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Unravelling the roles of lysine acetylation in neural development

Backround

The goal of our proposal is to elucidate new fundamental mechanisms that regulate neurogenesis in the developing nervous system with a focus on the cerebral cortex and the inner ear. In these structures, the generation of mature cells requires a tight coordination of multiple cellular activities including specification, cell cycle exit, migration and differentiation whose achievement relies on the implementation of transcriptional and post-transcriptional/translational events. Indeed, reversible post-translational modifications (PTM) of proteins play pivotal roles for the establishment of the nervous system (Creppe et al., 2009; Zhao et al., 2008). Numerous PTM have been identified among which some are responsible for the addition of functional groups (e.g. phosphorylation, addition of phosphate group), the addition of proteins or peptides (e.g. ubiquitination, addition of ubiquitin proteins), the modification of the chemical nature of amino acids (e.g. citrullination, conversion of arginine to citrulline), or for structural change (e.g. formation of disulfide bridges, covalent linkage between two cysteine amino acids). These chemical modifications occur after translation and regulate the activity, stability, localization or function of proteins. Lysine side chains of proteins are subjected to different rivalling and reversible PTM, including acetylation, methylation, ubiquitination, sumoylation or ADP-ribosylation (Merrick and Duraisingh, 2007). In vertebrates, lysine acetylation sites are as conserved as those in phosphorylated proteins, suggesting a selective pressure to maintain this protein modification. Recent data also indicate that such modification occurs on almost 2000 proteins (Choudhary et al., 2009), which is close to the size of the phosphoproteome. Although this PTM was until recently exclusively associated with transcriptional activation (through neutralization of positive charges of core histone tails lysines (Ren and Gorovsky, 2001)), there is now growing evidences to support lysine acetylation of a broad range of non-histone proteins (Choudhary and Grant, 2004; Close et al., 2010; Kim et al., 2006). This modification is promoted by lysine acetylases (KATs) and requires acetyl-CoA as the acetyl donor. It is believed that lysine acetylation regulates the activity, localization, specific interaction as well as stability/degradation of proteins, therefore controlling a variety of cellular processes such as apoptosis, proliferation and differentiation (Spange et al., 2009). Recent studies suggest that acetylation of cytoplasmic substrates contributes to brain development (Creppe et al., 2009; Reed et al., 2006) and, that disruption of this process is associated with various progressive neurological disorders (Dompierre et al., 2007; Hempen and Brion, 1996). Although it is widely accepted that a tight interplay between lysine deacetylases (KDACs) and KATs acts antagonistically (Creppe et al., 2009) to control protein acetylation, the enzymes that catalyse such modification on non-histone proteins remain often unknown. Thus, identifying KATs and KDACs as well as proteins whose dynamic acetylation regulates neurogenesis is pivotal to better understand the development of the central nervous system and in particular the cerebral cortex and the inner ear. This fundamental knowledge will be required to develop new therapeutic strategies for neurological and hearing disorders.

Aims of the scientific programme

The goal of this proposal is to uncover new protein substrates that undergo lysine acetylation during the development of the nervous system. For this purpose, we will use complementary approaches to identify putative candidates. Thus we will try to understand how the acetylation of specific proteins contributes to cerebral cortical neurogenesis (Aim 1) or inner ear development (Aim 2). For this purpose

we will use a combination of genetic and molecular technologies to validate their contribution to the development of these structures.

The following report summarizes the work performed the past year thanks to the generous funding from the FMRE/GSKE and provides the perspectives of our future research.

Aim 1: Defining how protein (de)acetylation regulates cerebral cortical neurogenesis

The cerebral cortex contains neurons that are distributed within layers and are regionally organized into specialized areas that underlie sophisticated motor, cognitive and perceptual abilities (Rash and Grove, 2006). Cortical lamination follows an « inside-out » sequence of neuronal placement and maturation that arises from the sequential birth and orderly migration of pyramidal projection neurons born in the dorsal telencephalon (Gupta et al., 2002) and, GABAergic interneurons generated in the ventral forebrain (Anderson et al., 1997). The projection neurons undergo radial migration along radial glia fibers to settle in the cortical plate, while interneurons migrate tangentially from the medial and caudal ganglionic eminences (MGE and CGE, respectively) to reach the cortical wall. More generally, the development of the cortex progresses through several stages including, neural proliferation, neuroblast migration and neuronal differentiation. Disrupting the completion of one or several of these steps often cause cortical malformations that can lead to severe learning disabilities, mental retardation and epilepsy (Bielas et al., 2004; Gupta et al., 2002). Thus, identification of new molecular pathways that promote the formation of the cortex is critical to interpret the pathological mechanisms that contribute to the onset and the progression of these disorders. Acetylation of α-tubulin in microtubules has recently been associated with the maturation (Creppe et al., 2009) and survival of neurons (Dompierre et al., 2007) and such modification is likely to occur on various protein substrates that are required for neurogenesis. While recent works revealed the existence of hundreds of acetylated cytoplasmic and mitochondrial proteins (Choudhary et al., 2009; Kim et al., 2006), some being expressed in neurons and their progenitors, the role of such modification and the identity of the KATs and KDACs that are responsible for the (de)acetylation of these substrates often remains unknown (Choudhary et al., 2009). Elongator is a multiprotein complex composed of 6 subunits (Elp1-6), which is expressed both in the nucleus and the cytoplasm where it plays multiple functions. It promotes acetylation of histones in the nucleus and thus contributes to transcript elongation (via the KAT domain). In addition, it promotes paternal genome demethylation (via the SAM domain). In the cytoplasm, it contributes to exocytosis and tRNA modification, and it has been shown in our laboratory that its acute loss resulted in alpha tubulin acetylation defects in microtubules. This posttranslational modification contributes to the migration and differentiation of cortical projection neurons. We ***searched for additional cytoplasmic candidate proteins that are acetylated by Elongator and that promote the development of the cerebral cortex*** For this purpose, we combined a candidate-based approach with a proteomic screen to compare the cortical acetylome (proteome of acetylated proteins) of WT and cKO Elp3 mice. One of the candidate proteins is a connexin. We found that this connexin is enriched in the developing cerebral cortex and is massively acetylated. Western blot analyses performed on cortical extracts from Elp3 conditional knockout (Elp3lox Foxg1:Cre) E12 mouse embryos showed a dramatic reduction of the level of acetylation of the connexin. In addition, co-immunoprecipitation assays with cortical tissue extracts demonstrated an interaction between the connexin and Elp1, the scaffold subunit of the Elongator complex. Thus, this connexin is a strong candidate for acetylation by Elongator. These results have been confirmed in several mouse and human cell lines. In addition, we found that HDAC6 is a KDAC responsible for the deacetylation of this connexin. We are currently assessing the putative role of connexin acetylation in corticogenesis, focussing on radial migration and the control of the interkinetic nuclear movement (INM). These biological events have previously been linked to connexin expression but not its acetylation. We

are currently identifying the lysine residues targeted by Elp3 with mass spectrometry on N2A cells, that expressed or not Elp3. We will perform mutations to important lysine residues by arginines. Plasmids coding for various lysine mutant forms will be engineered and delivered together with Cre-expressing vectors (to remove the endogenous Elp3) by in utero electroporation into cortical progenitors from cKO Elp3 embryos. Finally, will performed time-lapse recordings to study radial migration as well as the INM kinetics.

To investigate the ***role(s) of Elongator in tangential migration*** of cortical interneurons, we used an Elp3 flox; Dlx5,6 Cre-GFP mouse line (Elp3 cKO) newly generated in our laboratory. Real-time experiments on Elp3 cKO or WT MGE explants were performed on interneurons to analyse their migration as well as their cell shape modifications. After 24 hours of culture, control interneurons that have migrated out of MGE explants exhibited a polarized morphology with branched leading process. Our preliminary data indicated that interneurons that lack Elp3 expression had a significant decrease of migration velocity as well as a reduced frequency and amplitude of nuclear translocations. In addition, only 40% of Elp3 cKO migrating interneurons displayed a swelling. Real time imaging also indicated that the formation and division of growth cones (that underlie the production of new branches) were both, less frequent and less stable in Elp3 cKO interneurons, as compared to control experiments. Furthermore, immunolabeling of E12.5 embryo sections showed that the loss of Elp3 expression resulted in abnormal cellular shape and in a significant reduction of the number of GABAergic interneurons that entered into the cortex. This observation suggests that the conditional removal of Elp3 resulted in a tangential migration delay and supports the time-lapse results on MGE explants. Collectively, our data describe a novel role for Elp3 in the control of nucleokinesis kinetics, branching dynamics of interneurons, growth cone splitting and stability of newly formed neurites. To determine how Elp3 controls these parameters, we will combine time–lapse recording of MGE explants from Elp3 cKO GABAergic interneurons with rescue experiments. For this, we will electroporate vectors coding for Elp3 protein that lack either the Histone acetyl transferase (HAT) or the DNA methyltransferases (SAM) domains. In order to untangle the molecular mechanisms triggered by Elp3 to control tangential migration, we will FACS MGE- and CGE-derived GFP-positive interneurons and we will perform microarray experiments and mass spectrometry analyses to identify new genes or proteins regulated by ELP3.

Aim 2: Defining how protein (de)acetylation regulates the development of the inner ear

The development of the inner ear involves multiple processes including proliferation (in mice, ranging from E12.5 to E14.5 in the cochlea and between E12.5 and P2 in the vestibule) and specification of progenitors (between E15.5 and P4) into hair cells, the highly specialized mechanoreceptors, and supporting cells of the sensory epithelia. Concomitantly, epithelial neuroblasts delaminate from the otic epithelium to form the neurons of the cochleo-vestibular ganglion, which innervate the otic sensory elements (Rubel and Fritzsch, 2002). In the mammalian inner ear, similarly to the central nervous system (CNS), the regenerative capacity of hair cell and/or cochleo-vestibular neurons is lost during adulthood and no functional compensation is achieved. Consequently, deafness or balance dysfunctions, commonly resulting from lesion of the hair cells and/or of the neurons of the auditory or vestibular part of the inner ear, respectively, are permanent. There are currently no treatment designed to halt or prevent the progression of hearing loss or vertigo, therefore, understanding the molecular signals that control the number of progenitors, their differentiation and their tissular organization in the inner ear is a prerequisite for developing new strategies to promote hair cell regeneration and partially restore hearing. The main objective of this part of the project is to uncover the role of lysine acetylation during the inner ear development. We first focused our attention on the role of Elongator complex.

We first unravel the temporal and spatial expression of Elp3 and Elp1, two main members of the Elongator complex. Elp1 and Elp3 mRNA transcripts have been detected in the developing inner ear

and have a strictly overlapping pattern of expression. At E11.5, the first stage studied, they are present in the entire otic vesicle and absent in the surrounding mesenchyme. Later, there expression became mainly restricted to neurons in the cochlea-vestibular ganglion and to the sensory epithelium in the cochlea and the vestibule.
Using the newly created mouse line Elp3loxp/loxp, we generated FoxG1-cre conditional Elp3 knockout mice (referred to as Elp3 cKO) allowing the deletion of Elp3 in the entire otocyst at ≈E8.5. Although they were viable, these mice exhibited balance-related behavioral phenotype characterized by a tilted position of the head, circling movements, and a marked tendency to walk backwards when placed outside their cages. In addition, in the tail-hanging reflex, which normally induces a forelimb extension to reach the ground, they tended to bend ventrally and curl up their tail. We also analyzed Elp3 cKO mice at the cellular level both in the sensory epithelium and the cochlea-vestibular ganglion. Preliminary results showed that the kinocilium, a specialized primary cilium, is disorganized and that the adjacent stereocilia are misaligned in Elp3 cKO mice. Taken together, these results are in favor of a role of Elp3 in planar cell polarity. In the spiral ganglion (innervating cochlear hair cells), loss of Elp3 is associated with a massive neuronal apoptosis at E14.5. There is also a conspicuous decrease of the number of fibers that innervate hair cells. In addition, numerous remaining fibers present aberrant projections towards inner hair cells. Altogether, these results indicate that Elp3 seems to be involved in neuronal survival and axonal guidance in the cochlea.

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