up to eight cytoplasmic extensions per cell (Figures 6 to 9, E and F, I).

Additionally, in the case of SK-N-SH cells, this induced phenotype seems to be more pronounced than in SH-SY5Y cell line. In the latter, neuron-like features appear not to be so accentuated: connectivity is lower and extensions are shorter (Figures 7 and 9, B and C, I). Likewise, astrocyte-like features are less noticeable: cells have less neurite extensions and are more clustered (Figures 7 and 9, E and F, I).

4.1.3. ANKK1 overexpression correlates with a change in microtubule detyrosination levels.

To study the effect of ANKK1 overexpression upon detyrosination, we performed immunofluorescences in neuroblastoma cell lines using anti tyrosinated α-tubulin. Regardless of the ANKK1 isoform and cell line, we observe the expected pattern of microtubule tyrosination in the cells: detyrosinated tubulin enriched in the proximal segments of axons (Figures 6 and 7) (16). Also, both proteins colocalizate in the cytosol with a variable intensity (Figures 6 and 7, IV).

On the other hand, the phenotypes induced after the overexpression made impossible the comparation of tyrosination pattern between overexpressed cells and the negative control. Therefore, we performed a semi-quantitative analysis consisting of a western blot and a densitometry analysis (Figure 10).

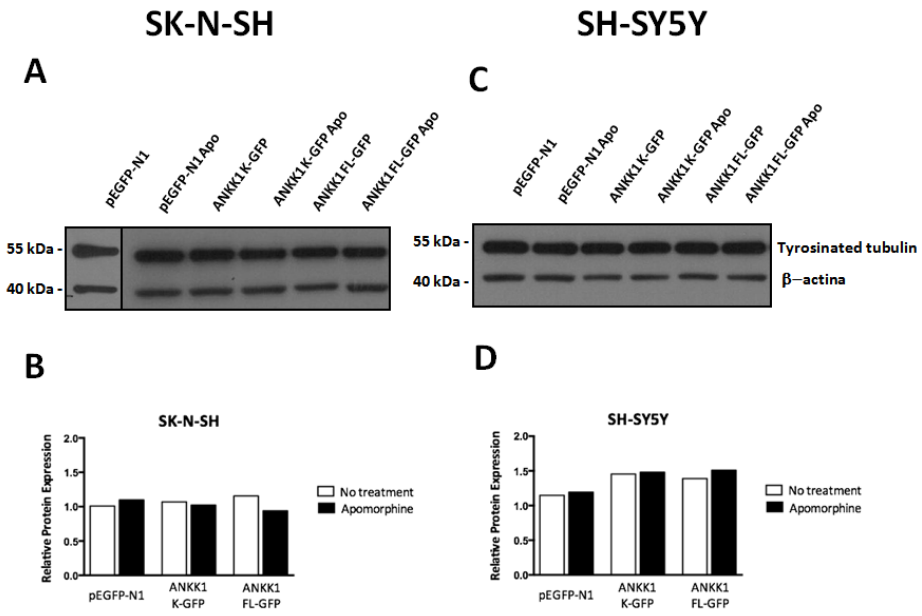

Figure 10. ANKK1 overexpression effect over microtubule tyrosination levels. **A and B**, immunobloting and densitometry analysis of SK-N-SH cells transfected with Pegfp-n1, ANKK1 K-GFP and ANKK1 FL-GFP with and without apomorphine treatment. **C and D,** immunobloting and densitometry analysis of SH-SY5Y cells transfected with Pegfp-n1, ANKK1 K-GFP and ANKK1 FL-GFP with and without apomorphine treatment.

We observed some differences in tyrosination levels. SH-SY5Y cells have greater tyrosination levels compared to SK-N-SH cells (Figure 10, A and B, C and D). Taking into consideration each cell line, SK-N-SH cells transfected with ANKK1 FL-GFP isoform show an increase of 7% in microtubule tyrosination (Figure 10, A and B). SH-SY5Y cells also show greater tyrosination levels, in this case for both ANKK1 isoforms transfections (21,09 % for ANKK1-K and 17,46 % for ANKK1-FL) (Figure 10, C and D).

Considering the effect of apomorphine treatment over tyrosination levels of transfected cells, apomorphine seems to have no effect in none of the transfected cell lines (Figure 10).

4.1.4. ANKK1 overexpression correlates with a change in microtubule acetylation pattern.

To study the effect of ANKK1 overexpression upon acetylation, we performed immunofluorescences in neuroblastoma cell lines using anti acetylated α-tubulin. For

this PTM, we observe differences in the acetylation pattern related to cell line and apomorphine treatment (Figures 8 and 9).

Regardless of the ANKK1 isoform overexpressed, in untreated SK-N-SH cells, acetylated α-tubulin microtubules are enriched in the proximal axonal region close to the soma (Figure 8, A and B, I). This pattern coincides with the one expected, specifically microtubule acetylation enriched at proximal axons rather than in cell bodies and growth cones (16). Cells treated with apomorphine show a more expanded acetylation pattern that covers both proximal and distal cell areas (Figure 8, E and F, I).

In the case of SH-SY5Y cell line, we observe a different acetylation pattern where the signal is located throughout the soma and the neurites, especially in the case of ANKK1 FL-GFP overexpression (Figure 9, B and C, I). Conversely, in the presence of apomorphine, ANKK1 K-GFP is the isoform that induces this different acetylation pattern.

To gain more insight in the microtubule acetylation pattern, we carried out a western blot and densitometry analysis (Figure 11).

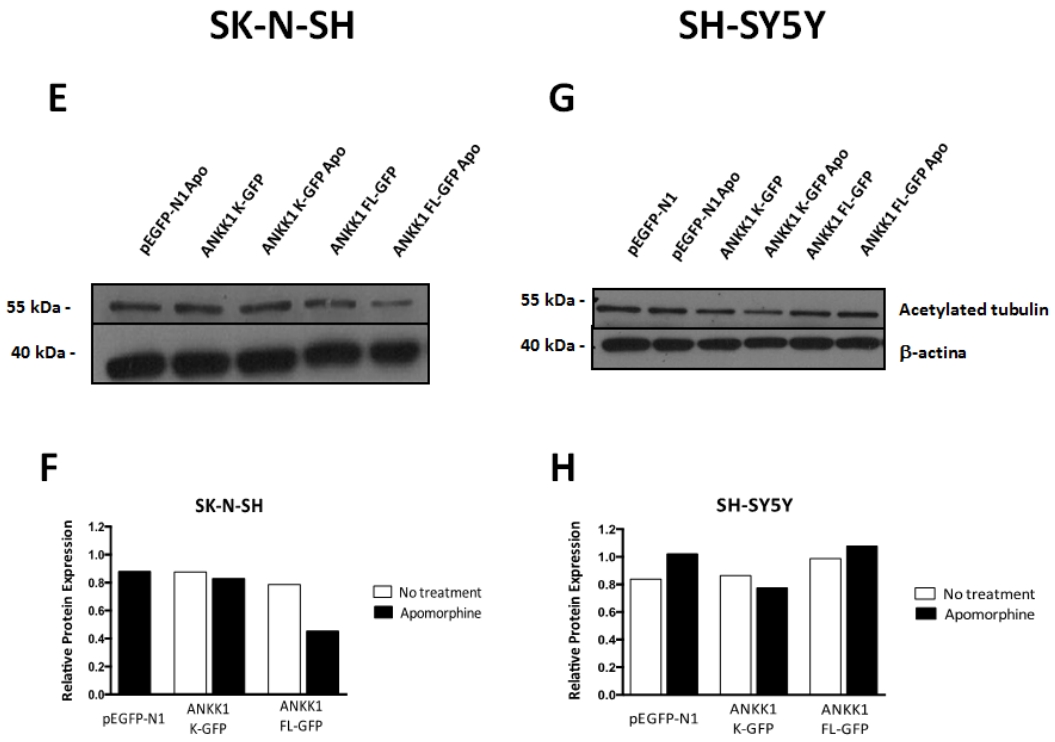

Figure 11. ANKK1 overexpression effect over microtubule acetylation levels. **E and F,** immunobloting and densitometry analysis of SK-N-SH cells transfected with Pegfp-n1, ANKK1 K-GFP and ANKK1 FL-GFP with and without apomorphine treatment. **G and H**, immunobloting and densitometry analysis of SH-SY5Y cells transfected with Pegfp-n1, ANKK1 K-GFP and ANKK1 FL-GFP with and without apomorphine treatment.

From this semi-quantitative analysis we can conclude that apomorphine has an effect itself upon the acetylation levels (Figure 11, C and D). On the other hand, this effect can be modulated by both ANKK1 isoforms overexpression (Figure 11, C and D).

5. DISCUSSION

The gene *ANKK1* that codes for the receptor interacting protein 5 (RIP5) has been widely associated to cognitive traits, psychiatric disorders and the dopaminergic functioning itself (3). However, the function of ANKK1 is still unknown, and there is a great interest in studying this protein and the signal pathways in which it is involved.

ANKK1 is known to be expressed in the radial glia cells of the developing central nervous system, which suggests that it might be implicated in neurodevelopment and plasticity of the brain (2). In these processes, neuronal microtubule cytoskeleton plays a crucial role. Here we study the relationship between ANKK1 and microtubule PTMs in order to comprehend the putative involvement of this protein into cytoskeleton dynamics.

To that purpose we investigated the effect of ANKK1-K and ANKK1-FL isoforms overexpression upon microtubules organization in neuroblastoma cell lines. Specifically, we have studied the post translational modifications (PTMs) that tune their dynamic functions. The use of two different neuroblastoma cell lines, one expressing Caspase-8 (SK-N-SH) and one that doesn´t (SH-SY5Y), adds another qualitative parameter to observe. Caspases are known to interact with RIP kinase protein family and to regulate their function (20). Indeed, RIP4 has been proved to have a caspase cleavage site in its interdomain and its function is modulated by Caspase-8 (21). Since ANKK1 is a RIP kinase structurally related to RIP4, it is likely that Caspase-8 would also have an impact upon its biological function.

In first place, after ANKK1 overexpression we observed an astonishing morphological change in neuroblastoma cells, regardless the protein isoform (ANKK1-K vS ANKK1-FL) and/or the cell line. The changes are towards a more neuronal-like phenotype, with features such as bipolar structure, increased connectivity and increased cytoplasmic projections akin to neuritis. Moreover, a 2 hours dopaminergic treatment with a non-selective dopamine receptor agonist changes drastically the cells morphology towards a more astrocyte-like phenotype. The astrocyte-like cells show features such as absence of bipolarity, low reticular connectivity and stellate morphology.

Interestingly, these induced phenotypes are more pronounced in the case of SK-N-SH cells, which are the ones that express caspase-8. Although there is a need to replicate these findings and further studies must be done, we think these results suggest that ANKK1 could be involved in the radial glia differentiation. Further, it is tempting to propose that ANKK1, that is located in the radial glia, participates in differentiation of neural lineages. On the other hand, since ANKK1 is expressed all along the neurodevelopment (neurogenesis and astrogliogenesis), our findings also suggest that dopaminergic stimulation might be a modulator of the protein function or the pathways where it is involved. To prove these hypotheses, further studies need to be carried out. First of all, immunolabelling experiments with neuronal and astrocyte specific markers would corroborate the observed induced phenotypes. Also, specific dopaminergic treatments would outline a possible transduction pathway that may be interacting with ANKK1.

Regarding the recombinant ANKK1 subcellular location, we observed that SK-N-SH and SH-SY5Y cell lines present overexpressed ANKK1 isoforms in both the nucleus and cytoplasm. Additionally, the subcellular location of the recombinant ANKK1 was affected by the apomorphine treatment as it was previously reported in other cell lines (1). To define more precisely this subcellular location, replicas should be done and subcellular fractions should be obtained and examined by quantitative methods.

Considering the microtubule PTMs, which was our matter of study, we found differences in terms of tyrosination levels and acetylation pattern of α-tubulin in neuroblastoma microtubule cytoskeleton.

Firstly, we found that α-tubulin tyrosination increases in SK-N-SH cells when the ANKK1-FL isoform is overexpressed and not for the ANKK1-K. In the case of SH-SY5Y cells, the overexpression of both ANKK1 isoforms causes the increment of α-tubulin tyrosination. It has long been known that stable microtubules, as compared with dynamic microtubules, are enriched in detyrosinated α-tubulin, which contributes to microtubule stabilization. Microtubules enriched in tyrosinated α-tubulin are the ones that suffer rapid growth and shrinkage to generate pushing and pulling forces in cells (15). Our results suggest that ANKK1 overexpression affect α-tubulin tyrosination that

is related to processes that require microtubule dynamics: neuritic outgrowth, growth cone advance, mitosis, cell migration and response to guiding cues. On the other hand, apomorphine seems not to have an effect upon α-tubulin tyrosination levels. Nonetheless, experimental replicas should be done to corroborate the differences found, since one individual semi-quantitative experiment is not enough to draw a conclusion.

Regarding α-tubulin acetylation, control cells show a high signal in proximal axons microtubules while it was less present in dendrites and growth cones. In contrast, when we overexpressed ANKK1-FL in SH-SY5Y cell line, we found a different pattern when compared with ANKK1-K and controls. Specifically, α-tubulin acetylation was detected throughout the soma both at proximal and distal areas suggesting an ANKK1 effect upon this PTM in microtubules. With the limitation of one single experiment, this suggests that ANKK1 function could affect α-tubulin acetylation, a PTM involved with processes that require a redistribution of microtubule acetylation, such as neuron polarization, neurite outgrowth, and cell proliferation. Moreover, apomorphine shows to have itself an impact on α-tubulin acetylation signal and acetylation levels, as we can see from the immunofluorescence and western blot respectively.

6. CONCLUSIONS

This work was a first approach to study a possible relation between ANKK1 function and neuronal cytoskeleton dynamics, using a neural cell model. Taking together our results, we came to several conclusions:

- Overexpression of both ANKK1-K and ANKK1-FL isoforms in neuroblastoma cell lines causes a morphological change towards a more neuronal-like phenotype.
- Overexpression of both ANKK1-K and ANKK1-FL isoforms together with apomorphine treatment causes a morphological change towards a more astrocyte-like phenotype.
- α-tubulin tyrosination levels increases in SK-N-SH neuroblastoma cell line after ANKK1-FL isoform overexpression. In SH-SY5Y neuroblastoma cell line, overexpression of the two ANKK1 isoforms increases α-tubulin tyrosination levels. No differences in α-tubulin tyrosination pattern are observed from immunofluorescence studies.
- α-tubulin acetylation signal change is observed in SH-SY5Y neuroblastoma cell line after the overexpression of ANKK1-FL isoform, specifically an expanded pattern is observed throughout the soma both at proximal and distal areas.

To confirm these conclusions, further studies need to be carried out. Specific dopaminergic treatments would outline the possible transduction pathway that may be interacting with ANKK1. Moreover, labelling with specific markers of different neural lineages would corroborate ANKK1 implication in neural differentiation processes.

Finally, there is still a long way to go in order to fully know the function of ANKK1.

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