Chapter 6

General discussion

tTG is a multi-functional enzyme involved in the regulation of many pathways both within cells as well as at the cellular surface. The best characterized function of this enzyme is its protein cross-linking activity leading to formation of protein complexes. Besides its cross-linking activity, tTG can also act as a GTPase, phosphorylate proteins and is shown to have PDI activity, at least *in vitro*. tTG is abundantly expressed throughout the (human) body, including the brain where it is thought to be catalytically inactive under physiological conditions. In the PD diseased brain though, enzymatic activation of tTG occurs in the SNpc, evidenced by the presence of tTG-mediated intra- and intermolecular cross-links in LBs and aggregated α-synuclein isolated from PD SN tissue, which implicates tTG in the pathogenesis of the disease (Andringa et al., 2004; Junn et al., 2003; Nemes et al., 2009).

At the start of this project, however, besides some additional data obtained mainly in biophysical test tube experiments, not much else was known about either the localization of tTG in SNpc neurons or its role in α-synuclein aggregation in PD. The results of our study into the latter subject are described in **chapter 2,** where a clear connection between induction of experimental PD and tTG-mediated transamidation and multimerisation of α-synuclein in cultured human neurons is demonstrated. In fact, our data for the first time showed formation of both intramolecularly cross-linked α-synuclein monomers and intermolecularly cross-linked α-synuclein polymers under PD-mimicking conditions in a cellular setting. Moreover, these data were in line with both results obtained previously by others in PD brain tissue (Andringa et al., 2004; Nemes et al., 2009) as well as data obtained by us in the present study in test tube experiments and, as such, provided an ideal set-up to test in parallel fashion the *in vitro* and cellular effect of novel tTG inhibitors on tTG-mediated α-synuclein cross-linking and aggregation. Results from these experiments demonstrated the efficacy of the potent and cell membrane permeant peptidergic tTG inhibitor Z006 to (almost) completely block tTG-induced cross-linking and aggregation of α-synuclein in an elaborately-characterized cellular model of PD. Z006 may therefore function as a lead compound for further *in vitro* (e.g. primary human neuron cultures) and *in vivo*, i.e. in animal model(s), evaluation of tTG as a therapeutic target against protein aggregation and neurotoxicity in PD. This notion of tTG as a target for neuroprotective treatment in PD is supported by recent data obtained from transgenic mice overexpressing human α-synuclein, in which coexpression of tTG promoted both synuclein aggregation and toxicity (Grosso et al., 2014). However, perhaps the most striking finding of the studies described here, is the discovery of a novel neuronal location of tTG; it is abundantly expressed at the ER. In **chapters 3 - 5** it is revealed that particularly in melanized neurons in PD brain tissue as well as in a PD-mimicking neuronal model tTG is present at (the outside of) the ER where it likely modulates ER-function. Given the novelty of tTG's association with the ER, there is little available literature that helps to fully comprehend the role of tTG at the ER and the possible implications for PD induction and/or progression. Therefore, the main focus of the remainder of this discussion will be to cover the potential consequences of the tTG-ER interaction for PD pathogenesis.

tTG and the UPR

Our data, in combination with recent data from others, strongly support the notion that tTG is associated with the ER in various cell-types throughout the body (Piacentini et al., 2014). The physiological function of tTG expression at the ER is at present poorly characterized, but seems indicative of a normal physiological role. Unique to the melanized neurons studied here though, is the appearance of specific tTG- ER granula during UPR, a complex signal-transduction pathway that mediates cellular adaptation to restore ER homeostasis. These structures seem a hallmark for stressed catecholaminergic neurons and are exclusively observed in SNpc and locus coeruleus (LC) of the PD-affected brain (**chapter3**). An intriguing question remains why this phenomenon seems restricted to this specific neuronal phenotype. At the moment there is no clear answer for this observation, but it is tempting to speculate that the stress-prone nature of these neurons (e.g. high ca^{2+} signalling, catecholaminergic phenotype, α-synuclein expression) could be key in this process.

The homeostasis of the ER can be altered by a series of conditions, such as calcium depletion from its lumen, oxidative stress, and mutations in proteins that traffic through the secretory pathway, among other events. All of these perturbations can result in disruption of the folding process in the ER, leading to the accumulation of misfolded/unfolded proteins inducing ER stress. ER stress, in turn, activates the UPR. In the last couple of years, the involvement of the UPR has become increasingly recognized in the pathogenesis of PD and other neurodegenerative diseases, such as AD and HD (Halliday and Mallucci, 2014; Mercado et al., 2013). The activation of the PERK pathway of the UPR during PD was initially characterized by Hoozemans and coworkers (Hoozemans et al., 2007) and these results were recently strengthened by analogous observations in various cellular and animal PD-models (Chung et al., 2013; Colla et al., 2012a, 2012b; Egawa et al., 2011; Hashida et al., 2012). PERK represents one of the three arms by which the stressed ER can induce the UPR. The stress sensor PERK acts by phosphorylating eukaryotic initiation factor 2α (eIF2α) which, in turn, halts translational repression to help alleviate the overload of unfolded proteins inside the ER. However, whereas short term activation of PERK may be protective in nature, more prolonged PERK activation results in the activation of C/EBP Homology Protein (CHOP) and the induction of apoptosis (Han et al., 2013). More recent studies hint also at activation of the other arms of the UPR at least during experimental PD (Hoozemans et al., 2012). For example, increasing X-box binding protein (XBP1) expression in the inositol-requiring enzyme 1 (IRE1α) -mediated pathway in both mice and cultured catecholaminergic neurons decreased neuronal loss upon MPTP and MPP$^+$ treatment, respectively (Sado et al., 2009). Also, deletion of activating transcription factor 6 (ATF6α) in mice diminished neuronal survival upon MPTP exposure (Egawa et al., 2011; Hashida et al., 2012). The observation of PERK activation in post-mortem PD-brain as described in **chapter 3** is thus in close agreement with these studies. In addition, as described in **chapter 4,** PERK activation was also observed in the MPP$^+$-treated catecholaminergic SH-

SY5Y cells. This suggests that the pathogenic events that occur in PD could be highly similar, if not identical, to the phenomena observed in our *in vitro* PD-mimicking culture model. In this human neuronal model, the induction of ER-stress with various PD and/or more specific ER-stress inducing toxins always coincided with the appearance of tTG at the ER in the form of tTG-positive ER-granules. This co-occurrence of UPR and sequestration of tTG at the ER therefore raises the interesting possibility that both phenomena could be tied together during the progression of PD.

Activation of neuronal tTG

A potentially important discovery described in **chapters 4 and 5** is the finding of enzymatic tTG activity at the ER-membrane upon PD induction. The enzymatic activity of tTG under these conditions is evidenced by the tTG mediated crosslinking of ER-associated proteins calnexin and beclin 1 (although, admittedly, our data do not completely exclude the possibility that beclin 1 is transamidated in the cytosol). These findings couple enzymatic tTG activity directly to ER-functioning, as to be discussed later on. Interestingly, the finding that tTG is enzymatically active at the ER contrasts with the prevailing view that cytoplasmic tTG is enzymatically inactive. In the cytoplasm, abundant GTP and low Ca^{2+} levels are thought to keep the enzymatic activity of tTG in check, and only extreme, apoptotic, stress-conditions induce tTG activity (Smethurst and Griffin, 1996). Which factors might therefore then contribute to the tTG-mediated PTM of several ER-proteins as found in **chapters 4 and 5**? There are several factors known to dramatically decrease the calcium requirement for activation of tTG (Király et al., 2011). For example, the binding of lipids to tTG has been shown to reduce this requirement (Nemes et al., 2009; Singh and Cerione, 1996). Moreover, it has been postulated that the cytosolic face of membranes could serve as calcium stores where the locally accumulated calcium is present in a higher effective concentration than in the surrounding cytoplasm (Nemes et al., 2009). Induction of the UPR through chemical inhibitors of protein folding (Caputo et al., 2013; Lee et al., 2014), or via specific A-gliadin peptides that induce ER-stress, have been reported to induce enzymatic tTG activity (Caputo et al., 2012). It is therefore certainly conceivable that the (stressed) ER membrane may facilitate an environment where tTG activation could occur. Interestingly, the ER is known to interact closely with mitochondria via specialized microdomains called the mitochondria-associated membrane (MAM). These domains are characterized by locally increased Ca^{2+} levels in the micromolar range (i.e., suitable for tTG activation), facilitated by activation of IP3R1 receptors located near the MAM. Moreover, these ER MAM domains are enriched in calnexin, calreticulin, and BiP, classical ER-chaperones strongly linked to regulation of ER Ca^{2+} pools (Hayashi and Su, 2007; Myhill et al., 2008; Rizzuto et al., 1993). In **chapter 4** it was observed that upon stress induction in neurons, tTG not only colocalizes with a number of these MAM-associated ER-chaperones, but also with IP3R1 receptors in granular structures. This observation makes it tempting to speculate that these tTG-ER granules could

represent MAMs, which may provide the right high Ca^{2+} conditions for enzymatic activation of tTG. Although highly interesting, at present this hypothesis remains subject for future investigations. An interesting tool that may help to answer this question would be the use of fluorescent labeled tTG inhibitors, which exclusively bind to the catalytically active 'open' form of tTG but not to the inactivated form and thus exclusively identifies activated tTG (Dafik and Khosla, 2011).

tTG-mediated post translational modification of ER-proteins

It is highly likely that tTG-mediated protein modifications at the ER are not without consequences for ER functioning. In this respect, there is a growing list of tTG-mediated PTM that can drastically alter the function of proteins (Hummerich et al., 2012; Walther et al., 2003, 2011). The massive tTG-mediated transamidation of proteins observed at the ER-surface during the experimental PD conditions described in **chapters 4-5,** even hint at the possibility that these modified ER proteins represent the tip of an iceberg. Important evidence for such a possibility has been put forward by Hamada and coworkers, who recently confirmed that tTG is able to transamidate the IP3R receptor (Hamada et al., 2014). In their study, the tTG-mediated PTM induced a covalent modification of a glutamine residue at position Q2746 of the IP3R receptor leading to attenuated Ca^{2+} release by the ER and, as a consequence, impaired Ca^{2+} signaling (Hamada et al., 2014). These data unequivocally pair tTG activation and its resulting transamidation of the IP3R receptor to altered functionality of the ER. In addition, IP3R1 receptor dysfunction is shown to increase neuronal vulnerability to ER-stress (Higo et al., 2010). In **chapter 4**, tTG was actually observed to colocalize with the IP3R receptor, hinting at the possibility that this receptor could also be targeted by tTG in a PD-like setting. Interestingly, under these experimental conditions the ER-resident Ca^{2+} transporters SERCA2 and the sodium/calcium exchanger NCX1 were actually found to be a substrate of tTG in our PD neuronal model (data not shown). Even though the functional implications for PD pathogenesis of tTG-mediated PTM in these Ca^{2+} transporters remain to be elucidated, they hint at the possibility that tTG may influence Ca^{2+} homeostasis via protein modification of Ca^{2+} transporters. This idea is supported by data on the consequences of other post translational modifications in these transporters. For example, oxidation and phosphorylation in SERCA2 are known to influence Ca^{2+} homeostasis (Vangheluwe et al., 2005). Post-translational modifications within calnexin are also coupled to alterations in ER-mediated Ca^{2+} homeostasis. For instance, phosphorylation of calnexin attenuates SERCA2 mediated calcium signaling and enhances protein quality control in the ER (Bollo et al., 2010; Cameron et al., 2009; Roderick et al., 2000). Additionally, calnexin is shown to modulate Ca^{2+} homeostasis via a palmitoylation-dependent interaction with SERCA2 (Lynes et al., 2013). An interesting question remains whether there are more protein candidates at the ER that are prone to tTG-mediated PTM. For example, is it possible that tTG-mediated PTMs occur within the ER-stress sensors or perhaps proteins down-stream in the ER-stress signaling pathway?

In order to identify such tTG-modified proteins on the ER elaborate screening is required in future studies.

tTG and PD neuronal vulnerability

Disruption of Ca^{2+} homeostasis is an important cause of UPR (Verkhratsky and Toescu, 2003) and has been implicated in the pathophysiology of various chronic neurodegenerative diseases, such as Amyotrophic lateral sclerosis (ALS), prion disorders, Huntington's and Alzheimer's and PD (Fonseca et al., 2013; Guo et al., 1997; Prell et al., 2013; Torres et al., 2010; Vidal et al., 2011). In fact, impairment of Ca^{2+} homeostasis is believed to be one of the main factors that underlie the specific vulnerability of the dopaminergic cells in the SNpc to neuronal death. These neurons have constantly increased basal Ca^{2+} levels, which are necessary for the autonomous pacemaking activity required to maintain a basal DA tone in the striatum(Calì et al., 2014; Guzman et al., 2009; Surmeier and Schumacker, 2013). In addition, these neurons have a low buffer capacity for Ca^{2+} that reduces their ability to cope with cytotoxic levels of Ca^{2+} (Damier et al., 1999; German et al., 1992). The use of Ca^{2+} rather than monovalent cations makes the neuron to consume more energy to maintain normal function, thereby further increasing the metabolic load. Mitochondria and the ER are the principal organelles involved in sequestering of Ca^{2+} in neurons and Ca^{2+} homeostasis in general. Thus in PD, where mitochondrial dysfunction is also evident, these properties are thought to undermine the ability of the dopaminergic neurons in the SNpc to cope with stress (Calì et al., 2013; Schapira, 2011). As mentioned before, Hamada and coworkers have explicitly shown that tTG has a pivotal role in IP3R mediated Ca^{2+} signaling (Hamada et al., 2014). They have shown that treatment of B-lymphocytes derived from HD patients with the tTG-activity inhibitor Z006 attenuated IP_3R-mediated Ca^{2+} release, thereby hinting at the possibility that pharmacological inhibition of enzymatically active tTG could be used to regulate Ca^{2+} homeostasis in neurons. These data make it tempting to speculate that inhibition of enzymatic tTG activity could alter the regulation of Ca^{2+} homeostasis in PD dopaminergic neurons and is, without doubt, an interesting concept that should be explored in follow-up studies.

tTG and autophagy regulation

Another aspect of stimulation of enzymatic tTG activity is the modulation of autophagy (which could be related to tTG-mediated PTM in beclin 1) as described in **chapter 5**. Autophagy is coupled to ER-functioning, as it is an important cellular degradation mechanism that helps alleviate ER-stress (Ogata et al., 2006; Pankiv et al., 2007). It is well known that pathological conditions that induce ER-stress, followed by the induction of UPR, eventually also lead to stimulation of autophagy (Fouillet et al., 2012; Verfaillie et al., 2010). Both the PERK/eIF2α and IRE1 arms of the UPR, for example, have been implicated in the regulation of autophagy (Ito et al., 2010; Kouroku et al., 2007; Ogata et al., 2006). Switching on autophagy in this manner can be

protective, as 'priming' neurons with moderate ER-stress, via induction of UPR by pretreatment of cells with non-lethal concentrations of ER-stressors (e.g. tunicamycin or thapsigargin), can actually prevent neuronal death. This response is called ER-hormesis, in which the induction of autophagy offers protection to cellular death (Matus et al., 2012; Mollereau, 2013; Petrovski et al., 2011). As such, induction of hormesis prevented degeneration of DA-neurons in the SN of 6-OHDA treated mice, and in SH-SY5Y cells, whereas inhibition of autophagy completely abrogated this protective effect in the SH-SY5Y cells and a drosophila model of PD (Fouillet et al., 2012). However, prolonged stimulation of autophagy may be associated more with cell death than survival. In **chapter 5,** a clear connection between tTG and the regulation of autophagy is shown, which adds to and extends the current available literature suggesting that tTG is an important regulator of autophagy (Decuypere et al., 2011; Hamada et al., 2014; Luciani et al., 2010). However, the exact molecular mechanism by which tTG regulates autophagy in PD-affected neurons and the functional consequences thereof remains to be elucidated.

UPR and tTG in PD: connecting the dots

Involvement of the UPR during PD is not thought of as a process that is only visible in the end-stages of the disease process. A popular view is that this mechanism is already activated in the early stages of PD (Hoozemans et al., 2012). This supposition is corroborated by the occurrence of chronic ER stress in neuronal cultures generated from PD-derived pluripotent stem cells (Chung et al., 2013). Additionally, UPR has been observed in early-symptomatic animals overexpressing α-synuclein where an association was found with the presence and formation of α-synuclein oligomers at the ER lumen (Colla et al., 2012a, 2012b). The potential ability of tTG to interfere with Ca^{2+} homeostasis as well as with autophagy, which are both essential tools for the neuron to cope with UPR, may therefore be decisive in controlling whether the stressed neuron survives or, eventually, dies in both the initial and later stages of the disease. This putative process is illustrated in figure 1, in which tTG enzymatic activation and the resulting tTG-mediated modification of important ER proteins (Fig. 1A), points towards a role of tTG in UPR induction and perhaps duration. It is therefore tempting to speculate that aberrant tTG activation may aggravate the outcome for the PD-affected neuron by interfering with cellular pathways that alleviate neuronal stress (Fig. 1B). This interesting supposition could, for example, be tested in studies that focus on the function of the UPR pathway upon (enzymatic) inhibition of tTG activity (e.g. via specific enzymatic inhibition with Z006). Together, the data presented in the chapters of this thesis provide a clear incentive for further study of the possible role of the enigmatic enzyme tTG in PD pathogenesis and future treatment.
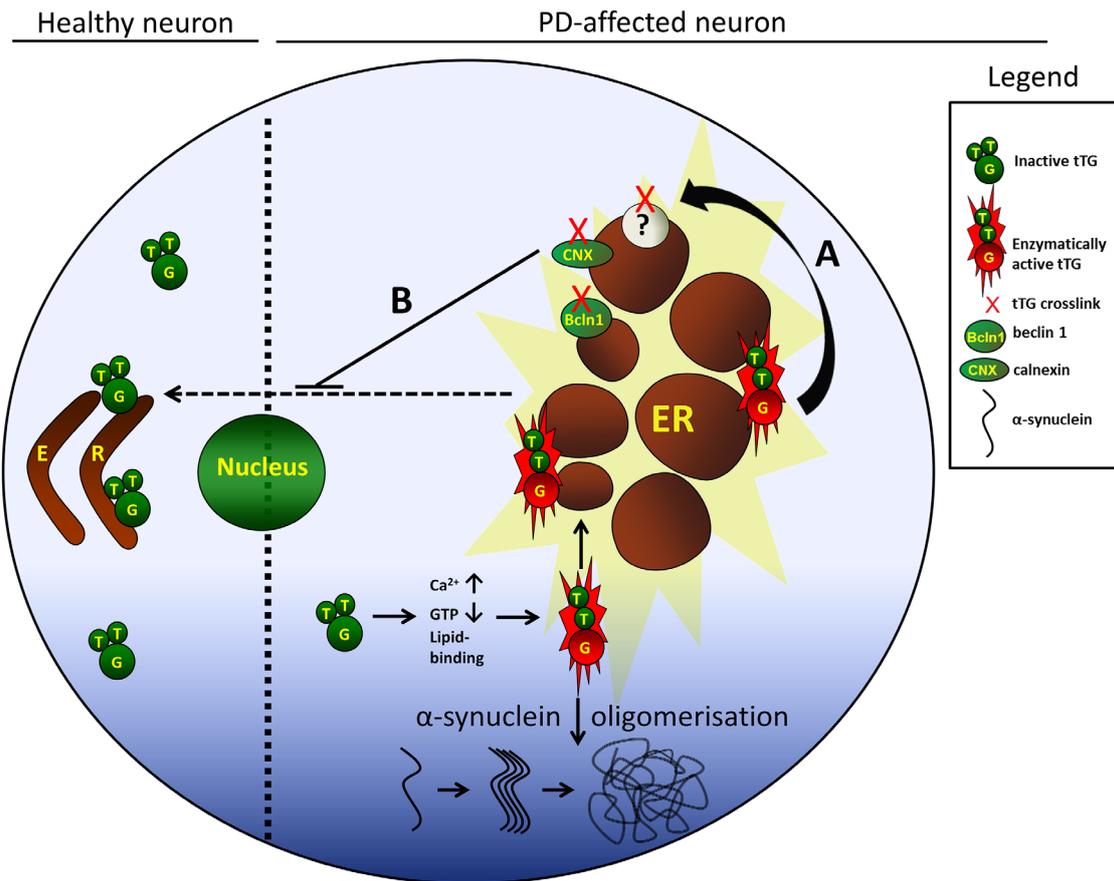
Fig. 1. Putative implications of tTG in PD pathogenesis. In the PD-affected neuron, cellular stress factors that lead to the activation of cellular tTG, results in the tTG-mediated misfolding and aggregation of α-synuclein. Additionally, the diseased melanized neurons undergo ER-stress which is characterized by the accumulation of tTG at the ER. (A) At the ER, tTG crosslinks (X) several key ER proteins involved in autophagy and Ca^{2+} homeostasis, e.g. beclin 1 (bcln1) and calnexin(CNX), and/or conceivably could regulate other neuronal processes via the crosslinking of other, yet-unidentified ER proteins (?). (B) ER-stress results in the induction of UPR in order to return ER homeostasis (dotted arrow), promoting neuronal survival. Activation of tTG could, for instance, disturb this rescue mechanism by interfering with autophagy and disruption of Ca^{2+} homeostasis.