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Tau protein aggregation and α-synuclein dysfunction: development of new *in vitro* and *in vivo* models to study neurodegenerative diseases

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Résumé

Les signatures histopathologiques de principales maladies neurodégénératives - maladie d'Alzheimer et la maladie de Parkinson - sont les enchevêtrements neurofibrillaires formés par la protéine tau et les corps de Lewy, formés par l'α-synucleine agrégée. La formation et l'accumulation d'inclusions tau provoquent des perturbations fonctionnelles du cytosquelette, et conduit à une dégénérescence neuronale. Les mécanismes précis du repliement et de l'agrégation de ces protéines, pour la protéine tau comme pour l'α-synucleine, ne sont pas totalement compris à ce jour.

Ici, nous nous sommes intéressés à cette question en utilisant des modèles in vitro et in vivo. En étudiant l'agrégation tau in vitro, nous avons mis en évidence un nouvel auto-assemblage réversible de tau, qui dépend de la température et de la présence d'ions zinc, et qui est a priori différent de l'agrégation de tau en présence d'inducteurs d'agrégation tels que l'héparine. Ce processus pourrait néanmoins être impliqué dans les premières étapes de l'agrégation pathologique de tau. Dans une deuxième partie nous avons développé des modèles murin pour étudier les dysfonctionnement de l'α-synucleine. Nous avons montré que l'α-synucleine est directement impliquée dans le développement embryonnaire de régions spécifiques du système nerveux, et qu'elle a des propriétés modulatrices seulement sur les neurones dopaminergiques de la substantia nigra, qui sont touchés dans la maladie de Parkinson.

Les résultats obtenus dans nos études de deux protéines qui subissent une agrégation pathogène et forment des inclusions intracellulaires ont contribué à la compréhension des processus moléculaires et cellulaires associés à la dégénérescence neuronale, ce qui fournira de nouvelles pistes pour développer de nouvelles stratégies de thérapies de maladies neurodégénératives.

Abstract

The histopathological hallmarks of the most common neurodegenerative diseases – Alzheimer's disease and Parkinson's disease are neurofibrillary tangles formed by tau protein and Lewy bodies inclusions formed by aggregated α-synuclein. The formation and accumulation of these proteins into inclusions cause functional disruptions of the cytoskeleton and leads to neuronal degeneration. The precise mechanisms of tau and synuclein misfolding and aggregation leading to those cellulare incluses, even though very studied, are not fully understood neither for tau protein nor for α-synuclein.

Here we have addressed this question using both in vitro and in vivo models. Investigating tau aggregation in vitro, we have found a reversible self-assembly of tau, which depends on temperature and is induced by zinc ions, which is different from the tau aggregation in the presence of aggregation-inducers such as heparin. This process could be implicated in the first steps of tau pathological aggregation. In a second part, we have developed a mouse model for studying the α-synuclein dysfunction. We have shown that α-synuclein is directly involved in the embryonic development of the specific regions of the nervous system, and that it has modulating effect only on the populations of dopaminergic neurons of substantia nigra, which are affected in Parkinson's disease.

Results obtained in our studies of two proteins that undergo pathogenic aggregation and form intracellular inclusions contributed to understanding of molecular and cellular processes associated with neuronal degeneration, which is important for the development of new disease-modifying therapies of neurodegenerative disorders.

2. Results

2.1. *In vitro* model of tau aggregation

Zinc is one of the most abundant transition metal in the body that serves to regulate many vital functions such as cell signaling, brain development and the responses of immune cells (Barr and Burdette, 2017; Fraker et al., 2000; Grabrucker et al., 2011). Despite vast importance, zinc, especially excess amounts of zinc, is neurotoxic, thus induce neuronal injury and cause cell death (Wani et al., 2017). Growing evidence reported that zinc may contribute to neurodegenerative diseases by initiating proteins aggregation. *In vivo* study showed that dyshomeostasis of intracellular zinc leads to lysosomal dysfunction and thus promotes α-synuclein accumulation (Tsunemi and Krainc, 2014). In addition, zinc was shown to induce α-synuclein aggregation and specifically its fibrillation (Breydo et al., 2012). Our recent data showed that zinc also binds to TDP-43 and cause its aggregation, pointing to potential role of zinc in the development of ALS (see annex).

Tau, the main component of neurofibrillary tangles in AD also appeared affected by zinc. Tau hyperphosphorylation, that believe to be an initiating event in the genesis of paired helical filaments, can be triggered by zinc dependent activation of the glycogen synthase kinase-3beta and inactivation of phosphatase like protein phosphatase 2A (Boom et al., 2009; Kwon et al., 2015). However, a growing body of evidence demonstrated that zinc directly binds to tau and thus induces its aggregation (Hu et al., 2017). It has been reported that zinc dramatically accelerate the fibrillization of tau fragment (244-372) and pathological mutant dK280 of human tau, associated with FTDP-17, via bridging Cys-291 and Cys-322 (Mo et al., 2009). In vitro studies confirmed the ability of zinc to bind to another human tau mutant hTauR406W, also associated with some cases FTDP-17 (Huang et al., 2014). A recent study showed that zinc interact with cysteine in R3 repeat of tau and significantly accelerate aggregation of three repeat tau construct (R1-R3-R4) (Jiji et al., 2017). All these results introduced zinc as an important factor in tauopathy and as a target for therapy development.

However, much uncertainty still exists about the process of zinc promoted aggregation of tau leading to development of neurodegeneration. It is worthy to note that all present studies about tau aggregation performed using mutant forms of tau protein and/or in presence of polyanions, that artificially accelerate aggregation of tau. Here we have investigated aggregation of full-length human hTau40 isoform in the presence of zinc ions in a wide range of temperatures using turbidimetry, isothermal titration calorimetry, dynamic light scattering and transmission electron microscopy. We found that contrary to zinc induced aggregation of TDP-43 and zinc accelerated aggregation of tau in the presence of heparin, zinc-initiated oligomerization of tau in absence of artificial aggregation inducers is a reversible process, which depends on temperature and concentration of zinc ions.

2.2. *In vivo* modelling of α-synuclein dysfunction

A progressive loss of DA neurons that occurs mostly in the SN is the hallmark of PD. It is believed that α-synuclein is involved in this process because its dysfunction caused by mutations, abnormal post-translational modifications and aggregation is associated with DA neurons pathology. In addition to dysfunction and death of DA neurons caused by toxic gain of α-synuclein function, it is feasible that the loss of this protein function also contributes to the disease pathogenesis.

To prove this idea, several mouse lines with inactivation of the gene encoding α-synuclein have been produced. However, studies of these lines provided convincing evidence that the constitutive removal of α-synuclein has a very limited effect on the nervous system of genetically modified mice. Since the synuclein family includes three proteins, which have high degree of similarity to each other and potentially, partially overlapping functions, the possibility of replacing the function of genetically inactivated α-synuclein by two other functionally active members of the family cannot be excluded. Such functional redundancy is possible during embryonic development at the stage of ontogenetic selection, when the plasticity of the nervous system is exceptionally high.

Previous studies revealed reduced complement of dopaminergic neurons in the SN of early postnatal and adult α-synuclein knockout mice originally produced by Abeliovich et al. (2000) compared to their wild type littermates (~15% reduction), whereas dopaminergic neurons of the ventral tegmental area (VTA) were not affected in these mice (Robertson et al., 2004). These results were later confirmed and extended to C57Bl6s line with a large deletion encompassing *Snca* gene (Garcia-Reitboeck et al., 2013). It has also been shown that this reduction is already obvious at embryonic day 13 (Garcia-Reitboeck et al., 2013).

To study this phenomenon in more details, we compared the number of dopaminergic neurons in SN and VTA of α-synuclein knockout and wild type mice at earlier stages of embryonic development, starting from the embryonic day 11 (E11), the earliest time point that allow confidently discriminate these two neuronal

populations. Serial histological sections through the mesencephalon region of E11, E12 and E13 embryos were prepared and immunostained with antibodies against tyrosine hydroxylase (TH), a rate-limiting enzyme in the process of DA synthesis and a marker commonly used for identification of dopaminergic neurons. Stereotaxic counting of TH-positive neurons confirmed the previous observation about the decreased number of these neurons in the SN of knockout mice. However, at E12 no difference in the number of TH-positive neurons was observed in the SN and at E11 the observed difference was opposite, more neurons were found in the SN of α-synuclein knockout mice than of wild type mice. At the same time, no statistically significant difference in the number of TH-positive neurons was observed in the VTA of these two groups of animals at all three studied developmental time points.

However, if α-synuclein deficiency in presynaptic terminal was indeed developing in neurons of PD patients as hypothesised, significant loss of its function shall occur only in adult or even ageing nervous system, whose compensatory ability is much lower comparing to the developing nervous system. Thus, in a relevant model of α-synuclein deficiency in PD, expression of α-synuclein should remain normal during development to prevent activation any compensatory mechanisms at that stage and be switched off in adult animals. This could be achieved in a sophisticated mouse model that combines conditional knockout of *Snca* gene and chemically triggerable expression of an activator of the gene deletion. Production of such system is described in (Ninkina et al., 2015) and the enclosed paper (Roman et al., 2017).

In the genome of a core mouse line the first coding exon of the *Snca* gene has been flanked by LoxP-sites ("floxed"), which allowed Cre-recombinase-driven deletion of the flanked region leading to complete inactivation of α-synuclein expression. Animals of this line can be bred with animals of another line that carries a transgenic cassette in which expression of Cre-recombinase is driven by a tissue- or cell-specific promoter, for example neuron-specific enolase (NSE) promoter to restrict the Cre-expression to neurons or TH promoter to restrict the Cre-expression to catecholamine neurons only. Moreover, transgenic lines expressing Cre-recombinase modified by conjugation with estrogen receptor (ER) could be used. The modified Cre is sequestered in the cell cytoplasm and therefore is inactive until binding estrogen analog tamoxifen, which triggers its translocation to the cell nucleus

where it can exert its recombinase activity. In our study we used a transgenic mouse line expressing modified Cre-ERT2 recombinase under control of NSE promoter, which allow deletion of "floxed" sequences in neurons only after tamoxifen administration to experimental animals. For this type of conditional inactivation experiments it is vital to have an ability to control the efficiency of the the LoxP/Cre recombination and identify cells in which this recombination took place. This is commonly achieved by introducing into genome of animals with "floxed" gene of interest of a reporter locus, for example a "floxed-stop"-lacZ cassette into mouse endogenous *Rosa26* locus. For further functional experiments it was important to generate mouse lines that will carry modifications of *Snca* gene (either "floxed" or completely inactivated) and a reporter *Rosa26* locus. Production of such lines was complicated by localization of *Snca* and *Rosa26* at the same mouse chromosome 6, approximately 54 Mb apart. To overcome this problem, we carried out a large breeding and selection programme to obtain founder animals with *Snca* and *Rosa26* appear on the same chromosome *in cis* as the result of natural chromosome crossover in meiosis. Two lines with desired chromosomal arrangement of *Snca* and *Rosa26* loci have been produced. One of these lines carried the *Snca* gene with "floxed" first coding exon and in another one this exon has been already deleted by LoxP/Cre recombination in the previous breeding program and therefore, this line represented a new variant of constituent α-synuclein knockout. Importantly, deletion of the first coding exon, either constitutive or conditional, left only minimal "footprint", i.e. only one 22-nucleotide LoxP site in the *Snca* gene intron and as the result expression of no other gene is affected in mice carrying these genomic modifications, which makes them significantly better models of α-synuclein deficiency than other lines previously produced in other laboratories.

3. Discussion & perspectives

3.1. A new Tau self-assembly model

So far most of the *in vitro* studies about tau aggregation into PHFs have been performed using established models which involve aggregation cofactors such as heparin or congo red. While it might be a good model to study the impact on this process of potential inhibitors, or the effect of some post-translational modification on tau, we believe that it might not be the best model to study the process itself. Indeed, heparin and congo red by accelerating the aggregation process may also modify it and thus mask or enhance the impact of known modulators of aggregation such as zinc ions. This is why we decided to work in a buffer devoid of any aggregation co-factors, in reducing conditions. In these conditions we have been able to get 3 new insights about zinc impact on tau self-assembly.

3.1.1. Existence of two types of zinc binding sites on tau

Mutations in *MAPT* gene, abnormal PTM and interaction with some aggregation-prone proteins, polyanions and metal ions can induce tau protein aggregation. Recently, *in vitro* studies revealed that metal ions, especially zinc, could impact tau fibrillization (Hu et al., 2017; Mo et al., 2009). In 2009 Mo et al. (Mo et al. JBC 2009) showed that Zn^{2+} accelerates the fibrillization of a tau peptide covering the the 244 to 372 amino acids. In this study conducted in the presence of Heparin they conclude to the existence of 1 type of binding site. They also show that Cys291 and Cys322 play a central role in this binding. While they conclude to the existence of one high affinity binding site (K_d = 3.8 µM), they observe a biphasic effect of zinc. While at low micromolar concentrations Zn^{2+} dramatically accelerates fibril formation, higher concentrations of Zn^{2+} inhibit it. A more recent study conducted this time a one full length tau with a point mutation (ΔK280) still in presence of heparin also concluded that Zn^{2+} dramatically accelerated tau aggregation (Hu et al. BBA 2017). In this study they also determined a single binding site with a high affinity (K_d = 0.7 µM).

Nevertheless, to obtain the fitting they had to remove many experimental points from their isothermal titration calorimetry data. In their discussion they even admit that the possibility that Zn^{2+} has two independent sets of binding sites for tau : a strong binding site at lower molar ratio (they call "specific") and a weak binding site at high molar ratio (they call "non specific").

This is why we conducted a similar ITC experiment but with more experimental points (for this we fused two consecutive ITC titrations). Our results clearly demonstrate that zinc bind to tau 2 types of sites, one site with a high affinity (K_d = 0.5 μM) and three sites with a low affinity (K_d = 16 μM), which could have different functions. First site would correspond to Cys-291 and Cys-322. Other three sites might implicates His (H-268, H-299, H-329, H-330 or H-362) which have been shown to be involved in tau binding (Mo et al., 2009). This interaction, as clearly demonstrated by our DLS data lead to significant structural changes in tau. Indeed, binding of the first zinc ion leads to compactization of the initially disordered protein, which reflects in the drop of the hydrodynamic diameter of Tau species from 12.2 to 10.8 nm. Moreover, it is accompanied by a decrease of polydispersity index, indicating that the population of tau conformations in solution becomes more homogeneous. The conformational changes of tau upon first zinc binding has been also shown by Yunpeng Huang et al. using CD experiments that allowed the authors to suggest a potential physical link between zinc and tau (Huang et al., 2014). The further zinc binding to low affinity sites results in even more compact structure of tau with hydrodynamic diameter about 8 nm, allowing us in turn to hypothesize the existence of physiological role of low affinity binding sites. This role could be significantly different from the one of high affinity site, but to reveal these roles, further investigations are necessarily.

This study raise new questions about mechanism of zinc-induced tau aggregation in particular about the role, played by high and low affinity sites in this process, the localization of the low affinity sites, and which amino acids participate in zinc chelation. The structuration of tau we observed upon zinc binding to both high and low affinity sites opens the challenge of resolution of 3D structure of zinc binding sites using NMR, which could be possible at low temperatures.

3.1.2. Zinc induces fast reversible non-PHF aggregates

Contrary to the well described fibrillary structure of PHFs described in the literature, the aggregates we have observed do not seem to have a homogenous structure. They are thioflavin negative, which mean they do not have beta sheets structure, and consistently with our DLS result they appear as huge granular aggregates. Yet it is not the first time that such granular aggregates are observed with tau constructs. Indeed, it has been shown that $tau_{244-372}$ form similar granular aggregates in presence of high concentrations of zinc (Mo et al., 2009). Similar structures have even been observed from cell preparations (Hu et al., 2017).

One of the main differences between these granular aggregates compared to PHF is that they are fully reversible. Removing of Zn^{2+} using EDTA induces a immediate disassembly of these aggregates. In addition, the fact that the process is also fully reversible when decreasing the temperature seems to indicate that the self-assembly is a intrinsic property of tau. In that sense Zn^{2+} impact on tau structure and self-assembly could have a physiological role, in a similar fashion that Mg^{2+} impacts tubulin self-assembly. Interestingly, hydrophobic/hydrophilic balance within molecule changes during tau aggregation, that could even lead to transition of aggregates in separate phase and formation of droplets (Wegmann et al., 2018).

Figure 22 (see next page) summarize the physiological hypothesis of this new pathway. Indeed, due to its reversibility, the formation of these oligomers could be induced in cells by zinc signals under physiological conditions and play a role in tau regulation. As we have shown the existence of two type of sites, both impacting tau structure, would permit a fine regulation of tau functions, would that be its interaction with tubulin or some of its less studied targets such as DNA for example (Asadollahi et al., 2017).

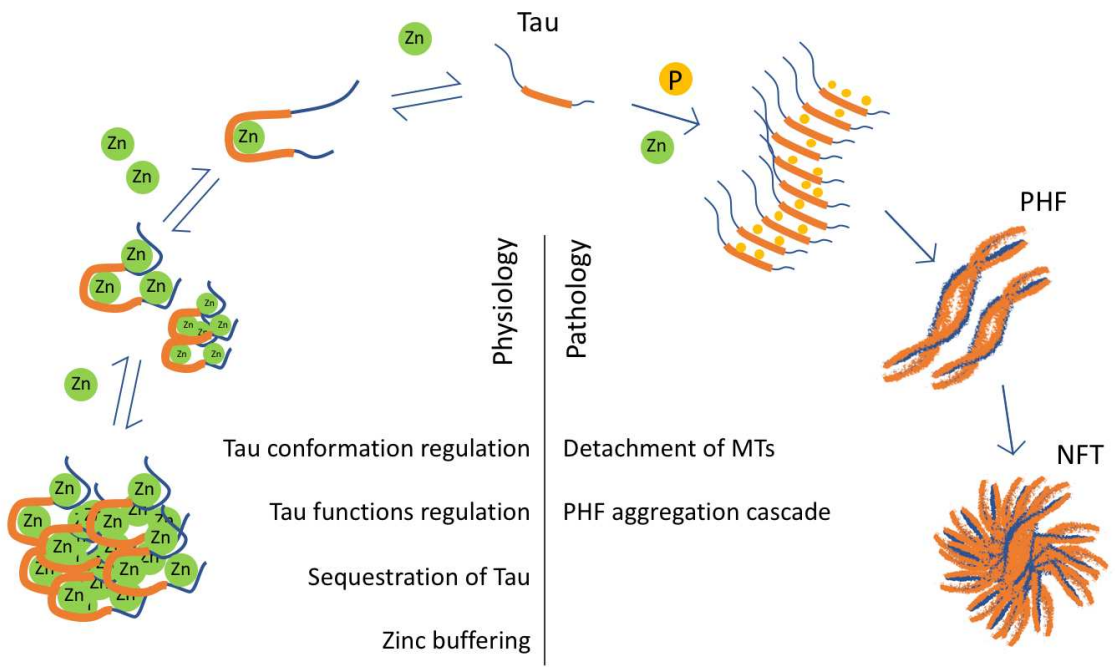

Figure 22 : Zinc-induced reversible self-assembly vs. PHF formation. *The right part of the scheme corresponds to the pathological pathway leading to PHFs formation and NFT formation. This pathway is globally irreversible and favored by post translational modifications sur as (hyper) phosphorylation of Tau, and Zn^{2+}. The left part of the scheme corresponds to a physiological pathway which is fully reversible. In the presence of low Zn^{2+} concentrations, Tau undergoes compaction; in the presence of high Zn^{2+} concentrations it leads to the formation of granular Tau oligomers.*

Another hypothesis would be that this new pathway could be a way of sequestering tau in order to prevent it from going into the PHF formation irreversible pathway. Or on the opposite it could be a way of buffering zinc into the cell so that it does not reach concentration that would that would be toxic for cell functions. Of course, we cannot exclude that those granular aggregates are part of the reversible initial steps leading to PHF formation, but the fact that similar mechanism have been proposed for other prone-to-aggregate proteins such as Amyloid beta is another argument in favor of our hypothesis.

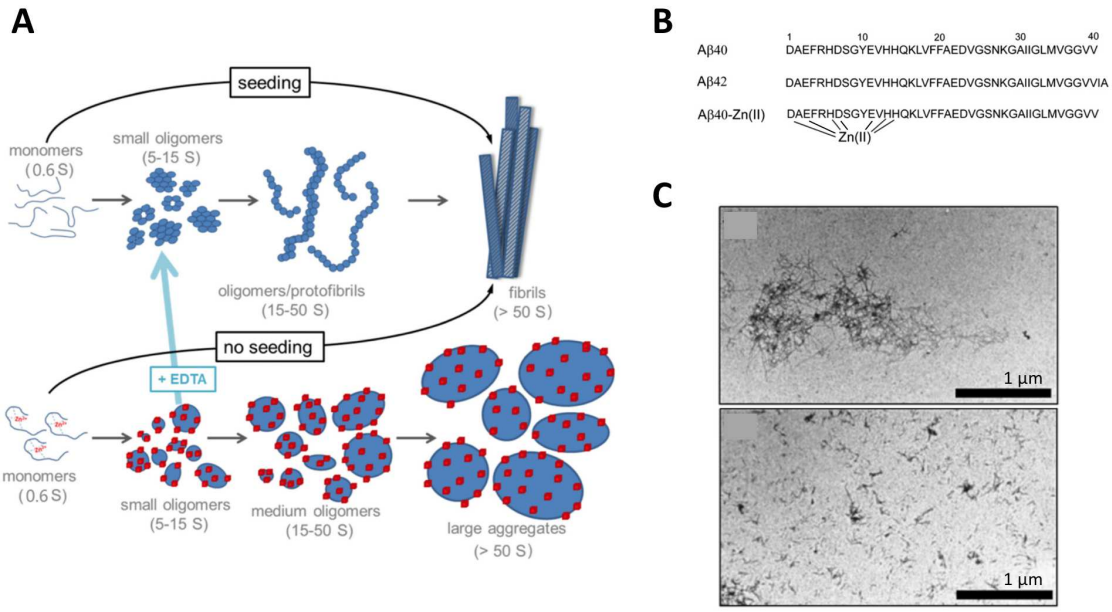

Figure 23. Effect of zinc on amyloid-beta structure. *[A] Amyloid-beta peptides form ordered fibrillar aggregates. In presence of zinc amyloid-beta peptides form amorphous aggregates. [B] Amino-acid sequence of amyloid-beta 40 and amyloid-beta 42, with the main coordination sites of zinc; [C] Long fibrillar structures formed by amyloid-beta 42 (top) and disturbed amyloid aggregates of amyloid-beta 42 formed in presence of zinc (bottom). Adopted from (Mithu et al., 2011; Rezaei-Ghaleh et al., 2011; Zhang et al., 2018).*

Zinc is one of the most abundant metal ion in human body where it is involved in various physiological functions. However, abnormal zinc accumulation is found in NDD. Increased level of zinc observes in amyloid plaques and NFTs in AD, point to a role of zinc in formation of pathogenic inclusions. Recent evidence demonstrate that zinc promotes amyloid-beta aggregation in non-fibrillary way. Equimolar concentration of zinc effect to aggregation kinetics of amyloid-beta, however, zinc leads to changes in structure and morphology of amyloid-beta aggregates (Figure 23).

Thus, an interesting perspective of this work would be to test our hypothesis that this tau reversible aggregation is indeed a concurrent pathway to PHF formation. One of the first things to do would be to test whether tau with specific post translational modifications (for example PHF specific phosphorylation) is still capable of undergoing such a zinc induced reversible self-assembly. Another possible experiment would be to see what happens when heparin is added to the granular aggregates.

3.2. Modelling of α-synuclein pathology

In contrast to tau the modelling of α-synuclein pathologies is more challenging (Benskey et al., 2016). Animal models of overexpression often do not recapitulate all specific features of PD pathology such as selective loss of dopaminergic neurons, substantia nigra degeneration and decreased dopamine level which are key pathological events in PD (Giasson et al., 2002; Gomez-Isla et al., 2003; van der Putten et al., 2000). Knockout models that reproduce synaptic deprivation of α-synuclein as a consequence of changed compartmentalization caused by its cytoplasmic aggregation, have demonstrated mild abnormalities in presynaptic terminals function (Giasson et al., 2002; Gomez-Isla et al., 2003; van der Putten et al., 2000). Knockdown of α-synuclein expression by viral delivery of α-synuclein-specific shRNA into SN of adult animals led to the degeneration in this region (Collier et al., 2016; Gorbatyuk et al., 2010; Kanaan and Manfredsson, 2012) that indicates an important role of this protein in maintaining the functional state of SN in normal and its involvement in the pathology. Genetic association between decreased α-synuclein expression and risk of PD has been also demonstrated (Markopoulou et al., 2014). Therefore, it is feasible that the loss of function of α-synuclein caused by its cytoplasmic aggregation and withdrawing from the synaptic terminals play the pathogenic role in the development of PD. The less prominent pathology in case of constituent gene knockout comparing to gene knockdown in adult animals could be explained by functional compensation for α-synuclein loss during embryonic development when the plasticity of nervous system is very high.

3.2.1. α-synuclein participate in the development of DA neurons in substantia nigra

To reveal the involvement of α-synuclein in embryonic development of dopaminergic neurons we have carried out comparative morphometric analyses of the dopaminergic neurons in the SN and VTA of α-synuclein knockout and wild type mice. SN and VTA are two neighboring anatomical structures that contain populations of dopaminergic neurons. Importantly, in PD brains the loss of

dopaminergic neurons predominantly accrue in SN whereas VTA dopaminergic neurons are much less affected. We have studied the number of dopaminergic neurons at days 11.5, 12.5 and 13.5 of mouse embryogenesis, after the onset of α-synuclein expression at E10.5. At this stage the post mitotic precursor cells are migrated from the ventricular zone at the border of the midbrain and forebrain into the anatomical area of SN and VTA where they undergo further differentiation into mature neurons that express a specific set of marker proteins including TH. During this period of embryonic development, a progressive increase in expression of α-synuclein is observed in both studied structures (Abeliovich and Hammond, 2007).

Our results have shown that the dynamics of the appearance of TH-positive neurons in the embryonic mesencephalon is different for α-synuclein knockout and wild type mice. In the presence of α-synuclein, a sharp increase in the number of TH-positive neurons at E12.5 followed by a moderate decline at E13.5 was observed in the SN, whereas in knockout mice the number of TH-positive neurons appeared to be higher at E11.5 but did not change as much as in wild type SN over the next two embryonic days. As the result, at E13.5 the number of TH-positive neurons in α-synuclein knockout mice becomes lower than in wild type mice, which is consistent with the previously published observations (Garcia-Reitboeck et al., 2013). A possible explanation of these observation is that precursors of SN neurons lacking α-synuclein migrate faster to the mesencephalon but their consequent differentiation into mature neurons is partially compromised.

Significant loss of dopaminergic neurons in the SN have been observed in PD patients whereas the pathology of dopaminergic neurons in VTA is not so profound (Alberico et al., 2015). We did not find statistically significant differences in the number of VTA neurons between α-synuclein knockout and wild type mice at any of the three studied embryonic days. These results suggest that dopaminergic neurons of SN are intrinsically more sensitive to changes of α-synuclein expression than VTA neurons, which is consistent with differential sensitivity of these two groups of neurons to PD pathology (Duda et al., 2016; Maingay et al., 2006; Mosharov et al., 2009).

Obtained data revealed a prominent modulating effect of α-synuclein on the early development and maturation of dopaminergic neurons in the SN and no such

effect on dopaminergic neurons in VTA, suggesting that the loss or ameliorating of α-synuclein function in the adult nervous system might have negative effect specifically on SN neurons and might contribute to their differential dysfunction in PD. This further emphasizes an urgent need for transgenic models recapitulating a late-onset depletion of α-synuclein, which better recapitulates pathological changes in the nervous system of PD patients.

3.2.2. Generation of new mouse lines for conditional inactivation of *Snca*

Recently, a core mouse line for conditional inactivation of *Snca*, α-synuclein encoding gene, has been produced (Ninkina et al., 2015). In studies that involve conditional inactivation of any gene by LoxP/Cre recombination it is important to monitor the efficiency of recombination and identify cells in which this recombination has actually happened. To make this monitoring possible for studies with conditional inactivation of *Snca* gene, we produced two new mouse lines that carry in their genomes both modified *Snca* gene, either "floxed" or already fully genetically inactivated, and a "floxed" reporter cassette in the *Rosa26* locus. The availability of these two new lines makes it possible to produce mice carrying various combinations of genetically modified *Snca* gene and *Rosa26* locus together with Cre-expressing transgenic cassettes. This shall allow easy generation of experimental and control animal cohorts for conditional inactivation of *Snca* gene by LoxP/Cre recombination that can be monitored using a reporter construct in the *Rosa26* locus for fast identification of cells with inactivation of *Snca* and therefore, depleted of α-synuclein.

This conditional α-synuclein knockout models will be a very useful tool for studying various aspects of α-synuclein function and dysfunction, such as seeding/transmission of its aggregation. It will be instrumental for revealing in detail how the loss of normal α-synuclein function that occurs in the adult brain contributes to the development of the nigrostriatal pathology in PD. To date we are doing big in vivo study using our new conditional knockout models. We already produced large cohorts of animals, in part of them we induced depletion of α-synuclein in adult age (6 months). General phenotype and motor behavior of experimental mice are

controlled at regular intervals. Brain samples from representative groups of experimental and control animals will be taken at 4, 8, 12 and 18 months after 6 month injection point. Expression of α-synuclein will be measured by qRT-PCR, Western blotting and immunohistochemistry (in parallel with beta-Gal staining of brain sections to monitor efficiency of Cre/LoxP recombination). Expression of various neuronal and synaptic markers in the brain of animals with special attention to markers of nigrostriatal system function will be performed by qRT-PCR, Western blotting and immunohistochemistry. Levels of dopamine and its metabolites in the striatum will be measured by HPLC. Possibly, loss of α-synuclein functions will lead to loss of dopaminergic neurons. We will investigate this event by morphometric analysis of neurons on a series of sections across the whole anatomical structure of SN and VTA. Thus, we will know what the impact of α-synuclein depletion in the adult brain is.